

Detecting pigments from colourful eggshells of extinct birds

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Abstract The known chemical basis of diverse avian eggshell coloration is generated by the same two classes of tetrapyrrole pigments in most living birds. We aimed to extend the evolutionary scope of these patterns by detecting pigments from extinct birds' eggs. In our samples biliverdin was successfully extracted from subfossil shell fragments of the blue-green egg-laying upland moa *Megalapteryx didinus*, while protoporphyrin was extracted from the beige eggs of two other extinct moa species. Our data on pigment detection from eggshells of other extant paleognath birds, together with published information on other modern lineages, confirm tetrapyrroles as ubiquitous

and conserved pigments contributing to diverse eggshell colours throughout avian evolution.

Keywords Egg matrix · Pigmentation · Radiation · Ratite

Introduction

Avian eggshells are some of the most diversely coloured natural materials (Kilner 2006). Despite analytical advances in the chemical characterisation of pigments, the

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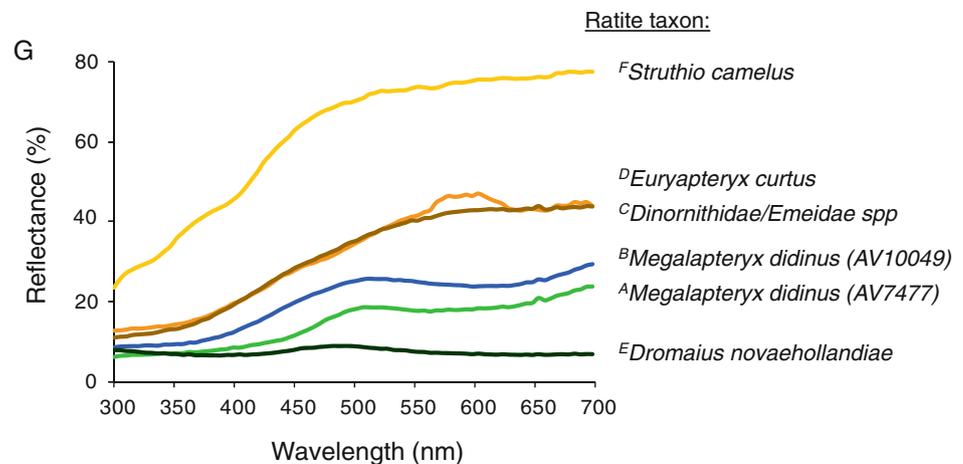
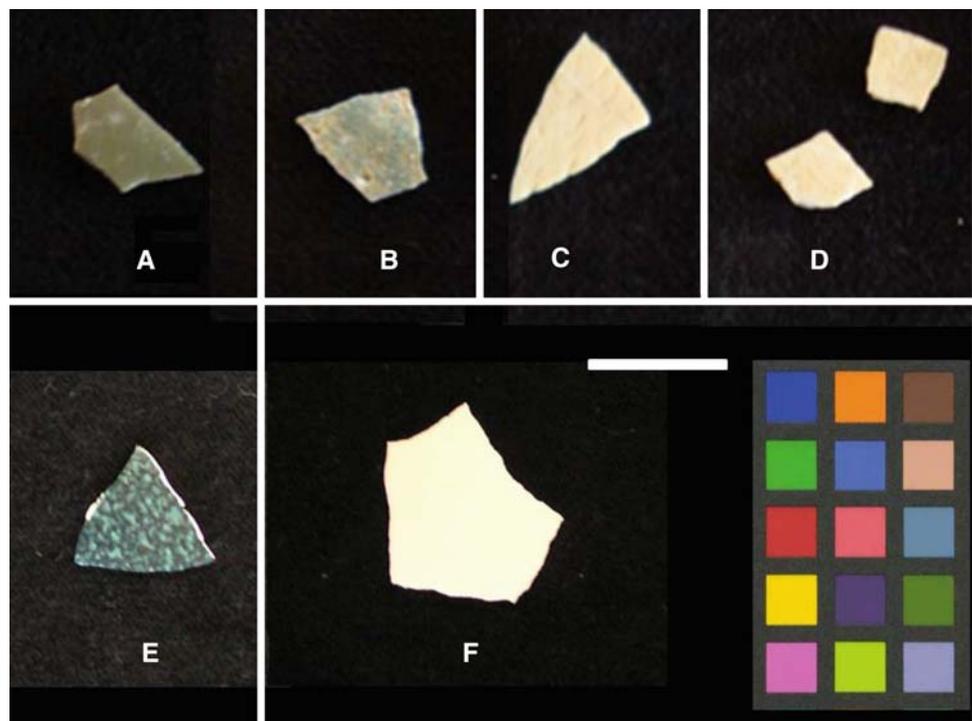
diversity of birds' egg colours has been solely attributed to pyrroles (reviewed in Gorchein et al. 2009). In birds, the bile pigment biliverdin produces the blue-green human-visible appearance of eggs while protoporphyrin (Miksik et al. 1996) is typically detected from red-brown maculated eggs. Immaculate eggs of diverse colours can contain either or both biliverdin and protoporphyrin, while white eggs may contain one, both, or no detectable pigments at all (Kennedy and Vevers 1976).

