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SYMPOSIUM

The Origin and Evolutionary Consequences of Skeletal Traits Shaped by Embryonic Muscular Activity, from Basal Theropods to Modern Birds

Alexander O. Vargas,[1,*] Macarena Ruiz-Flores,[*] Sergio Soto-Acuña,[*] Nadia Haidr,[†] Carolina Acosta-Hospitaleche,[‡] Luis Ossa-Fuentes[*] and Vicente Muñoz-Walther[*]

*Laboratorio de Ontogenia y Filogenia, Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Las Palmeras 3425, Santiago, Chile; [†]Instituto de Biología de Organismos Marinos, CONICET, Bvd. Brown 2915, U9120ACD, Puerto Madryn, Argentina; [‡]División Paleontología Vertebrados, Museo de La Plata, Paseo del Bosque s/n, (B1900FWA), La Plata, Argentina. CONICET.

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[1]E-mail: alexvargas@uchile.cl

Synopsis Embryonic muscular activity (EMA) is involved in the development of several distinctive traits of birds. Modern avian diversity and the fossil record of the dinosaur-bird transition allow special insight into their evolution. Traits shaped by EMA result from mechanical forces acting at post-morphogenetic stages, such that genes often play a very indirect role. Their origin seldom suggests direct selection for the trait, but a side-effect of other changes such as musculo-skeletal rearrangements, heterochrony in skeletal maturation, or increased incubation temperature (which increases EMA). EMA-shaped traits like sesamoids may be inconstant, highly conserved, or even disappear and then reappear in evolution. Some sesamoids may become increasingly influenced in evolution by genetic-molecular mechanisms (genetic assimilation). There is also ample evidence of evolutionary transitions from sesamoids to bony eminences at tendon insertion sites, and vice-versa. This can be explained by newfound similarities in the earliest development of both kinds of structures, which suggest these transitions are likely triggered by EMA. Other traits that require EMA for their formation will not necessarily undergo genetic assimilation, but still be conserved over tens and hundreds of millions of years, allowing evolutionary reduction and loss of other skeletal elements. Upon their origin, EMA-shaped traits may not be directly genetic, nor immediately adaptive. Nevertheless, EMA can play a key role in evolutionary innovation, and have consequences for the subsequent direction of evolutionary change. Its role may be more important and ubiquitous than currently suspected.

Introduction

Several skeletal traits of vertebrates are shaped by embryonic muscular activity (EMA). EMA has its effects at a complex post-morphogenetic stage, depending importantly on where embryonic muscles are placed and the forces they exert. Therefore, EMA provides a clear-cut example where understanding development requires analysis of an organismal system, beyond the cellular and molecular level. Perhaps because of this, it is difficult to discuss the origin and evolution of EMA-shaped traits in the terms of conventional evolutionary theory, which is largely focused on genes. Indeed, despite a truly vast literature on experimental embryonic paralysis, including insightful evolutionary discussions (Newman and Müller 2000; Müller 2003; Nowlan et al. 2010), EMA is rarely mentioned within mainstream topics of vertebrate evolution.

Interpretative challenges aside, EMA is known to be involved in the development of several distinctive traits of birds (Hall and Herring 1990). The fossil record on the origin of birds from dinosaurs is one of the best for any macroevolutionary transition, and is informative about the evolution of these traits.

Experimental embryonic paralysis is also technically easy in birds, and is known to produce "atavistic" traits that resemble the condition of ancient dinosaurs (Müller and Streicher 1989; Botelho et al. 2015a). These facts demand further inquiry into the evolutionary significance of EMA. To begin with, how can EMA-shaped traits get started in evolution? As we will show, there are several non-exclusive potential answers, but in most cases, they may originate as an indirect consequence or side-effect of other changes such as musculo-skeletal re-arrangements, shifting rates of skeletal maturation or changed conditions of embryonic incubation. This contrasts with "canonical" skeletal traits that are typically formed and patterned at early pre-morphogenetic stages, through direct action of molecular mechanisms such as Turing-type reaction-diffusion processes (Bhat et al. 2011; Raspopovic et al. 2014) and modulators of localized gene expression (Adachi et al. 2016; Nakamura et al. 2016). In contrast, because the mechanical forces of EMA are nongenetic, EMA-shaped traits may be perceived as being inconstant (not reliably formed) and non-selectable, making it easy to downplay their role in evolution. However, a more thorough analysis of specific cases, as reviewed below, may help dispel such a-priori assumptions.

The theropod fibular crest: a highly conserved EMA-shaped trait

The fibular crest on the tibia of most theropod dinosaurs (including birds, the only surviving lineage) has been one of the clearest examples for discussing the origin and evolution of an EMA-shaped trait (Müller and Streicher 1989). Theropods specialized in cursoriality and evolved a longer lower leg in which the outer fibula became much thinner than its medial neighbor, the tibia. Adult theropods present a bony crest projecting laterally from the proximal-anterior surface of the tibia, forming a rigid articular connection to the fibula that braces both bones together (Fig. 1A,B). The crest is at the insertion site of the musculus iliofibularis, that goes from the posterior region of the upper hip bone (ilium) to the posterior-proximal fibula, and plays an important role in bending the knee by pulling the lower leg backward and toward the body (Fig. 1C). In normal development, an independent cartilage arises at the future site of the crest between the tibia and fibula, that is then incorporated to the periosteum of the tibia before ossifying completely (syndesmosis tibiofibularis). Importantly, under experimental muscular paralysis, the crest is no longer formed (Müller and Streicher 1989).

A ready explanation for the origin of the fibular crest is that, as the fibula became narrower, the m. iliofibularis began projecting onto the tissues bridging the space between the tibia and fibula (Müller 2003). Since mechanical stimulation induces the cartilage formation pathway (Takahashi et al. 1998), it is readily conceivable how this could have led to the development of cartilage in between these bones. This new encounter led to the development of the crest in a way that may be compared to a callus: a reaction to nongenetic forces that stimulate its development. Importantly, the fibular crest is a highly conserved trait, that first made its appearance in advanced neotheropods about 200 million years ago, in the early Jurassic (Welles 1984) or perhaps even earlier, in the late Triassic (Colbert 1989); yet in modern birds, it continues to depend on non-genetic mechanical forces for its formation. If genes were more directly involved in the development of this trait, we might observe an at least partial formation of the crest upon paralysis, as occurs for other EMA-shaped traits (see below). The fibular crest demonstrates that a highly conserved EMA trait does not necessarily imply a more direct involvement of genetic-molecular mechanisms.

The reliable development and conservation of the fibular crest has had important evolutionary consequences: In several lineages, including birds, the fibula became shorter than the tibia, literally losing its distal portion and ending in a thin, splinter-like shape, with no distal articular surface and no contact with the ankle (Fig. 1B). If not for the fibular crest that braces the tibia against the proximal fibula, this trait would lead to a nonfunctional limb: the m. iliofibularis would pull on a proximal fibula with no skeletal connection to the rest of the lower leg, either at the ankle or tibia (Fig. 1C). Thus, reliable development of the fibular crest allowed subsequent reduction of the distal fibula, after which the presence of a fibular crest became indispensable (Müller and Streicher 1989; Newman and Müller 2000).

