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# Skeletogenesis of *Myiopsitta monachus* (Psittaciformes) and sequence heterochronies in Aves

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**SUMMARY** The ossification sequence of *Myiopsitta monachus* was determined. *Myiopsitta* has a similar sequence to other altricial birds, with delayed skeletons compared to precocial species. The hindlimbs ossify before the forelimbs, a condition that could be linked to altriciality. To determine the stability of the sequences of ossification across birds, we selected species of different groups of Aves and used event-pairing method and character mapping on a phylogeny. Our results show that the homogeneity in the development of birds was supported by 56.77% of the character states. Event-pair cracking phylogenetic method was applied to identify sequence heterochronies. Results reveal a high number of heterochronies and show that the long bones in limbs may behave as modules. In *Myiopsitta*, the ossa ectethmoidale and mesethmoidale ossify early. These bones provide the origin site of the Psittaciformes' novel adductor m. ethmomandibularis, associated with strong bite forces, and its acceleration in the sequence may correspond to the functional hypothesis. Also, the early appearance of some hyoid apparatus elements occurs, and could be related to the development of tongue in Psittaciformes and its role in handling food, and is in concordance with the functional and size hypothesis.

## INTRODUCTION

Embryonic development is a continuous process of morphological changes that can be treated as a series of discrete developmental events (Bininda-Emonds et al. 2002). In a comparative context, heterochrony involves changes in time or event rates and/or developmental processes underlying the formation of the morphological characters, and it is the most common evolutionary developmental reprogramming process (also including heterometry, heterotopy, and heterotypy; Arthur 2000, 2004). This makes the study of heterochronies essential in evolutionary developmental biology (Evo-Devo), which seeks to identify the developmental mechanisms that cause evolutionary changes in phenotypes (Hall 2003). Heterochonies can provide insight into the changes associated with shape and size of features (known as grow heterochrony), or for seeking changes in the order of occurrence of the events between taxa (known as sequence heterochrony) (Reilly et al. 1997; Smith 2001; Bininda-Emonds et al. 2002).

During the last decade, advances in analytical methods now allow for explicit phylogenetic analyses of heterochrony sequences involving many types of events (cellular, molecular, genetic, and morphological) (Bininda-Emonds et al. 2002; Smith 2003). Ossification sequences of birds are little known, even when the information they provide could be useful in the determination of homologies, phylogenetic relationships, identification of taxa, or heterochronies (Feduccia and Nowicki 2002; Maisano 2002a,b; Maxwell 2008a,b,c; Maxwell and Larsson 2009; Mitgutsch et al. 2011). In addition, ossification in fossil embryos enables identification of morphological patterns in extinct groups (Organ et al. 2015). Available information is mainly focused on the chicken and quail, because they are considered as model species and because their commercial importance (Maxwell 2008b), but information is also available for a few species orders such as Paleognathae, Galliformes, Anseriformes, Charadriiformes, and Passeriformes (e.g., Nakane and Tsudzuki 1999; Maxwell 2008a,b,c, 2009; Maxwell and Harrison 2008; Atalgin and Kürtül 2009; Maxwell and Larsson 2009; Maxwell et al. 2010; Mitgutsch et al. 2011).

Until now, there has not been a comprehensive study of the relative timing of ossification of skeletal structural units in a Psittaciformes species. The parrots are one of the most homogeneous avian orders, and are characterized by their colorful feathers, robust and recurved beaks, zygodactyl feet,

advanced cognitive abilities, highly developed locomotor system and feeding behaviors, and by their altriciality. The only information previously available of any Psittaciformes is the skeletogenesis of the cranio–mandibular complex of the budgerigar *Melopsittacus undulatus* (Tokita 2003). The embryological developmental stages of the monk parakeet *Myiopsitta monachus* have been recently described (Carril and Tambussi 2015), providing useful information to evaluate sequence heterochrony in a comparative context with other birds. Our aim here is to determine the complete ossification sequence of *M. monachus* and to compare and contrast with observations of other birds available in the literature. To the best of our knowledge, this is the first thorough investigation dedicated to establishing the ossification sequence of the entire skeleton for any Psittaciformes species. We also aim to recognize sequence heterochronies among some of the major clades of Aves for the first time in birds using the event-pair cracking phylogenetic method, and to compare skeletogenesis patterns between altricial and precocial birds.

## MATERIALS AND METHODS

### Specimens and ossification sequence

The monk parakeet was selected because it is one of the few Neotropical parrots for which capture is not restrictioned, due to high abundance and its status as a pest species (Canavelli et al. 2013). These aspects facilitated sampling and allowed us to obtain sufficient individual samples for this study.

A total of 63 specimens of *M. monachus* from La Plata (Buenos Aires) and Dean Funes (Córdoba) Argentina, were obtained from nests during breeding seasons from 2011 to 2013. Individuals were sacrificed according to protocols approved by the animal care committee and adhering to the legal requirements of Argentina. Samples were fixed by immersion in 4% formaldehyde solution for 48 h and preserved in 70% alcohol. The assignment of embryonic stages (34 to 40+) was performed based on descriptions of Carril and Tambussi (2015) and the nestling ages (from newly hatched to +21 days old) was calculated using the equation of the length of the hindlimbs' digit III, as proposed by Aramburú (1997).

We used a staining procedure (Dingerkus and Uhler 1977) to visualize and identify the condition of skeletons in embryos of different stages and in nestlings at different ages. This technique differentially stains cartilage with alcian blue and bone with alizarin red, and digests soft tissues with the enzyme trypsin. For permanent preservation, specimens were immersed in glycerine. Each specimen was observed under binocular microscope Leica S6D and images were acquired with a Nikon D-40 digital camera.

The skeletogenesis description was performed for each developmental stage and pooled by the age of the nestlings, and by anatomical region (i.e., skull, postcranial axial skeleton, and pectoral and pelvic appendages). The first occurrence of ossification was recorded based on the presence of osteoclast spicules that appear as white on bone matrix because they do not have enough calcium to be stained by alizarin red (Maxwell 2008b). The osteological nomenclature follows Baumel and Witmer (1993).

### Event-pairing and event pair cracking methods

To better understand how monk parakeet ossification sequences compare to other bird species, we used the ossification sequences for 86 skeletal elements (both cranial and postcranial) for seven additional avian species for which sequences are known. We chose representative species of major bird lineages with different style of development (precocial to altricial) and provide the first attempt at identifying heterochronies in a phylogenetic context. Unfortunately, information on ossification sequences is extremely scarce, and when it exists, is heterogeneous, with different resolution levels, which makes this kind of comparison difficult.