We aimed to extend the comparative scope of prior published pigment analyses by examining subfossil fragments from an extinct avian lineage. Amongst paleognath birds, most species of New Zealand's moa (Dinornithidae/Emeidae) laid eggs whose remains appear grey to beige to

human observers (Gill 2007). In contrast, eggshell fragments attributed to the Upland Moa (*Megalapteryx didinus*; Megalapterygidae; Bunce et al. 2009), are typically pale to bright green to the human eye (Fig. 1a). One extraordinary specimen of this species is a near-complete skeleton of a female, confirmed by nuclear DNA sequences (Huynen et al. 2003), that surrounded an apparently unlaidd blue egg (Gill 2007).

Here we used modern chemical techniques to determine whether biliverdin and protoporphyrin could be detected in colourful subfossil moa eggs. Although all taxa of the diverse moa radiation have been extinct for over 500 years, their mating systems, size dimorphism patterns, and plumage colours have been the subject of several recent

Fig. 1 a–f Representative eggshell fragments (*white scale bar* 1 cm for **a–d** and 2 cm for **e, f**) and **g** reflectance spectra of extinct and extant ratites included in our analysis



discoveries using ancient DNA and microscopy-based techniques (e.g. Bunce et al. 2003, Turvey et al. 2005, Rawlence et al. 2009). This continued interest in moa renders them a highly desirable focal lineage for new analyses of the chemical basis of egg coloration. However, as eggshells' appearances both change over time, even when specimens are stored in museums (Starling et al. 2006), and vary with environmental conditions at the time of laying (Aviles et al. 2007), in the absence of a known time series of moa eggs and their colours, our tests necessarily remain exploratory.

Methods

Sample description

Three moa eggshell samples were kindly provided by Otago Museum, New Zealand: (1) green eggshell (AV7477), attributed to *Megalapteryx didinus*, from Chatto Creek, Central Otago, South Island; (2) blue eggshell (AV10049) of the unlaidd *M. didinus* egg from Mount Aspiring National Park, South Island; (3) beige moa eggshell (AV7371, species unidentified) from Central Otago, South Island. Upland Moa remains are known only from the South Island (Tennyson and Martinson 2006), and so we sourced a fourth moa eggshell fragment from the North Island of New Zealand (Auckland Museum LB12048), beige, probably of *Euryapteryx curtus*, from Tokerau Beach, Northland.