Embryonic muscle degeneration and the origin of opposable toes

The grasping foot of birds offers another informative example on the origin and evolution of EMA-shaped traits. It differs from that of basal theropods in that the hallux (digit I, homologous to our big toe) is opposable. The original arrangement for modern birds, where only digit I is reversed, is called the anisodactyl foot, but several lineages have evolved an additional opposable digit: birds with zygodactyl feet (such as budgerigars) have opposable digits I

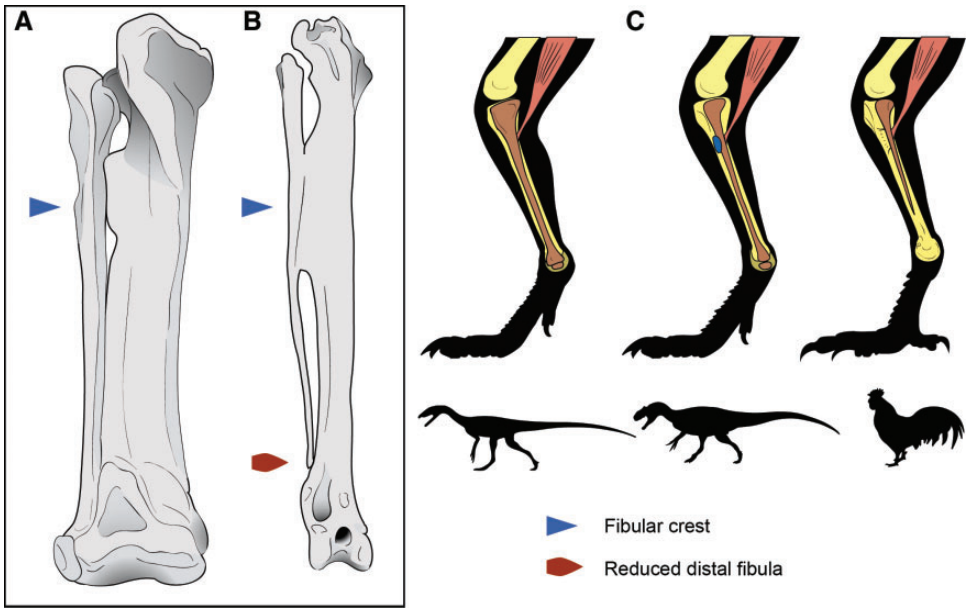

Fig. 1 The fibular crest of the tibia, an ancient EMA-shaped trait in the lower leg of birds. **(A)** The fibular crest (triangular arrow) originated in the remote ancestors of birds among the Neotheropod dinosaurs, at least 200 million years ago **(B)** The fibular crest persists in modern birds, who have also undergone further reduction of the fibula, losing its distal connection to the ankle (lanceolate arrow). **(C–E)** Evolutionary consequences of the fibular crest. **(**C**)** Before the origin of the fibular crest, the iliofibularis muscle pulled on the fibula, bending the knee. **(**D**)** In theropods, formation of the fibular crest (blue) braced the fibula against the tibia at the insertion site of the iliofibularis muscle. **(**E**)** In birds, the presence of the fibular crest allowed the fibula to lose its connection to the ankle. Without the crest, the fibula would have lost its skeletal connectivity with the rest of the leg, resulting in a non-functional limb. Schematic images based on Müller and Streicher (1989) and Newman and Müller (2000).

and IV, while birds with heterodactyl feet (such as trogons) have opposable digits I and II. These evolutionary innovations are typical examples of traits whose evolution has been chiefly discussed in terms of their adaptive value. However, recent research has revealed that these are EMA-shaped traits, which brings about a rather different narrative about their origin and evolution (Botelho et al. 2014; Botelho et al. 2015a, 2015b).

The foot of the budgerigar first develops as an anisodactyl foot: only later, digit IV acquires an additional metatarso-phalangeal articular surface (accessory trochlea), swinging toward lateral until it achieves its opposable orientation. This occurs along with a remarkable process of asymmetric degeneration of the intrinsic muscles of this digit (Botelho et al. 2014). In an anisodactyl basal neognath such as the quail, digit IV possesses a musculus extensor brevis digiti IV (EBDIV), running along the dorso-medial aspect of its metatarsal, and a musculus abductor brevis digiti IV (ABDIV) running along the latero-ventral aspect of the metatarsal (Fig. 2A). During embryonic development of the budgerigar, EBDIV becomes progressively thinner and disappears, as digit IV swings toward lateral (Fig. 2B). In experimental paralysis using decamethonium, there is no accessory trochlea or change in digit IV orientation. We may conclude that the asymmetric muscular force exerted by ABDIV is key to the development of an opposable digit IV (Botelho et al. 2014). In the primitive anisodactyl foot of the quail, a non-opposable digit IV probably reflects symmetric forces resulting from the sustained presence of both EBDIV and ABDIV. In zygodactyl feet, both muscles are formed in their normal positions, so there was no evolutionary change in early patterning or migration of their precursors. Rather, secondary degeneration could result from decreased nervous stimulation of muscles during development, which is known to have a trophic effect on muscle mass. The fact the nervous system may be involved is a reminder of just how indirectly EMA-shaped traits can be triggered.

Evolutionary variation in the adult musculature of bird feet (George and Berger 1966) can now be reinterpreted in the light of EMA. For instance, the Piciformes (woodpeckers) have independently evolved zygodactyly, and have lost the EBDIV muscle. In trogons with heterodactyl feet, digit II has become opposable by swinging toward medial: the laterally placed musculus adductor digiti II is lost, while the medially placed musculus abductor digiti II continues to exert its force: a similar but appropriately opposite pattern to that of zygodactyl feet. In Passeriformes like the zebrafinch, the foot is

