




## SYMPOSIUM

 $\delta^{18}\text{O}$ -DERIVED INCUBATION TEMPERATURES OF OVIRAPTOROSAUR EGGS

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**Abstract:** In order to determine the incubation temperature of eggs laid by non-avian dinosaurs, we analysed the oxygen isotope compositions of both eggshell carbonate ( $\delta^{18}\text{O}_c$ ) and embryo bone phosphate ( $\delta^{18}\text{O}_p$ ) from seven oviraptorosaur eggs with preserved *in ovo* embryo bones. These eggs come from the Upper Cretaceous Nanxiong Formation of Jiangxi Province, China. Oviraptorosaur theropods were selected because of their known brooding behaviour as evidenced by preserved adult specimens fossilized in brooding posture on their clutch. Incubation temperature of these embryos was estimated based on the following considerations: eggshell  $\delta^{18}\text{O}_c$  value reflects the oxygen isotope composition of egg water fluid; embryo bones precipitate from the same egg fluid; and oxygen isotope fractionation between phosphate and water is controlled by the

egg temperature. A time-dependent model predicting the  $\delta^{18}\text{O}_p$  evolution of the embryo skeleton during incubation as a function of egg temperature was built, and measured  $\delta^{18}\text{O}_c$  and  $\delta^{18}\text{O}_p$  values used as boundary conditions. According to the model outputs, oviraptorosaurs incubated their eggs within a 35–40°C range, similar to extant birds and compatible with the known active brooding behaviour of these theropod dinosaurs. Provided that both eggshell and embryo bones preserved their original oxygen isotope compositions, this method could be extended to investigate some reproductive traits of other extinct groups of oviparous amniotes.

**Key words:** oviraptorosaur, embryo, eggshell, incubation temperature, oxygen isotopes, China.

OXYGEN isotope fractionation between apatite, calcite or aragonite, and the aqueous fluids from which they have precipitated is temperature dependent and has been widely used in palaeobiological sciences to estimate the formation temperature of fossil vertebrate bones, teeth or invertebrate shell, thanks to the available oxygen isotope fractionation equations established for extant animals (Anderson & Arthur 1983; Kolodny *et al.* 1983; Grossman & Ku 1986; Lécuyer *et al.* 2013). Some of these isotopic studies focused

on the thermophysiology of non-avian dinosaurs using either stable oxygen isotope composition ( $\delta^{18}\text{O}$ ) of bone or tooth phosphate (Barrick & Showers 1994; Barrick *et al.* 1996; Fricke & Rogers 2000; Amiot *et al.* 2006) or using clumped isotope geochemistry ( $\Delta_{47}$ ) applied to dinosaur bones or eggshell calcites (Eagle *et al.* 2011, 2015). Both  $\delta^{18}\text{O}$  and  $\Delta_{47}$  values are in agreement with homeothermic and endothermic dinosaurs being able to raise and maintain their body temperature significantly above ambient

temperature, within, or close to, the known range of extant mammals and birds. These thermoregulatory capabilities allowed some non-avian dinosaurs to live at high latitudes (Rich *et al.* 2002) and to withstand seasonally or all year-round cold climates (Amiot *et al.* 2011). One interesting point is that some Late Cretaceous dinosaurs (hadrosaurids and non-avian theropods) were able to successfully reproduce at very high latitudes, as evidenced by the discovery of eggshell fragments recovered from the Maastrichtian Kakanaut Formation of Eastern Russia, located at a palaeolatitude of about 70–75°N (Godefroit *et al.* 2009). This discovery has raised questions about dinosaur reproductive capabilities and strategies in terms of incubation of their eggs. Fossil evidence has suggested that dinosaurs developed various strategies to incubate their eggs within a narrow temperature range by burying them, building nest mounds of decaying vegetation, active brooding, or using hydrothermal heat proximity (Seymour 1979; Clark *et al.* 1999; Deeming 2006; Grellet-Tinner & Fiorelli 2010; Varricchio *et al.* 2013; Tanaka *et al.* 2015). From a thermal perspective, these various strategies would result in different incubation temperatures and affect the geographical dispersal capabilities of these dinosaurs. In order to investigate the thermal relationship of dinosaur incubation strategies, we have tested a new method to infer embryo incubation temperature using the oxygen isotope compositions of both embryo bone phosphate and its eggshell carbonate.