Our analysis included data from previously published sequences of Palaeognathae (Ratite + Tinamide), Galloanserae and Neognathae: *Rhea americana* and *Eudromia elegans* (Maxwell 2009; Maxwell and Larsson 2009) as representatives of the Palaeognathae, *Anas platyrhynchos* as representative of the Anseriformes (Mitgutsch et al. 2011), *Gallus gallus* (Maxwell 2008b) as representative of the Galliformes, *Sterna hirundo* (Maxwell and Harrison 2008) as representative of the Charadriiformes, *Melopsittacus undulatus* (Tokita 2003) as representative of the Psittaciformes (in addition to our focal species in this study), and *Taeniopygia guttata* as representative of the Passeriformes (Mitgutsch et al. 2011). As mentioned, information about ossification sequences is disparate and varies widely, but each of the sequences selected here exhibits important data or in some cases uniquely available information, that deserve to be considered for this study. For example, the only Psittaciformes from which development has been previously described is *M. undulatus*, but information is restricted to the skull and hyoid apparatus. Also, *T. guttata* is the only altricial species which a complete ossification sequence has been published to date.

In order to identify changes in the ossification sequences and to establish the degree of sequence conservation in a phylogenetic context, the event-pairing method was used (Mabee and Trendler 1996; Smith 1996, 1997, 2001; Velhagen 1997; Jeffery et al. 2002, 2005). This method allows the codification of the sequence of development as an all-pairs comparison of the timing of each element relative to every other element (Harrington et al. 2013). The event-pairing method was proposed in the 1990s for the study of sequence heterochronies in the embryonic development of vertebrates (Mabee and Trendler 1996; Smith 1997; Velhagen 1997) and involves the dissociation of the development sequence into individual "events" (i.e., bone ossification). The "event pairs" are coded as: "0" if the event A occurs before the event B, "1" if the event A occurs at the same time as the event B, and "2" if the event A occurs after the event B. During development, it is

unlikely that events occur at exactly the same time and be assigned with the number 1. However, continuous sampling is difficult to carry out in practice, so the code "1" is the representation of a combination of unresolved pairs, where the sampling interval was long in relation to the real rate of development (Smith 1997).

Typically these events are represented in an event-pair matrix (Smith 1997, 2001, 2003), which ultimately it is a way to visualize the temporal relationships between events. The resulting event-pairs characters can then be mapped onto a known phylogenetic topology (Smith 1996, 1997; Maisano 2002b; Sánchez-Villagra 2002). Phylogenetic tree of species sampled in this study was taken from Jarvis et al. (2014) based on genomic data. Parsimony (characters states unordered) and Maximum Likelihood (Markov-K-state1, with equal probability for any particular character change) approaches were applied using MESQUITE package Version 3.02 (Maddison and Maddison 2015). Thus, those preserved characters, those characters with a defined pattern within a clade, and those characters with heterogeneous distribution can be identify (Jeffery et al. 2002). The advantages of this method includes the standardization of development regardless time or age of the organisms, and the comparison of all types of events (in addition to the size and shape) that can be analyzed as a whole and among several taxa at the same time (Smith 2001).

Finally, in order to identify sequence heterochronies, we reconstructed the artificial ancestral nodes' ossification sequences and compared ancestor and descendant sequences using the event-pair cracking method proposed by Jeffery et al. (2002). Heterochronies are recognized when the sequence position of an event changes relative to other events when comparing a taxa with the ancestral condition (Smith 1997, 2001). This method allows us to determine the direction and magnitude of changes of events and to identify coherent and significant synapomorphic changes.

Even though the reconstruction of the ancestral ossification sequence for Aves requires a non-avian outgroup which provide sufficient polarization at the base of the phylogeny, it has been postulated that the use of crocodilians (the only other living group of archosaurs) is inadvisable and may confound the analysis as they greatly differ temporally, morphologically, and developmentally from birds (Maxwell et al. 2010). Due to these considerations, we restricted our analysis to bird species.

First, the character states of the ancestral nodes resulting from the mapping are re-coded and the sign "?" is assigned to those characters with more than one possible character state (0, 1, and/or 2), and to those characters previously assigned with the number 1. A numerical value to each new character state of the ancestral nodes is assigned: to the state "0" a score 1, to the state "?" a score 0.5, and to the state "2" a score 0. For each event, the scores of all event-pair in which that event participates are added, and then the events are ordered from the highest to the lowest and the ancestral ossification sequence is determined. Second, an event-pair matrix for each ancestral node is performed, and event-pairs are compared between the ancestral nodes and their descendant. This comparison involves calculating the relative change for each character (RC = ancestral node state - descendant node state, multiplied by $-1$), where if RC = 0 there is no change between nodes, and if RC $\neq$ 0 there is change between nodes. The changes are selected and re-coded: positive RC's (1 and 2) with a value "+1", and negative RC's ($-1$ and $-2$) with a value "$-1$". From the RC's, the total relative change (TRC) is calculated as the sum of the states in which an event participated as part of a row less the sum of the states in which an event participated as part of a column. A positive TRC indicates that the change has been to later stages of the sequence, a TRC = 0 indicates that there was no change, and a negative TRC indicates that the change has been to early stages of the sequence. Third, the total absolute change (TAC) is calculated in the same manner as the TRC but using the absolute values. To identify those events actively moving in the sequence, the median of the TAC was calculated and the highest values were selected. Then are calculated the TRC and the TAC adjusted when discarded changes that are involved in another selected event and events moving apparently (called "*hitchhikers*", TAC = 0). Here the direction and magnitude of heterochronic changes are identified. Finally, the coherence of the movement is calculated with the index "J", where if J = $-1$ the event has moved at early stages of the sequence (acceleration), and if J = 1 the event has moved to late stages of the sequence (delay).

## RESULTS

### Skeletogenesis of *Myiopsitta monachus*

Every ossification event of all the 86 bony elements discriminated by anatomical region of *M. monachus* was designate with a stage number, in order of occurrence in the sequence of ossification for each state of development *in ovo* embryos or after birth. All this information is condensed in Table 1. Figures 1–3 show photos and schemes of *M. monachus* at different embryonic stages and posthatching ages with different skeletal elements present.

#### *Stage 34*

Ossification of the first elements begins. The neurocranium consisted of several cartilages, including the auditory capsules, the lamina orbitonasalis, and the ventrally oriented prenasal processes (Fig. 3A). The cartilaginous quadrate articulated with the mandible, and the Meckel's cartilages were elongated and linked rostrally (Fig. 3B). All cartilaginous elements of the hyoid apparatus were observed (Fig. 3C). In the postcranial axial skeleton, most cartilaginous elements were present. The first