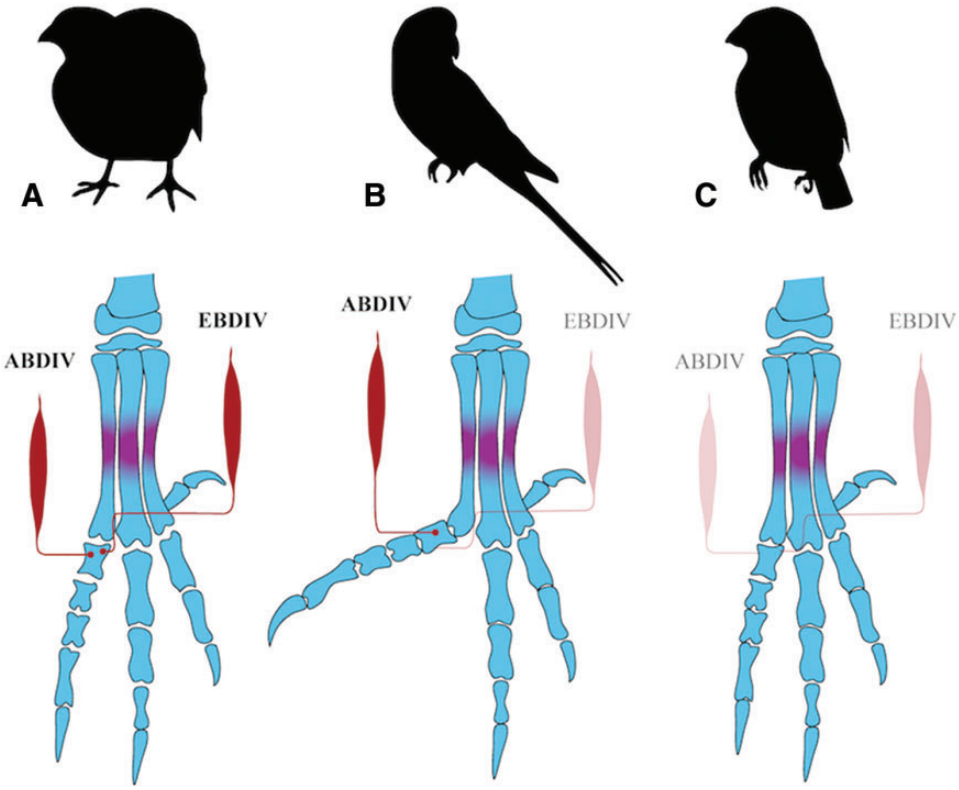

Fig. 2 Degeneration of intrinsic digit muscles and evolution of the avian foot. **(A)** In birds with primitive anisodactyl feet such as the quail, both intrinsic muscles associated with digit IV (ABDIV, abductor brevis digiti IV and EBDIV, extensor brevis digiti IV) are present in the adult. **(B)** In the budgerigar, the embryonic EBDIV muscle is formed but then degenerates, and digit IV swings laterally into its opposable orientation as a result of the unopposed action of ABDIV. Accordingly, the digit does not become opposable under experimental paralysis. **(C)** Passeriform birds are anisodactyl but their phylogenetic affinities suggest they evolved from ancestors with zygodactyl feet such as the budgerigar. Both muscles are formed but degenerate in zebrafinches. From a zygodactyl ancestor this could be achieved by additional degeneration of the ABDIV muscle. Because of the differences with quail, it may be more appropriate to refer to the foot of Passeriformes as "neo-anisodactyl."

anisodactyl, presumably retaining the primitive condition. However, unlike quail, both ABDIV and EBDIV are absent in adult Passeriformes (George and Berger 1966) This difference would be enigmatic in terms of purely functional explanations, but makes sense when we take EMA and evolutionary history into account. In embryos of the zebra finch (a Passeriforme) both muscles are formed, but then degenerate and disappear (Fig. 2C, Botelho et al. 2014). The closest relatives of Passeriformes among living birds and fossil taxa are zygodactyl: the Psittaciformes (parrots and allies) and the appropriately named Zygodactylidae (extinct). This suggests a possibly zygodactyl ancestor in the lineage leading to Passeriformes. If so, that ancestor could have undergone embryological degeneration of ABDIV. From that point, additional degeneration of EBDIV in the ancestors of Passeriformes would have been sufficient to trigger a secondary reacquisition of an anisodactyl foot (Botelho et al. 2014). Since this does not constitute an exact reversion to primitive anisodactyly (where ABDIV and EBDIV do not degenerate), it may be more appropriate to call the passerine foot "neo-anisodactyl".

Acquisition and then loss of opposability can be counterintuitive from an adaptive point of view. Passeriformes are not the only example suggesting low functional commitment to two opposable digits. Most woodpeckers are four-toed and zygodactyl, but some species of the genus *Picoides* have a derived three-toed foot, keeping the opposable digit IV, while losing the opposable hallux entirely (Spring 1965). The adaptive significance of having two opposable digits has been related to climbing on the trunk surface and pecking, but three-toed woodpeckers can do this with a single opposable digit, and several anisodactyl birds are adept at climbing. Climbing anisodactyl birds are unlikely to lose the hallux, which only occurs among ground dwellers. In the three-toed woodpecker, backup was provided by an additional opposable digit. Much like the aforementioned fibular crest, this case also shows how an

EMA-shaped trait has enabled subsequent reduction/loss in other skeletal structures.

Parental care, heterochrony, and EMA-shaped traits

Another important developmental observation is that the accessory trochlea is formed when the distal region of the metatarsal IV is composed of immature and proliferating chondrocytes, before cartilage maturation and ossification (Botelho et al. 2014, 2015b). Conceivably, immature cartilage is more plastic upon EMA, suggesting a developmental "window" for increased influence. This brought attention to a previously overlooked evolutionary correlation: namely, that new digit orientations have evolved only within altricial birds (Botelho et al. 2015b). Altricial birds are born with decreased mobility, remaining in the nest and depending highly on parental care: by comparison, precocial birds are able to walk and abandon the nest upon hatching. This is because embryonic development of the skeleton shows a remarkable heterochronic delay in altricial birds, being much less ossified upon hatching. Conceivably, delayed maturation may extend the time "window" in which the skeleton can be affected by EMA. Asymmetric muscle degeneration is also key, which readily explains why not all altricial birds have evolved other opposable digits. However, the fact that new opposable digits have never evolved in precocial birds suggests that altriciality may be an equally important requisite. EMA provides a reasonable causal link between altriciality and opposable digits. In contrast, adaptive uses of new opposable digits can vary greatly or may not always be evident (Bell and Chiappe 2011; Mitchell and Makovicky 2014) For instance, the genus *Geococcyx* is zygodactyl, but is a mainly terrestrial runner, like several anisodactyl birds. Perhaps the group with a most clear-cut use for zygodactyly is the Psittaciformes, where manipulation correlates with increased cognitive capabilities. The evolution of opposable digits may be chiefly driven by altriciality and changes in embryonic musculature, with adaptation as a secondary consequence that may never occur, and even then, may be easily lost.

The hallux is the most phylogenetically ancient opposable digit in the foot of birds, and also involves EMA. In basal theropods, the metatarsal of the hallux was a straight element, whereas in modern birds, the opposable orientation of this digit depends chiefly on the twisted shape of its metatarsal (Middleton 2001). Experimental paralysis in modern birds results in an atavistic non-opposable hallux with a straight mt1, resembling basal theropods (Botelho et al. 2015a). Some modern birds such as petrels and penguins also have straight metatarsals. Accordingly, they have lost the muscles extensor hallucis longus and flexor hallucis brevis (George and Berger 1966) that twist mt1 in other birds. This does not mean that these muscles were absent in ancient theropods. Their loss is derived among modern birds, and their insertion sites are present on mt1 of ancient theropods (Hutchinson 2002). No asymmetric muscle degeneration occurs during normal development of the opposable hallux. Rather, twisting of mt1 may relate to an ancient change of position within the foot. Mt1 in both basal theropods and modern birds is a short and proximally tapering element with no proximal articular surface, that cannot articulate to the ankle. Rather, it forms a non-synovial joint onto metatarsal 2. In ancient theropods, it attached to the medial side of mt2, but in basal birds, it shifted to the ventral side of mt2 (Middleton 2001). Assuming the muscles maintained their origin and insertion sites, this change may have led to altered forces twisting the metatarsal. However, basal ornithuromorpha (such as enantiornithine birds) show only partial change in the shape of mt1. Importantly, their fossilized embryos/hatchlings reveal that they hatched with a much greater degree of skeletal ossification than modern birds (Elzanowski 1981; Zhou and Zhang 2004; Chiappe et al. 2007; Kurochkin et al. 2013). The subsequent evolution of delayed skeletal maturation may have enabled a greater effect of EMA and a fully twisted mt1 (Botelho et al. 2015a).