As embryo bones mineralize from egg content at the egg incubation temperature, it is expected that the  $\delta^{18}\text{O}_p$  value of bone phosphate reflects both egg temperature and the  $\delta^{18}\text{O}_{ew}$  value of the water content in fluids from which it precipitates. A recent study has established a significant relationship between the  $\delta^{18}\text{O}_c$  value of bird eggshell carbonate and that of the egg albumen water, both originating from the mother's body fluids (Lizzerini *et al.* 2016). Therefore, it should be possible to estimate the oxygen isotope composition of the egg water from the eggshell  $\delta^{18}\text{O}_c$  value, and then calculate the bone formation temperature using the well-established oxygen isotope phosphate-water temperature scale (Lécuyer *et al.* 2013). In order to test this method on dinosaurs with a constrained incubation strategy, embryo bone and eggshell have been sampled from seven well preserved oviraptorosaur eggs recovered from the Upper Cretaceous Nanxiong Formation of Jiangxi Province, China. Oviraptorosaurs constitute a group of feathered maniraptoran theropods that existed during the Cretaceous on Laurasian continents (Osmólska *et al.* 2004). They are closely related to the avian lineage (Brusatte *et al.* 2014) sometimes even having been considered to be derived flightless birds (Maryanska *et al.* 2002; Osmólska *et al.* 2004). Direct evidence of active brooding in *Citipati* (Clark *et al.* 1999) and *Oviraptor* (Dong & Currie 1996), two representatives of the lineage, suggest that this behaviour was

common within the oviraptorosaurs, and thus narrows the expected incubation temperature to close to the mother's body temperature. Several factors control the embryo bone oxygen isotope composition of phosphate, including: water loss as vapour through the eggshell pores during incubation (Rahn & Ar 1974; Smart 1991), embryo metabolism (Hoyt *et al.* 1978), growth rate (Ricklefs 2010) and the timing of skeletal ossification (Maxwell 2009; Maxwell & Larsson 2009). Therefore we cannot simply use the temperature dependent phosphate–water oxygen isotope fractionation equation using bone  $\delta^{18}\text{O}_p$  and egg water  $\delta^{18}\text{O}_{ew}$  values estimated from eggshell calcite to get an accurate estimate of the temperature of embryo development. This is why a physiological model predicting the evolution of embryo bone  $\delta^{18}\text{O}_p$  value at various incubation temperatures and at changing  $\delta^{18}\text{O}_{ew}$  is proposed here. Our approach is based on, and scaled from, known fractionation factors and physiological traits of extant archosaurs (birds and crocodylians), and uses the measured bone  $\delta^{18}\text{O}_p$  values and egg water  $\delta^{18}\text{O}_{ew}$  values as boundary conditions. Predicted egg temperatures during incubation are then discussed in the light of expected incubation behaviour of oviraptorosaurs.

## MATERIAL AND METHOD

### Sample collection

Eggshell calcite and bone phosphate were sampled from seven elongatoolithid dinosaur eggs from the Upper Cretaceous Nanxiong Formation of Nankang District, Jiangxi Province, China (Fig. 1) containing embryonic skeletons. Five of these specimens are curated at the Institute of Vertebrate Paleontology and Paleoanthropology, Chinese Academy of Science, China (specimens IVPP V14723.2, V14723.3, V20182, V20183, V20184), one at the Geological Museum of China (GMV2212), and one at the Natural History Museum of Guangxi, China (NHMG10876). Three of these specimens, IVPP V20182, V20183 and V20184, have been described in a dedicated study and identified as belonging to the family Oviraptoridae (Wang *et al.* 2016).

To complement the existing dataset of oxygen isotope compositions of eggshell calcite and egg water from extant archosaurs, two eggs of the Nile crocodile *Crocodylus niloticus*, laid in 2012 at 'La Ferme aux Crocodiles', Pierrelatte, France, were sampled and analysed.