**Table 1. List of elements sorted according to anatomical region, indicating the event number assigned for each bone, the rank order of element ossification, and the embryonic stage or posthatching age in days where the elements ossify for *M. monachus***

| Element | No. of event | Rank | Stage | Age |
|---|---|---|---|---|
| Skull | | | | |
| Os basioccipitale | 1 | 22 | 40 | |
| Os exoccipitale | 2 | 21 | 40 | |
| Os supraoccipitale | 3 | 31 | | 0–5 |
| Rostrum parasphenoidale | 4 | 11 | 36 | |
| Ala parasphenoidalis | 5 | 14 | 36 | |
| Lamina basiparasphenoidalis | 6 | 14 | 36 | |
| Os laterosphenoidale | 7 | 32 | | 0–5 |
| Ossa otica | 8 | 12 | 36 | |
| Os squamosum | 9 | 7 | 35 | |
| Os parietale | 10 | 25 | 40+ | |
| Os frontale | 11 | 18 | 38 | |
| Os lacrimale | 12 | 12 | 36 | |
| Os ectethmoidale | 13 | 36 | | 6 |
| Os mesethmoidale | 14 | 34 | | 0–5 |
| Os nasale | 15 | 8 | 35 | |
| Os premaxillare | 16 | 8 | 35 | |
| Os maxillare | 17 | 7 | 35 | |
| Os palatinum | 18 | 7 | 35 | |
| Os pterygoideum | 19 | 6 | 35 | |
| Os jugale | 20 | 6 | 35 | |
| Os quadratojugale | 21 | 6 | 35 | |
| Os quadratum | 22 | 16 | 38 | |
| Os dentale | 23 | 3 | 35 | |
| Os supra-angulare | 24 | 3 | 35 | |
| Os angulare | 25 | 3 | 35 | |
| Os spleniale | 26 | 3 | 35 | |
| Os prearticulare | 27 | 4 | 35 | |
| Os articulare | 28 | 34 | | 0–5 |
| Paraglossum | 29 | 20 | 40 | |
| Basihyale | 30 | 34 | | 0–5 |
| Urohyale | 31 | 34 | | 0–5 |
| Ceratobranchiale | 32 | 10 | 36 | |
| Epibranchiale | 33 | 34 | | 0–5 |
| Postcranial axial skeleton | | | | |
| Vertebrae cervicales (corpus) | 34 | 26 | 40+ | |
| Vertebrae thoracicae (corpus) | 35 | 28 | 40+ | |
| Vertebrae synsacrales (corpus) | 36 | 30 | 40+ | |
| Vertebrae caudales (corpus) | 37 | 30 | 40+ | |
| Pygostylus | 38 | 37 | | 9 |
| Vertebrae cervicales (arcus) | 39 | 26 | 40+ | |
| Vertebrae thoracicae (proc. transversus) | 40 | 30 | 40+ | |
| Vertebrae synsacrales (proc. transversus) | 41 | 30 | 40+ | |
| Vertebrae caudales (arcus) | 42 | 33 | | 0–5 |
| Vertebrae synsacrales (arcus) | 43 | 32 | | 0–5 |
| Costa vertebralis | 44 | 13 | 36 | |
| Costa sternalis | 45 | 34 | | 0–5 |
| Processus uncinatus | 46 | 34 | | 0–5 |
| Pectoral appendege | | | | |
| Sternum | 47 | 39 | | 21 |
| Scapula | 48 | 9 | 36 | |
| Os coracoideum | 49 | 9 | 36 | |
| Clavicula | 50 | 9 | 36 | |
| Humerus | 51 | 1 | 34 | |
| Radius | 52 | 1 | 34 | |
| Ulna | 53 | 1 | 34 | |
| Os carpi radiale | 54 | 38 | | 21 |
| Os carpi ulnare | 55 | 38 | | 21 |
| Os metacarpale alulare (II) | 56 | 35 | | 0–5 |
| Phalanx digiti alulae | 57 | 24 | 40+ | |
| Os metacarpale majus (III) | 58 | 4 | 35 | |
| Phalanx proximalis digiti majoris | 59 | 17 | 38 | |
| Phalanx distalis digiti majoris | 60 | 19 | 38 | |
| Os metacarpale minus (IV) | 61 | 5 | 35 | |
| Phalanx digiti minoris | 62 | 35 | | 0–5 |
| Pelvic appendage | | | | |
| Ilium | 63 | 15 | 36 | |
| Ischium | 64 | 15 | 36 | |
| Pubis | 65 | 15 | 36 | |
| Femur | 66 | 1 | 34 | |
| Tibiotarsus | 67 | 1 | 34 | |
| Fibula | 68 | 1 | 34 | |
| Os metatarsale I | 69 | 27 | 40+ | |
| Phalanx I | 70 | 29 | 40+ | |
| Phalanx ungualis | 71 | 29 | 40+ | |
| Os metatarsale II | 72 | 2 | 34 | |
| Phalanx I | 73 | 29 | 40+ | |
| Phalanx II | 74 | 29 | 40+ | |
| Phalanx ungualis | 75 | 29 | 40+ | |
| Os metatarsale III | 76 | 2 | 34 | |
| Phalanx I | 77 | 28 | 40+ | |
| Phalanx II | 78 | 29 | 40+ | |
| Phalanx III | 79 | 30 | 40+ | |
| Phalanx ungualis | 80 | 29 | 40+ | |
| Os metatarsale IV | 81 | 2 | 34 | |
| Phalanx I | 82 | 23 | 40 | |
| Phalanx II | 83 | 35 | | 0–5 |
| Phalanx III | 84 | 35 | | 0–5 |
| Phalanx IV | 85 | 30 | 40+ | |
| Phalanx ungualis | 86 | 29 | 40+ | |

elements to ossify were those of the stylopod and the zeugopod of both limbs, whose ossification centers are found in their middle portions (Figs. 3G and I). Later in this stage, the ossa metatarsale II, III, and IV ossified, and the ossification centers were also observed in their middle portions (Fig. 3I).

### *Stage 35*

Almost all elements of the mandible began to ossify around Meckel's cartilage (i.e., os dentale, os supra-angulare, os