Successive delays in skeletal maturation along evolution could be related to increased pedomorphosis along the dinosaur-bird transition (Botelho et al. 2015a; Bhullar et al. 2016). As mentioned above, delayed skeletal maturation is also related to increased parental care: altricial birds can grow faster after hatching, because they are fed by their parents. Brooding, another form of parental care, was already present in maniraptoran dinosaurs (Varricchio et al. 2008), and could have increased incubation temperature, which is known to increase EMA (Oppenheim and Levin 1975). In crocodilians, increased incubation temperature leads to significant differences in size and morphology of the interclavicle as well as the growth of long bones, presumably due to increased EMA (Pollard et al. 2016, 2017). Several skeletal innovations have been argued to be a side-effect of non-shivering thermogenesis in birds, which evolved through muscle hyperplasia, conceivably leading to increased effects of EMA and increased incubation temperatures while brooding (Newman et al. 2013).

What is a "true" sesamoid? Categories versus evolutionary transitions

Until this point, we have dealt mainly with how EMA may alter the shape of a preexisting embryonic skeletal element. However, EMA also plays a role in the origin of new skeletal elements, especially the so-called "sesamoids." This is a rather ill-defined category of skeletal elements that are found associated with a tendon that reaches around a joint, such that the sesamoid acts like a fulcrum, providing leverage for the force exerted through that tendon. They often develop within the tendon itself at late stages (even post-hatching). Sesamoids can show some significant differences from canonical elements. For instance, sesamoid formation may require EMA, and the presence of a sesamoid may not be conserved in evolution: it can vary greatly among closely related clades, or they may even be intraspecifically "inconstant," varying among individuals (or even within a single individual, present on one side of the body, but not the other; Reviewed in Vickaryous and Olson 2007). Sesamoids that gather all the above characteristics could be considered "unequivocal" and will be called "Category I" sesamoids in this review. However, other elements often described as sesamoids may not fulfill one or more of the aspects listed above, generating controversy over whether they are "true" sesamoids. Consider the patella (knee cap), which is typically accepted as a sesamoid, but is constant, and highly conserved within large clades. Experiments in chicken have shown that the patella can develop in culture (Murray and Huxlay 1925; Niven 1933) or under conditions of paralysis, although it is smaller (Drachman and Sokoloff 1966), and may still frequently fail to develop (Hosseini and Hogg 1991). In humans, a set of specific genes seem to be required for its normal formation: mutations in the *LMX1B* gene lead to its agenesis or hypoplasia (in nail-patella syndrome; Dreyer et al. 1998), and mutations in the *ORC1*, *ORC4*, *ORC6*, *CDT1* or *CDC6* genes also lead to agenesis of the patella, although they are all correlated with severe growth problems (Meier–Gorlin Syndrome, types 1–5, respectively; de Munnik et al. 2015). Even so, in subadult humans with agenesis or hypoplasia of the patella, movements that imitate normal patterns of flexion and extension have been reported to rescue its development (Brunner 1891, cited in Vickaryous and Olson 2007). Consistent with this, upon surgical removal of the patella from the quadriceps femoris tendon of subadult dogs, it can regenerate if movement is permitted across the joint, but not if movement is impeded (Carey et al. 1927). Patella-like sesamoids with influences from both genetic variation and muscular activity will be referred to in this review as "Category II sesamoids."

At least some category II sesamoids may differ from other sesamoids in the mechanisms of their earliest formation. Arguably, only elements that form directly within a tendon are "true" sesamoids. However, recent studies on patella development in mouse have revealed a more nuanced scenario. Precursor cells of the patella are not found within the tendon, but in a unique population of cells on the surface of the cartilaginous femur, at the insertion site of the immature tendon (Eyal et al. 2015). These cells are unlike the cells of both femur and tendon, in that they express a unique combination of both *Sox9*, an early promoter of the cartilage pathway, and *Scleraxis (Scx)*, an early marker of the tendon pathway. Importantly, bony eminences of canonical bones have also been shown to derive from these unique *Sox9* + *Scx* populations at a tendon insertion site (Blitz et al. 2013). It is worth noting that in many cases, these bony eminences develop their own ossification centers ("traction epiphyses," see Barnett and Lewis 1958), which points to some modularity and independence from the main skeletal element. The main difference between the development of the patella and that of a bony eminence is that the patella then becomes a physically separate cartilage, a process that requires EMA. In absence of EMA, the population of *Sox9*-*Scx*-positive cells is formed anyway (consistent with reported independence of its formation), but the patella cartilage becomes completely continuous with that of the femur, resembling a bony eminence. Therefore, a significant difference between the patella and a bony eminence may be that EMA is required for the patella to develop as a separate skeletal element.

Previously, evolutionary patterns have been noted where sesamoids appear to have become bony eminences, or vice-versa (Barnett and Lewis 1958). For instance, in tree shrews, a sesamoid is found at the position that in other mammals is occupied by the lateral epicondyle of the ulna, where tendons of extensor muscles in the forearm attach (Barnett and Lewis 1958). The inverse process, in which a sesamoid has evolved to become a bony eminence, is represented by the hypertrophied "cnemial crest" observed in several shorebirds. It projects from the tibia and is associated with the triceps femoris extensor muscle, bearing striking resemblance in shape and position to the patella of other birds (Shufeldt 1884; Parsons 1904; Barnett and Lewis 1958). It is worth noting that a sesamoid may form at two

possible positions of a tendon bridging a joint (Barnett and Lewis 1958): near the proximal bone (in this case, the femur) or near the distal bone (the tibia). Unlike mammals, the patella of birds may be a distal sesamoid, which is consistent with fossil evidence that a patella has evolved independently in each lineage (reviewed in Vickaryous and Olson 2007). The newfound similarities in the development of bony eminences and sesamoids can now be used to interpret the evolutionary transitions between them. These suggest an important role for EMA: increased effect in the region of a bony eminence may lead it to develop as a separate sesamoid. Conversely, decreased EMA effects on sesamoid precursor cells may result in development as a bony eminence.

Although sesamoids are typically analyzed in terms of current function, this function may have changed since the origin of the sesamoid. For instance, in the forearm of most tetrapods, including birds, it is common for the ulna to present an olecranon process of the elbow, at the insertion site of the triceps brachii tendon (Fig. 3A). But in penguins there is no olecranon: instead, a separate sesamoid bone develops at the same position (Fig. 3B; Barnett and Lewis 1958). This is especially interesting since the adult wing is composed by very flat bones and is stiffened into permanent hyperextension, with essentially no movement at the elbow (Fig. 3B). The ulnar sesamoid has no possible function as a fulcrum, and any movements relevant for its development must occur only at embryonic stages. Although it may contribute to stiffening the wing, the presence of an ulnar sesamoid in penguins is likely a passive phylogenetic legacy: an ulnar sesamoid replacing the olecranon is already present in penguin outgroups such as petrels and albatrosses, which fly with hyperextended wings (Meyers and Stakebake 2005). Replacement of the olecranon for a sesamoid may have lifted a constraint on wing hyperextension. However, an ulnar sesamoid has also evolved independently in apodiformes (Stolpe and Zimmer 1939; Zusi 2013), which lack hyperextension and may present either continuous gliding (swifts) or hovering flight (hummingbirds). These divergent functional contexts suggest that adaptive significance can be acquired after the fact, and may not play a decisive role in the origin of the ulnar sesamoid.

