Distinct developmental pathways underlie independent losses of flight in ratites

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Recent phylogenetic studies question the monophyly of ratites (large, flightless birds incorporating ostriches, rheas, kiwis, emus and cassowaries), suggesting their paraphyly with respect to flying tinamous (Tinamidae). Flightlessness and large body size have thus likely evolved repeatedly among ratites, and separately in ostriches (Struthio) and emus (Dromaius). Here, we test this hypothesis with data from wing developmental trajectories in ostriches, emus, tinamous and chickens. We find the rate of ostrich embryonic wing growth falls within the range of variation exhibited by flying taxa (tinamous and chickens), but that of emus is extremely slow. These results indicate flightlessness was acquired by different developmental mechanisms in the ancestors of ostriches (peramorphosis) and the emu–cassowary clade (paedomorphosis), and corroborate the hypothesis that flight loss has evolved repeatedly among ratites.

1. Background

Recent phylogenomic analyses have revolutionized our understanding of avian evolutionary interrelationships (e.g. [1–3]). These studies have yielded numerous unanticipated phylogenetic insights, few of which are as consistently strongly supported as the hypothesis that flightless ratites are paraphyletic with respect to flying tinamous (figure 1a; [4–12]). Setting aside the unlikely scenario where flightlessness is ancestral for crown palaeognaths (the extant avian subclade to which only tinamous and ratites belong [13]), recent phylogenetic hypotheses for living and extinct palaeognaths invoke at least three independent losses of flight throughout ratite evolutionary history: along the respective stem lineages of ostriches (Struthionidae), rheas (Rheidae) and the kiwi–emu–cassowary clade (figure 1a). Studies incorporating sequence data from subfossil remains of recently extinct ratites (elephant birds and moas) suggest additional independent losses of flight among palaeognaths [4,6,7,10].

Although repeated losses of flying ability—and attendant anatomical changes—have evolved in other avian clades (e.g. dozens of times among rails [14,15]), the convergent loss of flight among ratites has, thus far, only been closely investigated from phylogenetic and genomic perspectives [1,3,6–11,16]. Such studies have convincingly established the general pattern of palaeognath interrelationships, and therefore the pattern by which flight must have been lost deep in ratite evolutionary history. However, the mechanistic developmental processes underlying such repeated losses of flight have not been subject to investigation.

Here, we draw on an embryonic developmental series to determine whether heterochronic changes in the developing wing may offer clues into how flight was repeatedly lost in palaeognaths. Our embryonic dataset includes ontogenetic sequences from two ratite lineages inferred to have lost flight independently:
ostrich (Struthio camelus) and emu (Dromaius novaehollandiae), as well as the flighted tinamou Eudromia elegans, and the neognath chicken (Gallus gallus; representing a flighted outgroup). Our data suggest different heterochronic mechanisms led to independent losses of flight in ostriches and emus.

2. Material and methods

Full details on incubation, specimen acquisition, staining protocols, specimen selection and measurement methodologies are presented in the electronic supplementary material.

(a) Embryo incubation

Eggs were incubated at Yale University following protocols selected to provide optimum survival of the embryos with maximum hatchability (although no eggs were incubated to hatching).

(b) Clearing and staining

Clearing and staining techniques were employed to differentially stain cartilage blue and calcified bone red, revealing an ontogenetic sequence of growing and calcifying embryonic skeletons.

(c) Data acquisition

Specimens were required to be grossly normal in appearance for their particular stage of development. Small specimens and small parts of larger specimens were measured using a microscope eyepiece reticle, calibrated against a steel ruler. Larger specimens and mounted skeletons were measured to the nearest 0.1 mm with Vernier calipers.

3. Results

Measurements taken for each embryo are illustrated in figure 2a. The full morphometric dataset is provided in the electronic supplementary material.

The relationship between total wing length and incubation age for the considered palaeognaths and chicken is illustrated in figure 1b. The volant taxa (chicken and tinamou) illustrate largely overlapping developmental trajectories, and broadly similar rates of wing growth (chicken \( y = 2.72x - 8.03, R^2 = 0.94 \); tinamou \( y = 3.65x - 19.75, R^2 = 0.92 \)). These proportional wing growth rates are similar to that exhibited by ostrich (\( y = 2.97x - 35.99, R^2 = 0.96 \)), and much higher than that exhibited by emu (\( y = 0.98x - 9.99, R^2 = 0.87 \)). Relative rates of wing growth with respect to chicken are 9% faster for ostrich, 34% faster for tinamou and 64% slower for emu (figure 1c). The rate of wing growth relative to incubation age for ostrich falls comfortably within the range of variation for the examined volant taxa. That for emu falls well below the range of volant birds. Relative to body length, the rate of wing growth for ostrich is intermediate between the faster rates exhibited by the flying taxa, and the slower rate exhibited by emu (figure 2b).

Additional allometric data are presented in the electronic supplementary material.