For a comparison with extant paleognath birds, we extracted pigments from eggshell fragments from extant ratites, including the following samples: (1) a fresh farmed ostrich egg *Struthio camelus*; (2) a fresh farmed emu egg *Dromaius novaehollandiae*; (3) two North Island Brown Kiwi *Apteryx mantelli* eggs hatched successfully in captivity at Rainbow Springs, Rotorua, (1)–(3) all sourced in New Zealand; (4) a farmed Greater Rhea *Rhea americana* egg purchased from a store in Berkeley, USA; (5) a fresh captive Chilean Tinamou *Nothoprocta perdicaria* egg from British Columbia, Canada, and (6) a fresh Great Tinamou egg *Tinamus major* from a nest at La Selva, Costa Rica (Brennan 2009).

Colour analyses

All eggshell fragments were washed with 70% ethanol and air dried. Each sample was photographed with a digital camera and, using Image J 1.40 (National Institute of Health, USA) to calculate surface areas. We collated published information on the human-perceived colours of each species' eggs (Walters 1994; Gill 2007). To record physical measures of appearance, prior to destructive

chemical analysis, the reflectance of eggshells for extinct moa and two extant ratites was also documented following Cassey et al. (2009) (Fig. 1).

Pigment analysis

We followed the extraction protocol for eggshell pigments given in Kennedy and Vevers (1976). Each eggshell sample of measured area (maximum $\sim 1 \text{ cm}^2$) was dissolved in fresh 5% sulphuric acid in methanol and steeped for 1–2 days (no longer than 2) before filtering through 1 mL barrier pipette tips (Axygen Biosciences) under slight pressure. The acidified methanolic filtrate was extracted into dichloromethane/methanol/water (1:2:1 v/v/v) three times recovering the lower phase of the organic layer each time, then once in 10% sodium chloride solution and twice with water, ensuring the pH of the final water wash was above 5. The organic solution was evaporated to dryness under a stream of nitrogen and dissolved in 1 ml of methanol. An aliquot was measured in an Agilent 8453 diode array spectrophotometer for its absorption spectrum from 220 to 1,000 nm versus methanol (as a blank).

An indicator of the pigments present was evident from these spectra and then confirmed by mass spectrometry. To assign the presence or absence of biliverdin and protoporphyrin we used, high-resolution accurate mass infusion mass spectrometry on a ThermoFinnigan LTQ FTMS ion cyclotron resonance (ICR) mass spectrometer operating in electrospray mode. We employed tandem mass spectrometry (MS^n) analysis with helium as the collision-induced dissociation gas on diluted methanolic samples using several diagnostic ions in the ion-trap (see below) followed by accurate mass using a resolution of 100,000 (at m/z 400) in the ICR cell on these ions when present in sufficient concentration. Several orders of magnitude of concentration ($\sim 10^6$) were discernable through this approach, confirming the identity of the pigments though their diagnostic daughter ion fragments and atomic composition with accurate mass values less than 2 ppm.

A quantitative assessment of both biliverdin and protoporphyrin was also conducted for a separate subset of the available shells using the same pigment extraction protocol followed by analysis on an ion-trap mass spectrometer. Samples were analysed by flow injection analysis using an Agilent 1100 series capillary HPLC, delivering 95% methanol with 0.1% formic acid at a flow rate of 20 $\mu\text{L}/\text{min}$ and coupled to an Agilent ion-trap mass spectrometer model SL with an electrospray ionization interface. Biliverdin IX α dimethyl ester and protoporphyrin IX dimethyl ester were quantified simultaneously by multiple reaction monitoring; simply protonated biliverdin IX α dimethyl ester was isolated at m/z 611.4 and was quantified using the fragment at m/z 311.1, fragments at m/z 209.1 and

m/z 283.2 were used as qualifier ions, simply protonated protoporphyrin IX dimethyl ester was isolated at m/z 591.3 and was quantified using the fragment of m/z 513.3, fragments at m/z 485.3 and m/z 445.3 were used as qualifier ions. Biliverdin IX α dimethyl ester and protoporphyrin IX dimethyl ester obtained from Frontier Scientific Inc. (Logan, Utah) were used as standards and they gave a linear response over the range 8 fmol to 2.4 pmol.