Sesamoideal traits of the pisiform and its re-evolution in birds

The pisiform is perhaps the best example of a bone whose sesamoideal nature remains controversial. It is placed at the posterior wrist and is associated with the tendon flexor carpi ulnaris, which is why it is readily described by anatomists as a sesamoid. However, many authors do not share this opinion: Indeed, the pisiform was not even listed in an important review of sesamoid bones (Vickaryous and Olson 2007). An influential argument is that the pisiform has been highly conserved in the wrist since early tetrapods, and should thus be considered a "true" carpal bone (Haines 1969; Reno et al. 2016). Recent work on chameleon development has also argued that their pisiform is not a sesamoid, because it apparently forms by segmentation from the ulnare, developing attachment sites for the flexor carpi

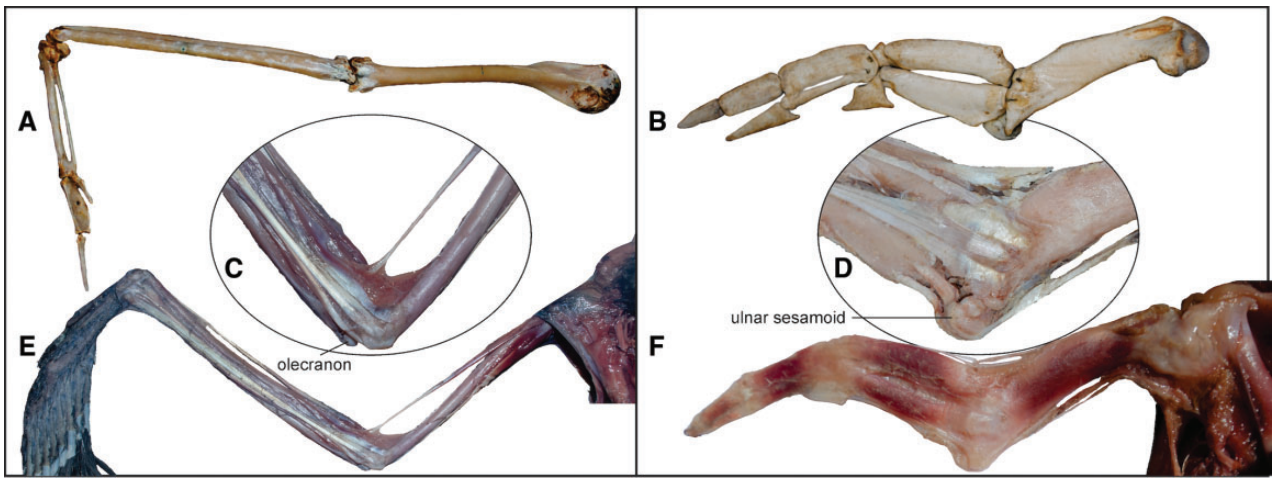

Fig. 3 Replacement of the olecranon process of the ulna for an ulnar sesamoid in penguins. **(A, B)** cranial (anterior) view of wing skeletal elements: (A) *Phalacrocorax brasilianus*, (B) *Pygosceslis adeliae*. **(C, D)** close up to humeral/zeugopodium articulation showing tendons. (C) anterior view of *Phalacrocorax atriceps*' femori extensor chick, (D) posterior view of a *P. adeliae* adult. **(E, F)** anterior view of dissected wing of (E) *Phalacrocorax atriceps*' femori extensor chick, (F) *P. papua* chick.

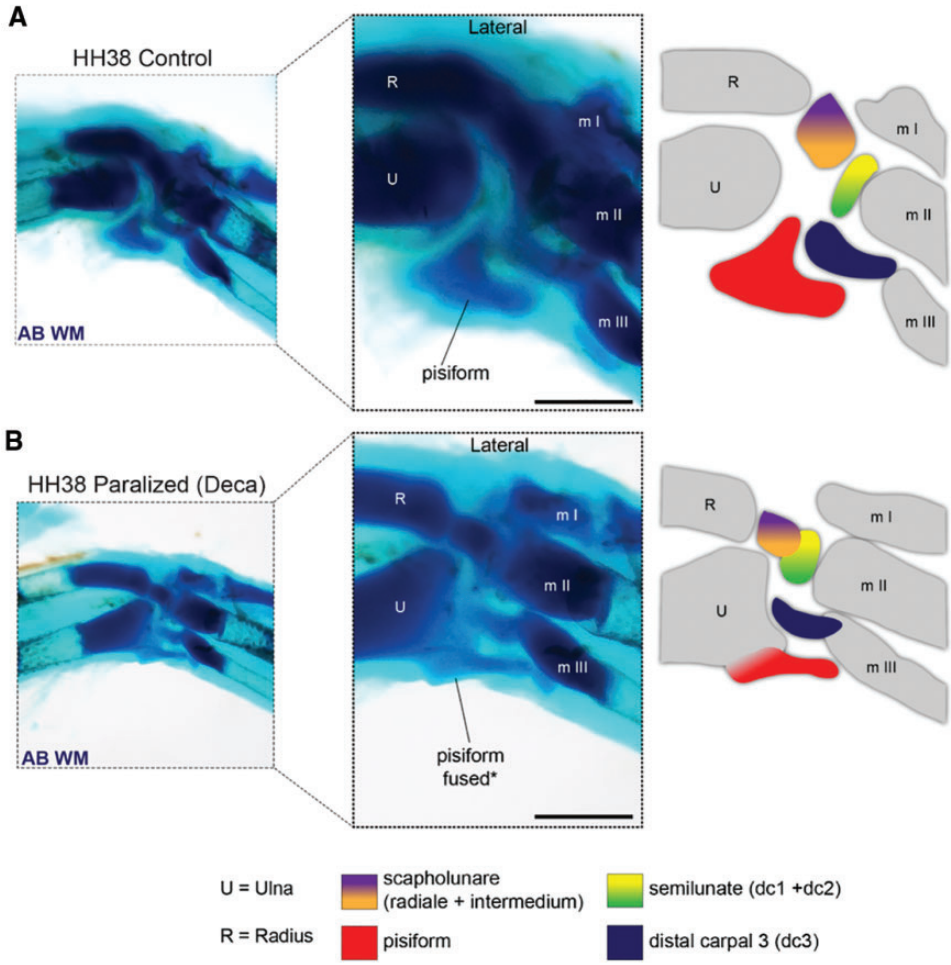

Fig. 4 Rigid paralysis modifies pisiform development. Following Botelho et al. (2014), we injected a diluted solution of decamethonium bromide (15 mg/mL in phosphate buffered saline 1×) at stage HH29 and we fixed and stained the embryos at stage HH38. **(A)** Control HH38 embryo with normal wrist cartilages showing the pisiform (red) fully separated from the distal part of the ulna. **(B)** HH38 embryo paralyzed with decamethonium bromide. In this experimental essay, we noted a smaller anomalous pisiform cartilage fused with the distal part of the ulna at the ventral–lateral side. We additionally conducted more paralysis experiments with injections at HH29 with similar results (= pisiform fused to the ulna) at HH42 and HH46. (See Supplementary Table for details). R, radius; U, ulna; mI, metacarpal 1; mII, metacarpal 2; mIII, metacarpal 3. For carpal element identification, see color details in the figure.

ulnaris tendon and the pisometacarpal ligament, rather than forming within a continuous tendon (Diaz and Trainor 2015).