4. Discussion

Early attempts to infer palaeognath interrelationships using biochemical data supported a monophyletic ratite clade sister to tinamous [17–22]. However, recent phylogenetic evidence is virtually unanimous in revealing a paraphyletic ratite topology, with some variation in the position of kiwis (Apterygidae) [4–11]. This new topological paradigm has inspired considerable work on macroevolutionary patterns across palaeognaths, with a particular emphasis on the origin of their peculiar biogeographic distribution.

Our revised understanding of ratite interrelationships conflicts with longstanding phylogenetic hypotheses based on comparative morphology (e.g. [23]), and reveals a surprising degree of homoplasy related to body size and flight-related
features of the pectoral apparatus [6]. This situation demands an attempt to unravel the developmental underpinnings of independent flight loss in ratites.

Various factors such as conservation priorities precluded the acquisition of developmental embryonic sequences for certain ratite subclades (e.g. kiwis). In this study, we analysed a comprehensive embryonic series for ostrich and emu, which are unanimously inferred as representing two independent transitions to flight loss in recent molecular phylogenies. This dataset is compared to embryonic sequences from the volant palaeognath *Eudromia elegans* (representing tinamous), and a volant terrestrial neognath (the chicken, *Gallus gallus*).

We demonstrate that rates of embryonic wing growth differ substantially between ostriches and emus, with those of ostrich falling within the range of variation exhibited by flying chickens and tinamous (figure 1c). These dissimilar developmental trajectories among ratites support the hypothesis that flightlessness in ostriches and emus was acquired independently, as a single flight loss event among ancestral ratites would be expected to manifest in similar patterns of forelimb development. Considering the decelerated allometric trajectories of the wing and slow overall growth rates exhibited by emus (electronic supplementary material), we suggest flightlessness among the ancestors of emus and cassowaries may have been acquired at small body size via reduced investment in wing development (neoteny), similar to that exhibited by the extant flightless cormorant, *Phalacrocorax harrisi* [28], and the extinct Stephen’s Island (Lyall’s) wren, *Traversia lyalli* [25], followed by acquiring large body size.

By contrast, the ostrich embryonic series demonstrates body growth by peramorphosis (electronic supplementary material); combined with large wings, this pattern may suggest ancestral stem ostriches were flying birds that grew too large to fly, while retaining large wings. Extant ostriches employ these functional wings for display [26]. Considering that recent phylogenies support independent flight losses for rheas (Rheidae) and ostriches, similarities in their skeletal proportions and display behaviours suggest rheas underwent a similar trajectory towards flightlessness along their ancestral stem lineage. Similar heterochronic mechanisms leading to flight loss have been proposed for flightless pigeons (*Raphus cucullatus* and *Pezophaps solitaria*) [27], and South American steamer ducks (*Tachyeres* sp.) [28].

5. Conclusion

Along with an improved understanding of avian phylogeny in general, recognition of the non-monophyly of extant ratites is among the most important recent advances in comparative ornithology. This study attempts to move beyond corroborating recent phylogenetic hypotheses for ratite interrelationships by investigating developmental mechanisms of convergent flight loss among ratites, and illustrates that the heterochronic mechanisms underlying flightlessness in ostriches and emus are different.

Future advances depend on the investigation of developmental sequences in other, less easily obtained, ratite taxa, as well delving into gene expression patterns in developing ratite embryos. Recent reconstructions of ancestral rates of genomic evolution indicate a relatively small common ancestor of all extant palaeognaths, which may have repeatedly given rise to large, flightless descendants [5,29]. However, direct evidence bearing on flight loss among palaeognaths can only be obtained via the discovery and accurate systematic interpretation of fossils from the stem lineages of the modern ratite subclades. Such discoveries have proven elusive, despite detailed studies of known fossil palaeognaths (e.g. [30–33]). Future fossil evidence will hopefully shed light on how and when the various ratite subclades lost flight and acquired large body size—such discoveries will ultimately be necessary for us to fully understand what has quickly become a classic example of convergent evolution.

**Ethics.** C.F. acquired and processed these embryos in the 1990s, when there was no IACUC regulation of avian embryos. The Yale Institutional Animal Care and Use Committee reviewed and approved this research for this project in 1996 but did not assign a protocol number. All of the data used in this paper was collected between 1996 and 1999, as part of the project approved in 1996.

**Data accessibility.** Complete dataset uploaded as electronic supplementary material, tables S1–S9.

**Authors’ contributions.** C.F. collected data. Both authors designed study, analysed data, wrote the paper and agree to be accountable for the material herein, and approve the final version of this manuscript.

**Competing interests.** We declare we have no competing interests.

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**Figure 2.** (a) Schematic of stained 12-day old tinamou (*Eudromia elegans*) embryonic skeleton with measurements indicated. BL, body length (cranial edge of the atlas to caudal edge of the ilium); WL, wing length (combined length-wise measurements of humerus, ulna, semilunate carpal, metacarpal III and phalanges of digit III); LL, leg length (combined length-wise measurements of tarsometatarsus, tibiotarsus and femur). (b) Developmental trajectory of wing length versus body length; colour scheme matches that of figure 1. Slope for ostrich (0.38) is intermediate between emu (0.21) and the flying taxa (tinamou = 0.56; chicken = 0.67).