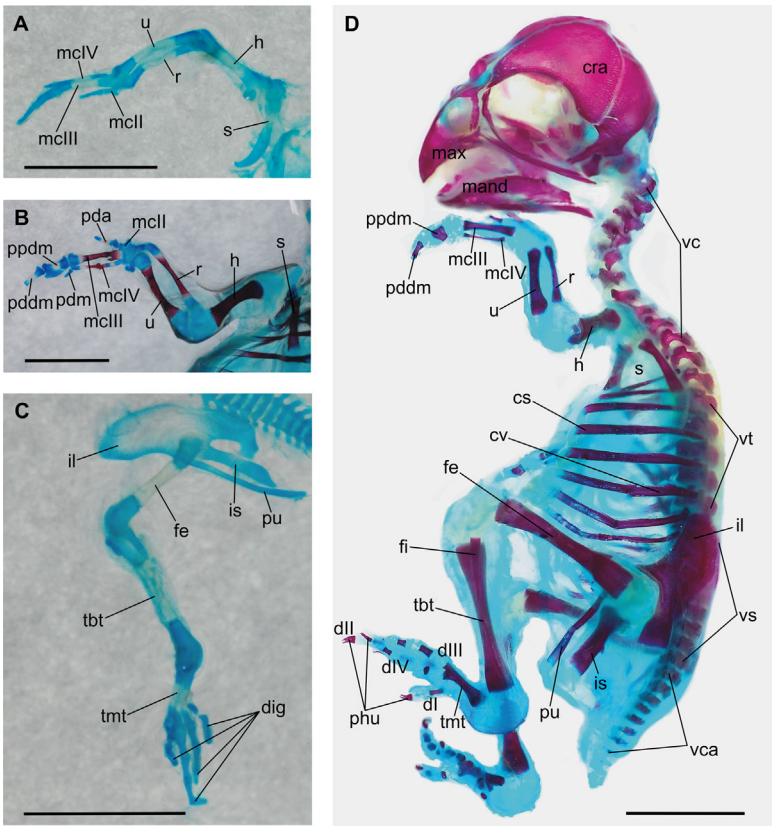


**Fig. 1.** Specimens of *M. monachus* double stained and cleared in left lateral view. A, forelimb of an embryo at stage 36; B, forelimb of a nestling of 0–5 days old; C, pelvic girdle and hindlimb of an embryo at stage 36; D, nestling of 0–5 days old. References: cra, ossa cranii; cs, costa sternalis; cv, costa vertebralis; dI, digit I; dII, digit II; dIII, digit III; dIV, digit IV; dig, digits; fe, femur; fi, fibula; h, humerus; il, ilium; is, ischium; mand, ossa mandibulae; max, ossa maxillae et palati; mcII, os metacarpale alulare (II); mcIII, os metacarpale majus (III); mcIV, os metacarpale minus (IV); pda, phalanx digiti alulae; pddm, phalanx distalis digiti majoris; pdm, phalanx digiti minoris; phu, phalanx ungualis; ppdm, phalanx proximalis digiti majoris; pu, pubis; r, radius; s, scapula; tbt, tibiotarsus; tmt, ossa metatarsale; u, ulna; vc, vertebrae cervicales; vca, vertebrae caudales; vt, vertebrae thoracicae; vs, vertebrae synsacrales. Scale = 1 cm.

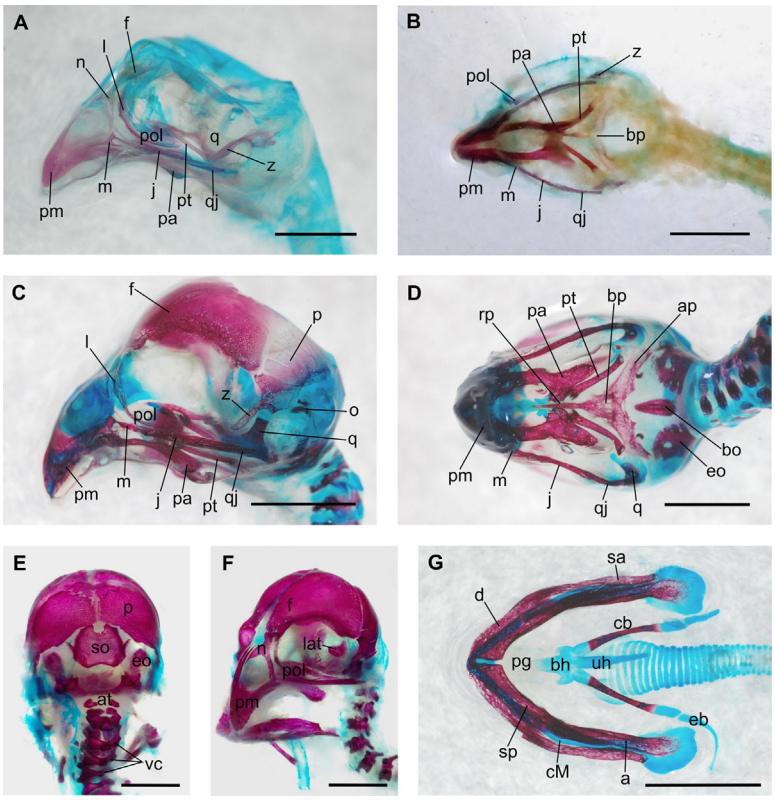


**Fig. 2.** Specimens of *M. monachus* double stained and cleared. A, Lateral left view of a skull of an embryo at stage 38; B, ventral view of a skull of an embryo at stage 36; C, lateral left view of a skull of a nestling of 0–5 days old; D, ventral view of a skull of a nestling of 0–5 days old; E, caudal view of a skull of a nestling of 0–5 days old; F, rostro-lateral view of a skull of a nestling of 0–5 days old; G, ventral view of a mandible and hyoid apparatus of a nestling of 0–5 days old. References: a, os angulare; ap, ala parasphenoidalis; at, atlas; bh, basihyale; bo, os basioccipitale; bp, lamina basiparasphenoidalis; cb, ceratobranchiale; cM, Meckel's cartilage; d, os dentale; eb, epibranchial; eo, os exoccipitale; f, os frontale; j, os jugale; l, os lacrimale; lat, os laterosphenoidale; m, os maxillare; n, os nasale; o, ossa otica; p, os parietale; pa, os palatinum; pg, paraglossum; pm, os premaxillare; pol, processus orbitalis of the os lacrimale; pt, os pterygoideum; q, os quadratum; qj, os quadratojugale; rp, rostrum parasphenoidale; sa, os supra-angulare; so, os supra-occipitale; sp, os spleniale; uh, urohyale; vc, vertebrae cervicales; z, processus zygomaticus of the os squamosum. Scale = 5 mm.