However, precursors of the mouse patella would appear to segment from the femur upon detaching from its surface (Eyal et al. 2015), so it is no longer clear if alleged segmentation automatically discards sesamoidal affinities. Instead, it remains possible that the pisiform could be a category II sesamoid. Here, we present some new experimental data from chicken that support this hypothesis. By applying decamethonium bromide at stage HH29, before earliest formation of the pisiform cartilage, rigid muscular paralysis results in a notable decrease in size of the pisiform, in contrast with the mild or null effects observed in the other canonical wrist elements (Fig. 4). Additionally, the pisiform was observed consistently fused to the ulna, resembling a projection from this bone. The pisiform of the chicken embryo is formed very close to the surface of the distal ulna during normal development (Botelho et al. 2014; it does not segment from the ulnare in birds, *contra* Diaz and Trainor 2015). This supports the possibility that its precursor cells originate at the surface of the ulna, much like the precursors of the mouse patella originate at the surface of the femur. The results emphasize the role of EMA in normal development of the avian pisiform, and support the notion that EMA allows the pisiform to become physically separate from the ulna, rather than developing as a bony eminence. Also, note that the pisiform resembles the patella in that it does not cease to form under paralysis, which suggests a more direct involvement of genetic-molecular mechanisms.

Remarkably, loss and regain of the pisiform have actually occurred along the evolutionary transition from dinosaurs to birds (Botelho et al. 2014). While the pisiform is functionally important for locomotion in quadrupedal animals, it became notably reduced in bipedal dinosaurs. An ossified pisiform is already undetectable in the vast majority of tetanuran dinosaurs, where basal forms are typical "carnosaurs" with reduced forelimbs. It then remained absent right up to maniraptoran dinosaurs closest to the origin of birds. If present at all, the pisiform was either non-ossified or too small to be preserved. From this condition, it re-appeared in birds as a large ossified carpal. Importantly, the pisiform functions in the wing downstroke of modern birds (Vazquez 1992), and its evolutionary reappearance coincided with the earliest establishment of unambiguous flight capabilities in basal Avialae (Botelho et al. 2014). A recent study has provided an independent case in which a category II sesamoid has disappeared and re-appeared in evolution. An ossified patella was present at the origin of crown marsupials, but was then lost in most modern forms, including the diverse Diprotodontia. However, the Tarsipedidae are well nested within Diprotodontia and possess an ossified patella, indicating its evolutionary reappearance (Samuels et al. 2017). Evolutionary reappearance of lost sesamoids is reminiscent of reported evolutionary variation, where a given sesamoid may be present in most individuals of a species, but just a few in another related species (Sarin et al. 1999). This suggests that a stage of inconstancy may precede the disappearance and/or reappearance of a constant sesamoid, a process that may involve variation in EMA, direct genetic effects, or a combination of both.

The pisiform of mammals and birds is homologous, in that it was already present in their most recent common ancestor (Reynolds 1897; Haines 1969). However, the mammalian pisiform has undergone some unique evolutionary changes: unlike other wrist bones, it develops a growth plate with its own separate epiphyseal ossification, specifically resembling the development of long bones such as those in the neighboring forearm. *HoxA-11* and *HoxD-11* genes in the chicken are normally expressed in the forearm and lower leg (the zeugopod region of limbs) but not the wrist or ankle bones. Their expression is absent from the pisiform, as observed in histological sections (Yokouchi et al. 1991). However, in the mouse, the expression of these genes extends into the pisiform (Reno et al. 2016). Expression of *HoxD-11* is also found in the carpal region of the alligator, who has a uniquely elongate ulnare and radiale, with epiphyseal growth plates (Vargas et al. 2008). Likewise, in *Xenopus*, *HoxA-11* extends into the tibiale ("astragalus") and fibulare, which show growth plates and elongate shape (Blanco et al. 1998). This suggests that expansion of *HoxA-11* and *HoxD-11* expression into the mesopodial region resulted in some wrist/ankle bones with traits usually found in long bones of the zeugopod (Reno et al. 2016). It could be argued that such a transformation would only be possible for canonical wrist elements, and thus the pisiform cannot be sesamoid. However, this idea would fail to integrate all relevant information. Instead, we suggest that *HoxD-11* and *HoxA-11* may be able to induce zeugopod-like traits in any carpal cartilage in which they become expressed, regardless of whether it originates as a canonical bone or a category II sesamoid. This would also confirm that sesamoids can come under the influence of the same genetic-molecular mechanisms that pattern canonical skeletal elements.

Perspectives on EMA research

The understanding of EMA in evolution has progressed rapidly in the last few years. Importantly, new research has introduced stages of skeletal development (maturation) as a potentially decisive factor. Different skeletal elements show intrinsic differences in their reaction to experimental paralysis depending on the day it commences, with short periods of sensitivity to movement that are intrinsic to each element (Pollard et al. 2017 and references therein). This could relate to the varying degrees of maturation for each element upon EMA, a possibility that can be further explored through histological and molecular characterization. For instance, mt1 of the avian hallux has been shown to twist as a largely immature element that expresses Col-II, before the onset of Indian hedgehog, and before cartilage hypertrophy and Coll-X (Botelho et al. 2015a). Another important new development is the demonstration of how embryonic muscles can show secondary degeneration, altering the forces of EMA and producing skeletal innovations. Avian musculature is not only highly modified with regard to that of other amniotes, but has also varied considerably within the evolution of modern birds. While adult studies may alert to the absence of a given muscle, there is often no reliable developmental data on whether it may form transiently in the embryo, and then degenerate.

Overall rates of skeletal maturation in birds are related to their degree of parental care. Both underwent significant evolutionary change during the

dinosaur-bird transition, which included the evolution of brooding. These factors have strong potential to increase the effects of EMA, which is involved in the development of the furcula, sternum, and other distinctive skeletal traits of birds (Hall and Herring 1990; Newman et al. 2013). The origin of each avian trait is often analyzed separately; This may create the impression that they have evolved in modular fashion, as if each trait was selected independently. However, biological systems are often highly integrated, such that changes in one component have consequences for others, either immediately, or in an evolutionary timescale, allowing future changes. The emerging scenario suggests that interrelated behavioral and heterochronic changes played an important role in the origin and early evolution of birds. This is further supported by the continued relevance of the altricial-precocial spectrum in modern birds, which appears to have had far-reaching evolutionary consequences for entire sets of traits (Botelho and Faunes 2015).