For the quantitative assessment measurements, we standardised the detected pigment concentrations to eggshell fragment size used in extractions by dividing measurements by eggshell sample area to calculate pigment concentration as pmol/cm². We consider this appropriate because pigments are predominantly found within the eggshell cuticle (Miksik et al. 2007). Presence of compounds is reported only for samples where detected concentrations were elevated relative to internal controls. To describe quantitative data, we compared the findings from moa explicitly with our result from eggs of emu (a known source of biliverdin, but not protoporphyrin) and ostrich (a known source of protoporphyrin but not biliverdin) (Kennedy and Vevers 1976).

Results

Detection of biliverdin

The result of our two detection techniques allowed us to identify the presence of biliverdin from shell samples of two different Upland Moa eggs, but not of the other two moa taxa in our sample (Table 1). The concentration of recovered biliverdin from the eggshell samples was 0.21 pmol/cm² from Upland Moa AV10049, which was

two magnitudes lower than that recovered from the emu (19.8 pmol/cm²) and 20 times higher than that from the ostrich (0.001 pmol/cm²).

Detection of protoporphyrin

We successfully detected protoporphyrin from the beige eggshells of both the South Island moa (0.30 pmol/cm²) and the North Island moa (0.37 pmol/cm²) samples. These concentrations were similar in magnitude to that of protoporphyrin detected from the ostrich eggshell (0.10 pmol/cm²). We did not detect protoporphyrin above instrumental threshold in either the green or the blue shells of Upland Moa or the emu sample.

Discussion

Despite the small number of taxa and shell fragments available to us, a necessary corollary of our methods to destructively sample museum specimens of extinct species, we detected the pigments biliverdin and protoporphyrin from different subfossil eggshells of extinct birds. All of the moa fragments were collected from different localities and had been stored in different storage units or facilities prior to analysis and we positively detected both classes of pigments from different specimens. Nonetheless, it remains possible that the reported patterns of pigment detection are due to environmental contaminants or chemical and physical changes to the pigments on and inside the eggshell matrix that occurred during the unknown period between egg formation and laying and the time of pigment extraction in this study. For example, there was disagreement in the positive detection of biliverdin between our two

Table 1 Outputs of two analytical detection protocols of biliverdin from extinct and extant paleognath bird eggshell samples available for destructive sampling

Taxon	High-resolution accurate mass infusion mass spectrometry	Liquid chromatography-mass spectrometry	Combined detection	Human-perceived eggshell colour
Dinornithidae/Emeidae spp. ^a	n/c	No	No	Beige
<i>Megalapteryx didinus</i> (AV10049) ^a	n/c	Yes	Yes	Blue
<i>Megalapteryx didinus</i> (AV7477) ^a	Yes	No	Yes	Green
<i>Euryapteryx curtus</i> ^a	n/c	No	No	Beige
<i>Struthio camelus</i>	No	No	No	Beige
<i>Apteryx mantelli</i>	No	n/c	No	White/blue
<i>Dromaius novaehollandiae</i>	Yes	Yes	Yes	Green
<i>Rhea americana</i>	Yes	n/c	Yes	White/blue
<i>Nothoprocta perdicaria</i>	Yes	n/c	Yes	Brown
<i>Tinamus major</i>	Yes	n/c	Yes	Blue

n/c refers to analyses not conducted

^a Extinct taxa

analytical methods when destructively sampling two different fragments of the green Upland Moa egg (Table 1), implying a sensitivity to sample identity of the detection protocols.