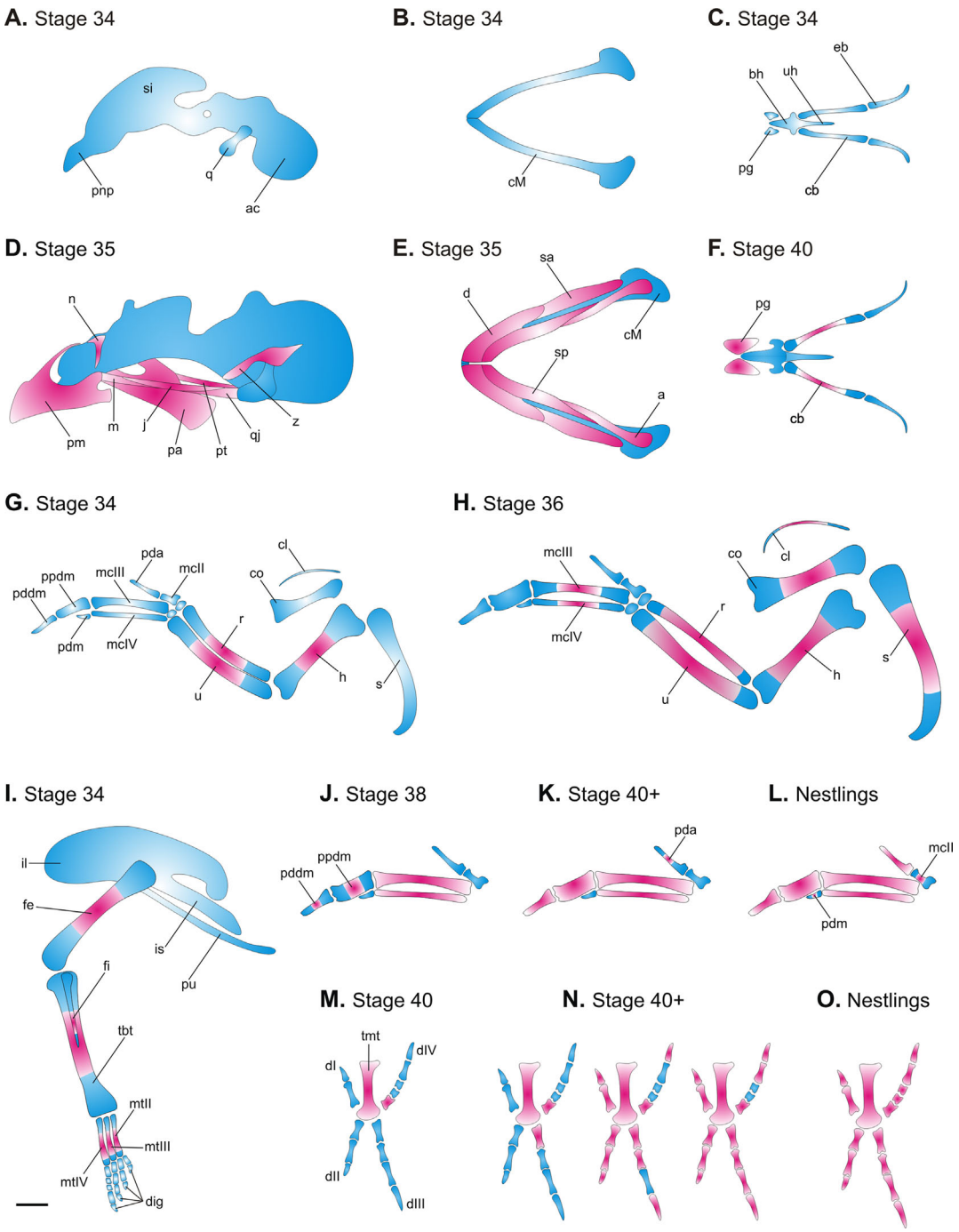


**Fig. 3.** Schemes of the skeletal regions of *M. monachus* showing cartilages (in blue) and bones (in red) present at different embryonic stages and nestlings. A, D, G–L, left lateral view; B, C, E, F, ventral view; M–O, dorsal view. A and D, Skull; B and E, mandible; C and F, hyoid apparatus; G and H, forelimbs; I, hindlimb; J–L, forelimb distal elements; M-O, hindlimb distal elements. References: a, os angulare; ac, auditory capsule; bh, basihyale; cb, ceratobranchiale; cl, clavicula; cM, Meckel's cartilage; co, os coracoideum; d, os dentale; dI, digit I; dII, digit II; dIII, digit III; dIV, digit IV; dig, digits; eb, epibranchiale; fe, femur; fi, fibula; h, humerus; il, ilium; is, ischium; j, os jugale; m, os maxillare; mcII, os metacarpale alulare (II); mcIII, os metacarpale majus (III); mcIV, os metacarpale minus (IV); mtII, os metatarsale II; mtIII, os metatarsale III; mtIV, os metatarsale IV; n, os nasale; pa, os palatinum; pda, phalanx digiti alulae; pddm, phalanx distalis digiti majoris; pdm, phalanx digiti minoris; pg, paraglossum; pm, os premaxillare; pnp, prenasal process; ppdm, phalanx proximalis digiti majoris; pt, os pterygoideum; pu, pubis; q, os quadratum; qj, os quadratojugale; r, radius; s, scapula; sa, os supra-angulare; si, septum inteorbitale; sp, os spleniale; tbt, tibiotarsus; tmt, ossa metatarsale; u, ulna; uh, urohyale; z, processus zygomaticus of the os squamosum. Scale = 1 mm.

angulare, and os spleniale; Fig. 3E). The os prearticulare was present in most analyzed embryos. Later in this stage, the first element to ossify in the forelimbs was the os metacarpale majus (III), followed by the os metacarpale minus (IV). Toward the end of this stage, the elements of the skull base began to ossify: first the os pterygoideum, os jugale, and os quadratojugale; then the os squamosum, os maxillare, and os palatinum; and finally the os nasale and os premaxillare (Fig. 3D). The ossification center of the os squamosum was located in the processus zygomaticus.

#### *Stage 36*

The elements of the pectoral girdle began to ossify at this stage (i.e., the scapula, os coracoideum, and clavicula, with ossification centers at their middle portions; Figs. 1A and 3H). In the hyoid apparatus, the ceratobranchiale ossified. In the skull base, the first element to ossify was the rostrum parasphenoidale, followed by the lamina basiparasphenoidalis, the ala parasphenoidalis, the ossa otica, and os lacrimale (Fig. 2B). The ossification center of the os lacrimale was located in the processus orbitalis, which was elongated and ventrally surrounded the orbit. In the postcranial axial skeleton, ossification centers were observed in the middle portion of the costa vertebralis. Later in this stage, the elements of the pelvic girdle ossified (i.e., the ilium, with a preacetabular ossification center, the ischium, and pubis; Fig. 1C).

#### *Stage 38*

In the skull, the first element to ossify is the os quadratum, and then the os frontale with its ossification center at the upper margin of the orbit (Fig. 2A). In the forelimbs, the first bone to ossify is the phalanx proximalis digiti majoris, and later the phalanx distalis digiti majoris (Fig. 3J).

#### *Stage 40*

Initially, the paraglossum in the hyoid apparatus ossified (Fig. 3F). In the skull, the first element to ossify is the os exoccipitale, followed by the os basioccipitale. In the hindlimbs, the phalanx I of the digit IV ossified (Fig. 3M).

#### *Stage 40+*

In the skull, the os parietale ossified. In the postcranial axial skeleton, the vertebrae ossified in an anterior to posterior direction: the corpora and arci of the vertebrae cervicales ossified first (although the atlas ossified after the vertebrae cervicales), the corpora of the vertebrae thoracicae ossified secondy, and finally the corpora of the vertebrae synsacrales and vertebrae caudales, together with the processus transversus of the vertebrae thoracicae and vertebrae synsacrales, ossified last. In the forelimbs, the phalanx digiti alulae (I) ossified (Fig. 3K). In the hindlimbs, the os metatarsale I ossified. Regarding the digits of the hindlimbs, the first phalanxes to ossify are the phalanx I of the digit III, then all the phalanxes of the digits I and II together with the phalanx II and phalanx ungualis of the digit III and the phalanx ungualis of the digit IV, and finally the phalanx III of the digit III together with the phalanx IV of the digit IV (Fig. 3N).