In this regard, it is worth researching how EMA can relate to the development of traits beyond those of the musculoskeletal system. For instance, the developing nervous system is related to muscle in an operational feedback through proprioception, so it is likely that EMA plays a role in the development of the central nervous system. Even epidermal traits may be affected by EMA. It has long been known that before hatching, ostriches develop calluses on the skin of their underside. This is commonly accepted as a case of genetic assimilation, since callus formation is usually triggered non-genetically, during post-hatching behavior (Waddington 1953; Gilbert 2000). However, these calluses may require EMA for their formation, rather than being directly induced by genetic-molecular mechanisms.

Much like skin cells have the potential to develop a callus, mesenchymal cells in general will respond to mechanostimulation by initiating *Sox9* expression and the cascade leading to cartilage formation (Takahashi et al. 1998). Conditions leading to increased mechanical stimulation in an embryonic region can thus lead to the formation of neomorphic structures, as illustrated by the fibular crest and sesamoids. It has even been proposed that in early metazoa, the entire skeleton was patterned by mechanical forces, which were subsequently replaced by the molecular-genetic mechanisms that now pattern most of the skeleton (Newman and Müller 2000). This hypothesis may be hard to test, but a similar process of genetic assimilation may be documented by the evolution of sesamoids, as suggested by possible evolutionary transitions from category I to category II sesamoids. In this regard, many category I sesamoids are known to develop at late stages and fully embedded within a tendon, rather than at the surface of an early cartilaginous element. Type II sesamoids are not found within a tendon, but are attached to the tendon of a muscle on their proximal aspect, and to a distal tendon/ligament with no muscle at their distal aspect (such as the patellar tendon, or the pisometacarpal ligament). It is interesting to find out if the precursors of type I sesamoids also show co-expression of *Sox9* and *Scx*, and exactly how types I and II differ in the early development of their associated tendons or ligaments. Common patterns, as well as differences, should be enlightening about hypothesized evolutionary transitions and the variable influence of genetic-molecular mechanisms.

While genetic assimilation and other similar processes are well-documented (West-Eberhard 2003), it is not an inevitable outcome in the evolution of EMA-shaped traits. If nongenetic factors are recurrent and reliably present during development, genetic assimilation may be superfluous; even if it occurs, it may then be easily lost since non-genetic factors would still be available to induce development of the trait. Traits like the fibular crest are so reliably produced and conserved, they are as much an endogenous part of development as any other heritable trait. In contrast, for some EMA-shaped traits, non-genetic factors may not always become available. As a result, their presence may be inconstant, as often occurs with sesamoids. In these cases, molecular-genetic factors can make the difference towards more constant formation, enabling the evolution of functional specialization and commitment around the trait. Another intriguing possibility for future research is that of genetic de-assimilation: namely, that molecular-genetic mechanisms may cease to participate in the development of an EMA-shaped trait. Such a process may have occurred in the evolutionary loss of type II sesamoids. Loss of functional commitment may have allowed decreased genetic influence and inconstant development. If EMA influences are also reduced, the sesamoid may then disappear as an ossified element.

Evolutionary studies of vertebrate structure tend to discuss genes for specific traits, and the selective pressures that may favor them. However, no all traits are directly selectable. Complex developmental mechanisms often intercede between genotype and phenotype, that are key to determine the possibilities of evolutionary change. The fact that research on EMA is not a preferred approach should not be confused with a lack of evolutionary relevance. We

anticipate that a combination of experimental and comparative work, including data from the fossil record, will continue to document the actual evolutionary importance of EMA.

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Supplementary data

Supplementary Data available at *ICB* online.

References

Adachi N, Robinson M, Goolsbee A, Shubin NH. 2016. Regulatory evolution of Tbx5 and the origin of paired appendages. Proc Natl Acad Sci U S A 36:24–8.

Barnett CH, Lewis OJ. 1958. The evolution of some traction epiphyses in birds and mammals. J Anat 92:593–601.

Bell A, Chiappe LM. 2011. Statistical approach for inferring ecology of Mesozoic birds. J Syst Palaeontol 9:119–33.

Bhat R, Lerea KM, Peng H, Kaltner H, Gabius H-J, Newman SA. 2011. A regulatory network of two galectins mediates the earliest steps of avian limb skeletal morphogenesis. BMC Dev Biol 11:6.

Bhullar BS, Hanson M, Fabbri M, Pritchard A, Bever GS, Hoffman E. 2016. 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.

Blanco MJ, Misof BY, Wagner GP. 1998. Heterochronic differences of *Hoxa-11* expression in Xenopus fore- and hind limb development: evidence for lower limb identity of the anuran ankle bones. Dev Genes Evol 208:175–87.

Blitz E, Sharir A, Akiyama H, Zelzer E. 2013. Tendon-bone attachment unit is formed modularly by a distinct pool of *Scx*- and *Sox9*-positive progenitors. Development 140:2680–90.

Botelho JF, Faunes M. 2015. The evolution of developmental modes in the new avian phylogenetic tree. Evol Dev 17:221–3.

Botelho JF, Ossa-Fuentes L, Soto-Acuña S, Smith-Paredes D, Nuñez-León D, Salinas-Saavedra M, Ruiz-Flores M, Vargas AO. 2014. New developmental evidence clarifies the evolution of wrist bones in the dinosaur-bird transition. PLoS Biol 12:e1001957.

Botelho J, Smith-Paredes D, Soto-Acuña S, Mpodozis J, Palma V, Vargas AO. 2015a. Skeletal plasticity in response to embryonic muscular activity underlies the development and evolution of the perching digit of birds. Sci Rep 5:9840.

Botelho JF, Smith-Paredes D, Vargas AO. 2015b. Altriciality and the evolution of toe orientation in birds. Evol Biol 42:502–10.

Brunner C. 1891. Ueber Genese, congenitalen Mangel und rudi- mentäre Bildung der Patella. Virchows Archiv Abteilung a Pathologische Anatomie 124:358–72.

Carey EJ, Zeit W, McGrath BF. 1927. Studies in the dynamics of histogenesis. J Anat 40:127–58.

Chiappe LM, Shu'An J, Qiang J. 2007. Juvenile birds from the early cretaceous of China: implications for enantiornithine ontogeny. Am Mus Nov 3594:1–46.

Colbert EH. 1989. The triassic dinosaur Coelophysis. Mus North Ariz Bull 57:160.

de Munnik SA, Hoefsloot EH, Roukema J, Schoots J, Knoers NV, Brunner HG, Jackson AP, Bongers EM. 2015. Meier–Gorlin syndrome. Orphanet J Rare Dis 10:114.

Diaz RE, Trainor PA. 2015. Hand/foot splitting and the 're-evolution' of mesopodial skeletal elements during the evolution and radiation of chameleons. BMC Evol Biol 15:184.

Drachman DB, Sokoloff L. 1966. The role of movement in embryonic joint development. Dev Biol 14:401–20.

Dreyer SD, Zhou G, Baldini A, Winterpacht A, Zabel B, Cole W, Johnson RL, Lee B. 1998. Mutations in *LMX1B* cause abnormal skeletal patterning and renal dysplasia in nail patella syndrome. Nat Genet 19:47–50.