We cannot be certain of either the original coloration of the extinct birds' eggs, nor the interpretation of negative chemical detection results in our analyses here. Nonetheless, following the directional predictions of published results on patterns of human-assessed coloration and biliverdin detection in the eggshells of modern bird species (Kennedy and Vevers 1976; Moreno et al. 2006), we also found a consistent association of human-perceived blue-green eggshell colours with the presence of biliverdin from the combined ratite and tinamou eggs in our samples at the species level ($G^2 = 2.8$, $p = 0.045$, 1-tailed; Table 1).

It is unknown whether moa, or their competitors, parasites and predators (Cassey et al. 2008), had evolved to perceive and behaviourally discriminate between diverse eggshell colours (Reynolds et al. 2009). Irrespective of any potential adaptive function of diverse egg colours, paleoneurological data already suggest that visual perception was more important for moa compared to their nocturnal kiwi relatives in New Zealand (Corfield et al. 2008), perhaps in the context of their diverse plumage colours (Rawlence et al. 2009) and/or herbivorous foraging tactics (Fadzly et al. 2009) of the different moa species. Our research thus contributes to recent advances in research on the behavioural and sensory ecology of extinct animal lineages, including genetic and microscopic analyses of sexual dimorphism in growth (Bunce et al. 2003), behavioural reconstruction of nesting patterns (Varricchio et al. 2008), and neuroanatomical correlates of sensory functions (Zelenitsky et al. 2009; Patek and Oakley 2003).

The discovery of biliverdin and protoporphyrin in moa eggshells is consistent with a ubiquitous role of tetrapyrroles generating eggshell colours throughout avian evolution (Kennedy and Vevers 1976; Gorchein et al. 2009). Physiologically and biochemically, the evolutionarily ubiquitous and conserved role of pyrroles contributing to eggshell coloration is mechanistically feasible because both classes of the dominant tetrapyrrole pigments of eggshells, are involved in the general synthesis and catabolism of vertebrate haem, and are likely deposited as de novo metabolites in the avian shell gland (Wang et al. 2009).

There are repeated suggestions, based on phylogenetic reconstruction and comparison with reptiles, that the ancestral avian eggshell was white and immaculate (reviewed in Kilner 2006), in parallel with the pigment-free white coloration of the calcium carbonate matrix of crocodilian eggs (our own unpublished data). Nevertheless, our results from extinct bird eggs expand on the scope of this conclusion as it seems likely that pyrrole eggshell pigments

are both ancient in origin and highly conserved throughout the diverse radiations of birds. It remains to be shown what the structural, chemical, and biological mechanisms are that result in pigmented eggshell types and what role these pigments play in the protein network of the eggshell matrix (Sharp and Silyn-Roberts 1984).