#### *Nestlings (Fig. 1D)*

Skull bones expand their areas of ossification. In the first 0–5 days posthatching, the os supraoccipitale, os laterosphenoidale, and os mesethmoidale ossified, and at 6 days after hatching, the os ectethmoidale ossified (Figs. 2C–F). In the mandible, the os articulare ossified. In the hyoid apparatus, the basihyale, urohyale, and epibranchiale ossified during the first 0–5 days after hatching. In the postcranial axial skeleton, the first elements to ossify are the arci of the vertebrae synsacrales and vertebrae caudales, followed by ossification of the costa sternalis and the processus uncinatus. Finally, the pygostylus ossified at 9 days after hatching. In the forelimbs, the os metacarpale alulare (II) and the phalanx digiti minoris ossified in the first 0–5 days after hatching (Figs. 1B and 3L), and the last elements to ossify are the ossa carpi radiale and ulnare at 21 days old. In the hindlimbs, the last phalanxes to ossify are the phalanxes II and III of the digit IV in the first 0–5 days after hatching (Fig. 3O). The latest element to ossify of the entire sequence is the sternum, which occurred at 25 days after hatching.

## Comparison of ossification sequences among Aves: conservative or changeable?

We used 3655 characters to construct the event-pair matrix (Table S1).

Characters mapping onto the phylogeny showed 56.77% of character states with homogeneous distribution, 21.81% of character reversions of terminal taxa including 30 autapomorphies for *M. monachus* (Table S2), and 9.66% of character states showed heterogeneous distribution. These patterns represented 88.24% of non-informative characters states. The characters states with a defined pattern within a clade are informative, and represent the remaining 11.76% of states. These include 188 states for Paleognathae, 23 for Neognathae, 109 for Galloanseres, 28 for Neoaves, 72 for Passerimorphae, and 10 for Psittaciformes (Table S3).

Figure 4 shows sequence heterochronies within Aves. These include several delays and accelerations in the ossification sequence of different nodes compared to their ancestors. We found evidence for: (i) nine delays and four accelerations in Paleognathae; (ii) 20 delays and 18 accelerations in *R. americana* and 18 delays and 13 accelerations in *E. elegans*, both species share the delay of limbs' elements and the acceleration of several skull bones; (iii) 13 delays and

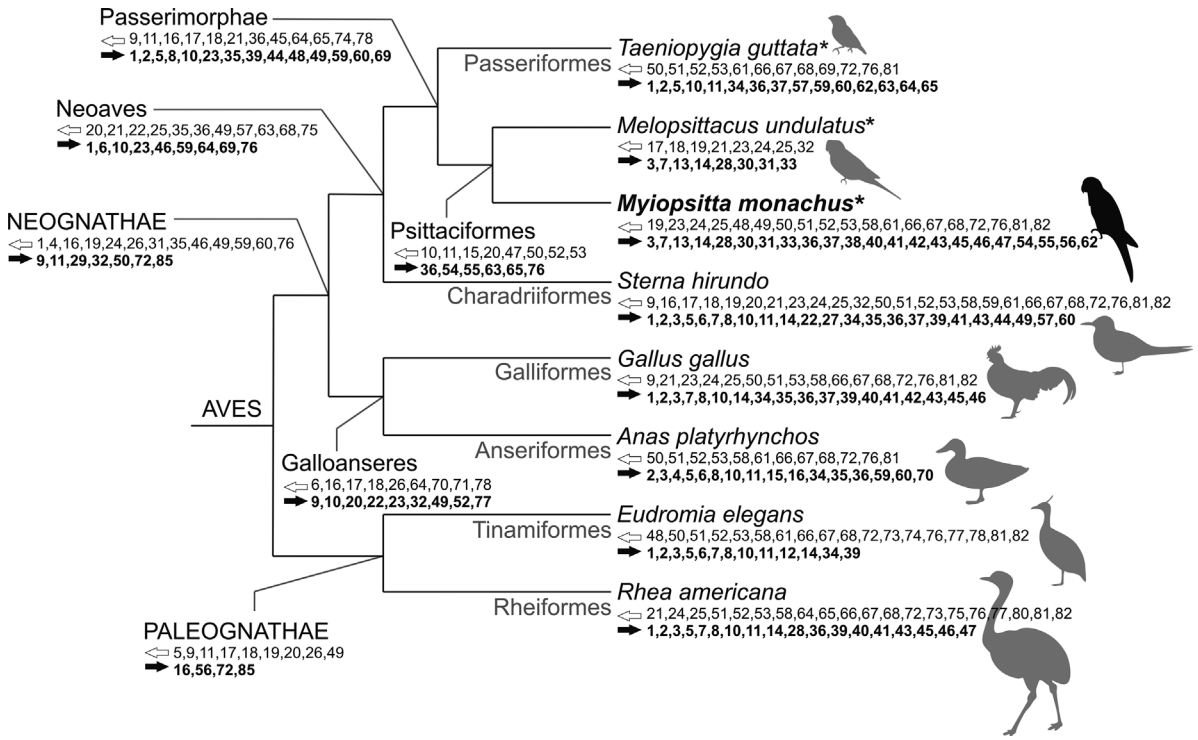


**Fig. 4.** Sequence heterochronies of ancestral and terminal nodes resulting from the event-pair cracking phylogenetic method. Numbers correspond to the number of event assigned for each bone (see the Table 1). Arrows and numbers in normal type show the delays in the sequence regarding the ancestral node, while arrows and numbers in bold type show the accelerations regarding the ancestral node. Altricial species are indicated with an asterisk.

seven accelerations in Neognathae; (iv) regarding the ancestral node Aves, the os squamosum and os frontale are delayed in Paleognathae while in Neognathae these elements are accelerated, in Paleognathae the os premaxillare is accelerated while in Neognathae is delayed, and both clades share the delay ossification of the os pterygoideum, os spleniale and os coracoideum, and the acceleration of the os metatarsale II and phalanx IV of digit IV; (v) among the Neognathae, nine delays and nine accelerations take place in the Galloanseres; (vi) 12 delays and 16 accelerations in *A. platyrhynchos* and 17 delays and 18 accelerations in *G. gallus*, both of these species show a delay in the ossification of some elements of the limbs and the acceleration of some skull and postcranial axial skeleton elements; (vii) 11 delays and nine accelerations in Neoaves; (viii) compared to the ancestral node Neognathae, the lamina basiparasphenoidalis and ischium are delayed in Galloanseres while in Neoaves they are accelerated, the os jugale, os quadratum, and os coracoideum are accelerated in Galloanseres while in Neoaves are delayed, and both clades share the acceleration in the ossification of the os parietale and os dentale; (ix) 25 delays and 23 accelerations in *S. hirundo*; (x) 12 delays and 14 accelerations in Passerimorphae; (xi) eight delays and six accelerations in Psittaciformes; (xii) 19 delays and 22 accelerations in *M. monachus*, and eight delays and eight accelerations in the skull of *M. undulatus*; and finally (xiii) we found 12 delays and 15 accelerations in *T. guttata*.