Elzanowski A. 1981. Embryonic bird skeletons from the Late Cretaceous of Mongolia. Paleontol Pol 42:147–76.

Eyal S, Blitz E, Shwartz Y, Akiyama H, Schweitzer R, Zelzer E. 2015. On the development of the patella. Development 142:1831–9.

George JC, Berger AJ. 1966. Avian myology. New York (NY): Academic Press.

Gilbert SF. 2000. Diachronic biology meets Evo-Devo: C.H. Waddington's Approach to Evolutionary Developmental Biology. Amer Zool 40:729–37.

Haines RW. 1969. Epiphyses and sesamoids. In: Gans C, editor. Biology of reptilia. New York (NY): Academic Press. p. 81–115.

Hall BK, Herring SW. 1990. Paralysis and growth of the musculoskeletal system in the embryonic chick. J Morphol 206:45–56.

Hosseini A, Hogg DA. 1991. The effects of paralysis on skeletal development in the chick embryo. I. General effects. J Anat 177:159–68.

Hutchinson JR. 2002. The evolution of hindlimb tendons and muscles on the line to crown-group birds. Comp Biochem Physiol A Mol Integr Physiol 133:1051–86.

Kurochkin EN, Chatterjee S, Mikhailov KE. 2013. An embryonic enantiornithine bird and associated eggs from the Cretaceous of Mongolia. Paleontol J 47:1252–69.

Meyers RA, Stakebake EF. 2005. Anatomy and histochemistry of spread-wing posture in birds. 3. Immunohistochemistry of flight muscles and the "shoulder lock" in albatrosses. J Morphol 263:12–29.

Middleton KM. 2001. The morphological basis of hallucal orientation in extant birds. J Morphol 250:51–60.

Mitchell JS, Makovicky PJ. 2014. Low ecological disparity in Early Cretaceous birds. Proc Biol Sci 281:20140608.

Müller GB. 2003. Embryonic motility: environmental influences and evolutionary innovation. Evol Dev 5:56–60.

Müller GB, Streicher L. 1989. Ontogeny of the syndesmosis tibiofibularis and the evolution of the bird hindlimb: a caenogenetic feature triggers phenotypic novelty. Anat Embryol (Berl) 179:327–39.

Murray PDF, Huxlay JS. 1925. Self-differentiation in the grafted limb-bud of the chick. J Anat 59:379–84.

Nakamura T, Gehrke AR, Lemberg J, Szymaszek J, Shubin NH. 2016. Digits and fin rays share common developmental histories. Nature 537:225–8.

Newman SA, Mezentseva NV, Badyaev AV. 2013. Gene loss, thermogenesis, and the origin of birds. Ann N Y Acad Sci 1289:36–47.

Newman SA, Müller GB. 2000. Epigenetic mechanisms of character origination. J Exp Zool 288:304–17.

Niven JSF. 1933. The development in vivo and in vitro of the avian patella. Wilehm Roux Arch Dev Biol 128:480–501.

Nowlan NC, Sharpe J, Roddy KA, Prendergast PJ, Murphy P. 2010. Mechanobiology of embryonic skeletal development: Insights from animal models. Birth Defects Res C Embryo Today 90:203–13.

Oppenheim RW, Levin HL. 1975. Short-term changes in incubation temperature: behavioral and physiological effects in the chick embryo from 6 to 20 days. Dev Psychobiol 8:103–15.

Parsons FG. 1904. Observations on traction epiphyses. J Anat Lond 38:248–58.

Pollard AS, Boyd S, McGonnell IM, Pitsillides AA. 2016. The role of embryo movement in the development of the furcula. J Anat 230:435–43.

Pollard AS, Charlton BG, Hutchinson JR, Gustafsson T, McGonnell IM, Timmons JA, Pitsillides AA. 2017. Limb proportions show developmental plasticity in response to embryo movement. Sci Rep 7:41926.

Raspopovic J, Marcon L, Russo L, Sharpe J. 2014. Digit patterning is controlled by a Bmp-*Sox9*–*Wnt* Turing network modulated by morphogen gradients. Science 345:566–70.

Reno PL, Kjosness KM, Hines JE. 2016. The role of *Hox* in pisiform and calcaneus growth plate formation and the nature of the zeugopod/autopod boundary. J Exp Zool B Mol Dev Evol 326:303–21.

Reynolds SH. 1897. The Vertebrate Skeleton. Cambridge: University Press. p. 1–559.

Samuels ME, Regnault S, Hutchinson JR. 2017. Evolution of the patellar sesamoid bone in mammals. Peer J 0–53.

Sarin VK, Erickson GM, Giori NJ, Bergman AB, Carter DR. 1999. Coincident development of sesamoid bones and clues to their evolution. Anat Rec 257:174–80.

Shufeldt RW. 1884. Concerning some forms assumed by the patella in birds. Proc U S Nat Mus 7:324–31.

Spring LW. 1965. Climbing and pecking adaptations in some North American woodpeckers. Condor 67:457–88.

Stolpe M, Zimmer K. 1939. Der Schwirrflug des Kolibri im Zeitlupenfilm. J Ornithol 87:136–55.

Takahashi I, Nuckolls GH, Takahashi K, Tanaka O, Semba I, Dashner R, Shum L, Slavkin HC. 1998. Compressive force promotes *Sox9*, type II collagen and aggrecan and inhibits IL-1beta expression resulting in chondrogenesis in mouse embryonic limb bud mesenchymal cells. J Cell Sci 111:2067–76.

Vargas AO, Kohlsdorf T, Fallon JF, VandenBrooks J, Wagner GP. 2008. The evolution of *HoxD-11* expression in the bird wing: insights from alligator *mississippiensis*. PLoS One 3:e3325.

Varricchio DJ, Moore JR, Erickson GM, Norell MA, Jackson FD, Borkowski JJ. 2008. Avian paternal care had dinosaur origin. Science 322:1826–8.

Vazquez RJ. 1992. Functional osteology of the avian wrist and the evolution of flapping flight. J Morphol 211:259–68.

Vickaryous MK, Olson WM. 2007. Sesamoids and ossicles in the appendicular skeleton. In: Hall BK, editor. Fins into limbs: evolution, development and transformation. Chicago (IL): Chicago University Press. p. 323–41.

Waddington CH. 1953. Experiments in acquired characteristics. Sci Am 189:92–9.

Welles SP. 1984. Dilophosaurus wetherilli (Dinosauria, Theropoda). Osteology and comparisons. Palaeontographica Abteilung A 185:85–180.

West-Eberhard MJ. 2003. Developmental plasticity and evolution. New York (NY): Oxford University Press.

Yokouchi Y, Sasaki H, Kuroiwa A. 1991. Homeobox gene expression correlated with the bifurcation process of limb cartilage development. Nature 353:443–5.

Zhou Z, Zhang F. 2004. A precocial avian embryo from the Lower Cretaceous of China. Science 306:653.

Zusi RL. 2013. Introduction to the skeleton of hummingbirds (Aves: Apodiformes, Trochilidae) in functional and phylogenetic contexts. Ornithol Monogr 77:1–94.