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References

- Aviles JM, Stokke BG, Moksnes A, Roskraft E, Moller AP (2007) Environmental conditions influence egg color of reed warblers *Acrocephalus scirpaceus* and their parasite, the common cuckoo *Cuculus canorus*. *Behav Ecol Sociobiol* 61:475–485
- Brennan PLR (2009) Incubation behavior of great tinamou (*Tinamus major*). *Wilson J Ornithol* 121:506–511
- Bunce M, Worthy TH, Ford T, Hoppitt W, Willerslev E, Drummond A, Cooper A (2003) Extreme reversed sexual size dimorphism in the extinct New Zealand moa *Dinornis*. *Nature* 425:172–175
- Bunce M, Worthy TH, Phillips MJ, Holdaway RN, Willerslev E, Haile J, Shapiro B, Scofield RP, Drummond A, Kamp PJJ, Cooper A (2009) The evolutionary history of the extinct ratite moa and New Zealand Neogene paleogeography. *Proc Natl Acad Sci USA* 106:20646–20651
- Cassey P, Honza M, Grim T, Hauber ME (2008) The modeling of avian visual perception predicts behavioural rejection responses to foreign egg colours. *Biol Lett* 4:515–517
- Cassey P, Ewen JG, Marshall NJ, Vorobyev M, Blackburn TM, Hauber ME (2009) Are avian eggshell colours effective intraspecific communication signals? A perceptual modeling approach. *Ibis* 151:689–698
- Corfield JR, Wild JM, Hauber ME, Parsons S, Kubke MF (2008) Evolution of brain size in the Palaeognath lineage, with an emphasis on New Zealand ratites. *Brain Behav Evol* 71:87–99
- Fadzly N, Jack C, Schaefer HM, Burns KC (2009) Ontogenetic colour changes in an insular tree species: signalling to extinct browsing birds? *New Phytol* 184:495–501
- Gill BJ (2007) Eggshell characteristics of moa eggs (Aves: Dinornithiformes). *J R Soc N Z* 37:139–150
- Gorchein A, Lim CK, Cassey P (2009) Extraction and analysis of colourful eggshell pigments using HPLC and HPLC/electrospray ionization tandem mass spectrometry. *Biomed Chromatogr* 23:602–606
- Huynen L, Millar CD, Scofield RP, Lambert DM (2003) Nuclear DNA sequences detect species limits in ancient moa. *Nature* 425:175–178
- Kennedy GY, Vevers HG (1976) A survey of eggshell pigments. *Comp Biochem Physiol B Biochem Mol Biol* 55:117–123
- Kilner RM (2006) The evolution of egg colour and patterning in birds. *Biol Rev* 81:383–406
- Miksik I, Holan V, Deyl Z (1996) Avian eggshell pigments and their variability. *Comp Biochem Physiol B* 113:607–612
- Miksik I, Eckhardt A, Sedlakova P, Mikulikova K (2007) Proteins of insoluble matrix of avian (*Gallus gallus*) eggshell. *Connect Tissue Res* 48:1–8
- Moreno J, Lobato E, Morales J, Merino S, Tomas G, Martinez-de la Puente J, Sanz JJ, Mateo R, Soler JJ (2006) Experimental

- evidence that egg color indicates female condition at laying in a songbird. *Behav Ecol* 17:651–655
- Patek SN, Oakley TH (2003) Comparative tests of evolutionary tradeoffs in a palinurid lobster acoustic system. *Evolution* 57:2082–2100
- Rawlence NJ, Wood JR, Armstrong KN, Cooper A (2009) DNA content and distribution in ancient feathers and potential to reconstruct the plumage of extinct avian taxa. *Proc R Soc Lond B* 276:3395–3402
- Reynolds SJ, Martin GR, Cassey P (2009) Is sexual selection blurring the functional significance of eggshell coloration hypotheses? *Anim Behav* 78:209–215
- Sharp RM, Silyn-Roberts H (1984) Development of preferred orientation in the eggshell of the domestic fowl. *Biophys J* 46:175–179
- Starling M, Heinsohn R, Cockburn A, Langmore NE (2006) Cryptic genes revealed in pallid cuckoos *Cuculus pallidus* using reflectance spectrophotometry. *Proc R Soc Lond B* 273:1929–1934
- Tennyson A, Martinson P (2006) *Extinct birds of New Zealand*. Te Papa Press, Wellington
- Turvey ST, Green OR, Holdaway RN (2005) Cortical growth marks reveal extended juvenile development in New Zealand moa. *Nature* 435:940–943
- Varricchio DJ, Moore JR, Erickson GM, Norell MA, Jackson FD, Borkowski JJ (2008) Avian paternal care had dinosaur origin. *Science* 322:1826
- Walters M (1994) *Birds' eggs*. Dorling Kindersley, London
- Wang XT, Zhao CJ, Li JY, Xu GY, Lian LS, Wu CX, Deng XM (2009) Comparison of the total amount of eggshell pigments in Dongxiang brown-shelled and Dongxiang blue-shelled eggs. *Poult Sci* 88:1735–1739
- Zelenitsky DK, Therrien F, Kobayashi Y (2009) Olfactory acuity in theropods: palaeobiological and evolutionary implications. *Proc R Soc Lond B* 276:667–673