## Sequence heterochronies of *Myiopsitta monachus*

Regarding the ancestral node Psittaciformes, *M. monachus* shows a delay in the ossification sequence of several bones including: the os pterygoideum in the skull; the os dentale, os supra-angulare, and os angulare in the mandible; the scapula, os coracoideum, clavicula, humerus, radius, ulna, os metacarpale majus III, os metacarpale minus IV in the pectoral appendage; and the femur, tibiotarsus, fibula, os metatarsale II, os metatarsale III, metatarsale IV, and phalanx I of digit IV in the pelvic appendage. We also found an acceleration in the ossification of the following bones: the os supraoccipitale, os laterosphenoidale, os ectethmoidale, os mesethmoidale in the skull; the os articulare in the mandible; the basihyale, urohyale, and epibranchiale in the hyoid apparatus; the corpora of the vertebrae synsacrales and vertebrae caudales, pygostylus, processus transversus of the vertebrae thoracicae and vertebrae synsacrales, arci of the vertebrae caudales and vertebrae synsacrales, costa sternalis and processus uncinatus

in the postcranial axial skeleton; and the sternum, os carpi radiale, os carpi ulnare, os metacarpale alulare (II), and phalanx digiti minoris in the pectoral appendage.

Patterns of acceleration and delay skull ossification showed that the mandible and the hyoid apparatus are shared between both compared Psittaciformes. The only exceptions are the os maxillare, os palatinum, os quadratojugale, and ceratobranchiale, which are delayed in *Melopsittacus undulates*.

## DISCUSSION

### Skeletal development and differences between altricial and precocial birds

Ossification sequences are influenced by several factors such as muscle development, embryonic movements, constraints and modularity, the sequence of chondrification and the source of osteogenic cells, sexual dimorphism, and ecological variables like temperature and humidity (Maxwell 2008c). On the contrary, other factors such as the size of the embryo, the incubation period, or the use of specimens collected from the wild or incubated in the laboratory field, do not influence ossification sequences (Maxwell 2008a,b,c, 2009). At the time of hatching, altricial birds present a lower degree of ossification than precocial birds. The ossification of skeletons of altricial birds are delayed compared to precocial birds (Starck 1993; Blom and Lilja 2004). Is not surprising that this was also observed in the altricial *M. monachus*. Young monk parakeets hatch with 23.25% of their skeletal elements still unossified, mainly skull bones, the hyoid apparatus, the postcranial axial skeleton, and the forelimbs.

In precocial birds, the columna vertebralis begins to ossify at embryonic stages 36–38, and at hatching, all vertebral elements are ossified including the pygostylus. In contrast, in altricial birds, these elements all ossify later and therefore cartilaginous elements are still present at hatching (Starck 1993). In *M. monachus*, the columna vertebralis begins to ossify at the last embryonic stage, starting with the vertebrae (stage 40+), and the last element to ossify is the pygostylus at 9 days after hatching. Also, the costa sternalis, sternum, os supraoccipitale, os articulare, and urohyale begin their ossification after hatching. Consistent with other altricial birds (and in contrast to precocial birds, Starck 1993), the elements of the pelvic girdle of *M. monachus* are not fused at hatching.

The ossification sequence during prenatal development of *M. monachus* generally follows the same pattern described for other birds (e.g., Tokita 2003; Maxwell 2008a,b,c, 2009; Maxwell and Harrison 2008; Maxwell and Larsson 2009; Maxwell et al. 2010; Mitgutsch et al. 2011). Developmental sequences seem quite conservative in birds. In this regard, the long bones of both limbs ossify first, followed by their distal elements. In the skull, bones at the base and jaw ossification precedes the ossification of the remainder elements. Mitgutsch et al. (2011) interpret that the ossified cranial roof late integration with other ossified complex could be related to the rapid postnatal growth. Phylogenetic explanations are not possible at the moment. The columna vertebralis ossification proceeds in a cranio-caudal gradient, but the atlas ossifies after the vertebrae cervicales as in some Paleognathae (Maxwell and Larsson 2009), and may be associated with the cervical musculature development of and/or the early head movements (Maxwell and Larsson 2009).

All the elements of the pectoral girdle except the sternum, ossify in ninth order in *M. monachus*. The sternum ossifies in thirty-ninth order, while the elements of the pelvic girdle ossify fifteenths. At the forelimbs level, belatedly ossify the os carpi radiale and os carpi ulnare at 21 days old, while in the hindlimbs are the phalanxes II and III of digit IV at first 0–5 days posthatching. Therefore, the forelimbs complete their ossification after the hindlimbs. In the tetrapods plesiomorphic condition, forelimbs develop earlier than the hindlimbs, a state retained or reversed among mayor clades (Bininda-Emonds et al. 2007; Richardson et al. 2009). For diapsids and even birds, synchronicity was postulated for both forelimbs and hindlimbs (Bininda-Emonds et al. 2007). However, our results show that the relative timing of forelimb versus hindlimb development varies within birds. For example, in precocial species such as *S. hirundo*, *G. gallus*, and *A. platyrhynchos*, the forelimbs complete their ossification first whereas in the altricial *T. guttata* and the precocials *E. elegans* and *R. americana*, they are synchronous. On the contrary, in the altricial *M. monachus* the hindlimbs complete their ossification first. Heterogeneous and phylogenetically distant groups show similar limbs' relative timing of ossification, and therefore, it does not appear to be phylogenetically constrained. Particularly in *M. monachus*, they could be linked due to requirements for active movement and the use of the hindlimbs inside the nest, but not the need to use them to fly until much later. This adaptive hypothesis could be tested with studies in other altricial species with better temporal resolutions of their ossification sequences.

During vertebrates' limbs development, morphogenesis and osteogenesis are disparate and independent phenomena, because the ossification do not follow a proximo-distal gradient as the morphogenesis (Maisano 2002a). This pattern is also observed in *M. monachus*, were distal elements of the autopodium ossified earlier than proximal elements (see Table 1). It is also interesting to note that last hindlimbs phalanges to ossify are the smaller ones in *M. monachus* (phalanxes II and III of digit IV, characters 83 and 84, respectively), in the same way as in some Ratites (Maxwell and Larsson 2009). In both cases, it may be associated with size hypothesis (see hypothesis below).

The arrangement of the hindlimbs digits of Psittaciformes and the other birds studied here is different as in the former retroversion of the toe IV occurred (zygodactyl feet), however, no pattern is observed in the sequence of ossification that may be associated with this. During the process of ossification, certain events can

occur at different rates or times but at the end, results are similar (e.g., the conformation of the skull roof) or deeper changes may also occur and result in markedly different structures (e.g., several types of feet). Accordingly, there are events in the ossification sequences that cannot be compared. Discussion rests on the concepts of modularity and homologies and we refer the reader to the work of Von Dassow and Munro (1999) for more information.

## How *Myiopsitta monachus* heterochronies are related to function and size

Two hypotheses are proposed in the ossification sequence analysis: (i) the functional hypothesis, suggesting that the functionally important bones ossify earlier in the sequence (Mabee et al. 2000; Maxwell 2008a) and (ii) the size hypothesis, first proposed by Huxley (1932), suggesting that the time of onset of an organ formation in the embryo is related to its adult size, where small elements ossify later in the sequence and large elements ossify earlier (Maxwell 2008a, Sánchez-Villagra et al. 2008).

*M. monachus* show the acceleration of ossification of the os ectethmoidale (character 13) and os mesethmoidale (character 14) in the sequence (Fig. 4). These bones form the rostral portion of the orbit and contribute to the formation of the septum inteorbitale, which lacks of foramen in Psittaciformes. Also, they provide the origin site of the Psittaciformes' novel adductor muscle ethmomandibularis associated with strong bite forces (Tokita 2003; Carril et al. 2015), and its early appearance may correspond to the functional importance hypothesis. In addition, based on the functional and size hypothesis, *M. monachus* shows the acceleration of the hyoid bones basihyale, urohyale, and epibranchiale (characters 30, 31 and 33; Fig. 4), and could be linked to the development of the Psittaciformes tongue and its role in handling food.

However, in relation to both hypotheses some expected results were not observed. For example, according to the size hypothesis, acceleration of the paraglossum and os palatinum would be expected, because they are distinctly large in Psittaciformes. Likewise, an acceleration of the os lacrimale and os squamosum would be expected, as their processus orbitalis and processus postorbitalis, respectively, form the exclusive arcus suborbitalis in some Psittaciformes and its where the novel adductor m. pseudomasseter attaches (Carril et al. 2015). It is notable that in *M. monachus*, the ossification center of the os lacrimale is located precisely in the processus orbitalis, which is elongated and surrounds ventrally the orbit.

## Phylogenetic analysis of the ossification sequences

During embryonic development the sequence of events plays an important role in adult morphology and in evolution, therefore, the ossification sequence may reveal clues about the evolutionary history of species (Maxwell 2008c). Ossification sequences comprise patterns of bone formation and show some degree of conservation among birds, but they are not invariant and intraspecific differences between related species and among higher taxa occur, and heterochronies can be recognized (Maxwell 2008c, Maxwell et al. 2010).

The study of sequence heterochronies has recently increased due to the creation of several new methods of sequence analysis (Maxwell and Harrison 2009), including the event-pair cracking phylogenetic methods used in this work. This method analyses the sequences in an evolutionary context and estimates the sequences at the ancestral nodes and, as the mapping of the ossification sequences is made on an established phylogeny, the results are based on the developmental data. This is an advantage over other methods, which build relationship among taxa based on hypotheses from event-pair characters, usually leading to unreasoned ancestral reconstructions (Bininda-Emonds et al. 2002).

Aves are an appropriate group for the study of the evolution of ossification sequences because there is consensus in the most basal divergence of the clades and, although they share a basic body plan, they have some degree of morphological and ecological differentiation, and how both affect the skeletogenesis can be tested (Maxwell 2008c).

In birds, the skeletogenesis begins in the last embryonic stages and continues after hatching in a pattern or sequence determined for each species (Maxwell 2008a,c, 2009). Based on our results of the event-pairing method, homogeneity in the development of birds is only supported by 56.77% of the character states with homogeneous distribution. Results of the event-pair cracking applied in the analysis of intraspecific variation of distantly related taxa showed a high number of heterochronies along phylogenetic history (Fig. 4), but it was not possible to establish a clear association between delays and/or accelerations with functional and size aspects and/or between precocial and altricial birds. The high levels of homoplasies in terminal nodes may reflect the rapid evolution of the sequences. However, it is possible that it could be the result of a methodological artifact as the characters are treated independently and hypothetical sequences are reconstructed in the ancestral nodes (Bininda-Emonds et al. 2003; Harrison and Larsson 2008). Similar patterns were obtained by other researchers (e.g., Bininda-Emonds et al. 2003) that have argued that the use of robust phylogenies, as in the present study, potentially reduces artefacts (Bininda-Emonds et al. 2002).

In the terminal nodes common heterochronies to all species were observed, like the delay of long bones of both limbs (characters 51, 52, 53, 66, 67, 68, 69, 72, 76, and 81). Not surprising, ossification of the elements of the limbs is coordinated and are thought to function as clusters or modules (Goswami et al. 2009), defined as sets of characters within an organism that possess some autonomy relative to other characters and undergo

separately developmentally and evolutionarily transformation (Poe 2004; Callebaut 2005). The modules can be the result of the action of shared developmental mechanisms or could form functionally integrated units (Schoch 2006). These groups of limb long bones form at about the same time in all the studied species and have been actively shifted back together on the sequences regarding their ancestral nodes. They represent the only cluster identified in our sequences analysis.

Finally, regarding heterochronies of internal nodes, previous studies using other methodologies (e.g., Parsimov, Maxwell 2008c), show some similarities with our results: (i) differences in the order of appearance of the os frontale (character 11) and ceratobranchiale (character 32) between Paleognathae and Neognathae regarding the ancestral node Aves; (ii) delay of lamina basiparasphenoidalis (character 6) and phalanx II of digit III (character 78) in Galloanseres regarding the ancestral node Neognathae; and (iii) acceleration of the lamina basiparasphenoidalis (character 6) and delay of the os quadratum (character 22) in Neoaves regarding the ancestral node Neognathae.

The several interpretations of ancestral states, resulting from various different methodological approaches, can influence the understanding of heterochronic patterns. Thus, different estimates of ancestral states may result in opposed reconstructions and dissimilar interpretations. We acknowledge the problems that may arise when comparing datasets from different sources, such as distinct ranking resolutions of the ossification sequences from which the ancestral sequences are constructed. However, the applied methodology takes into account the simultaneous events in the sequences as well as the missing data. Future studies including a greater number of species, better resolutions of their ossification sequences, and using different analytical methods to make comparisons are needed to obtain more reliable results.

## CONCLUSIONS

Although ossification sequence of *M. monachus* and *M. undulatus* is fairly similar, developmentally they differ in some aspects at skull level (delays of the os maxillare, os palatinum, os quadratojugale and ceratobranchiale in *M. undulatus*). More information is needed on the rest of the skeleton to confirm whether other heterochronic events occur between both. It is clear that interspecific variation is a topic that remains to be explored.

As expected, the ossification sequence of *M. monachus* shows a general pattern similar to that described for other birds. Almost 25% of their bones are not ossified at the time of hatching, supporting existing hypotheses that argue that skeletons of altricial birds are delayed compared to precocial birds (Starck 1993; Blom and Lilja 2004).

Some elements involved in the highly developed feeding behaviors capabilities of Psittaciformes (i.e., site of attachment of novel adductor muscles and hyoid bones) are accelerated in the sequence, and may correspond either to the functional and/or size hypothesis.

Our results of the event-pairing method show homogeneity in the development of birds only supported by 56.77% of the character states having homogeneous distribution.

The event-pair cracking phylogenetic method reveal a high number of heterochronies along phylogenetic history, and shows that limbs long bones may behave as modules.

This work of ossification sequences and spatial patterning of bone differentiation means an important step in the study of morphological evolution by increasing the data on the development of wild non-model Aves.

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## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

**Table S1.** List of the 3655 characters obtained from the construction of the event-pair matrix for *Myiopsitta monachus*.

**Table S2.** Autapomorphies for *Myiopsitta monachus*.

**Table S3.** Characters states having a defined pattern within the clade Psittaciformes.