### Thin-sections and the microstructure of oviraptorosaur eggshells

Thin-sections of eggshell from specimens IVPP V14723.2, V14723.3, GMV2212 and NHMG10876 were cut at



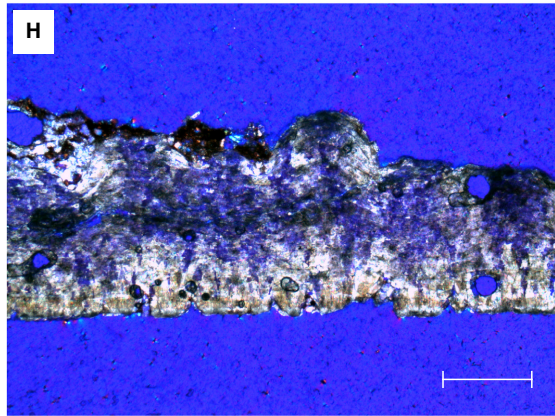
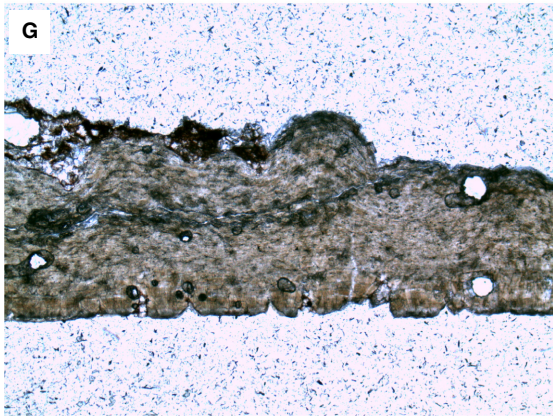
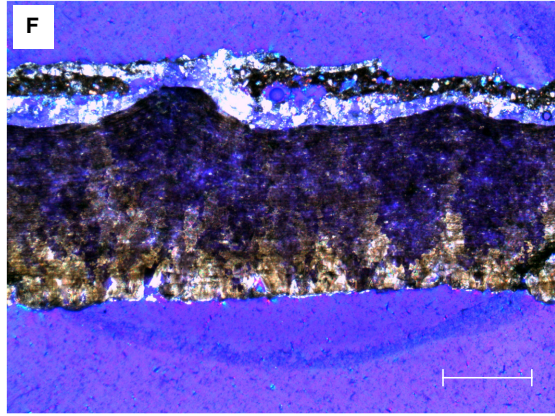
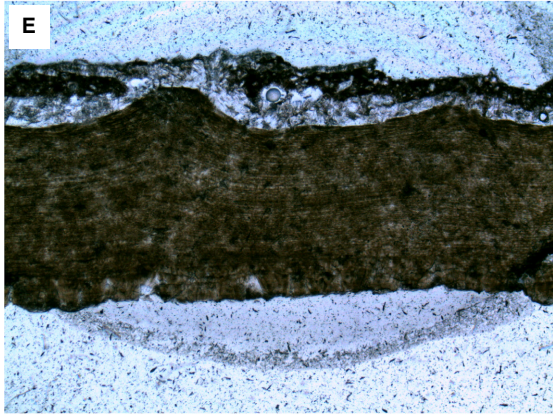
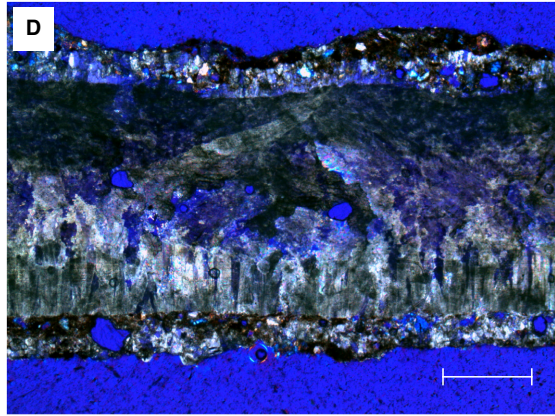
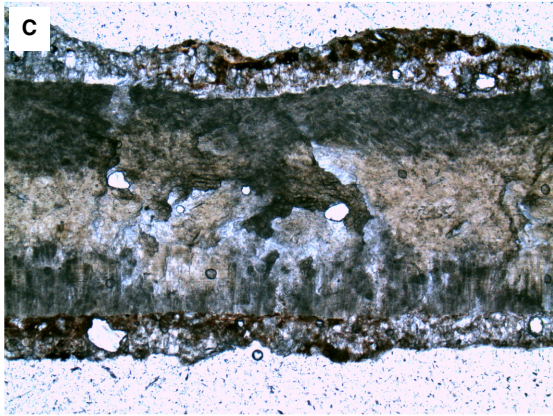
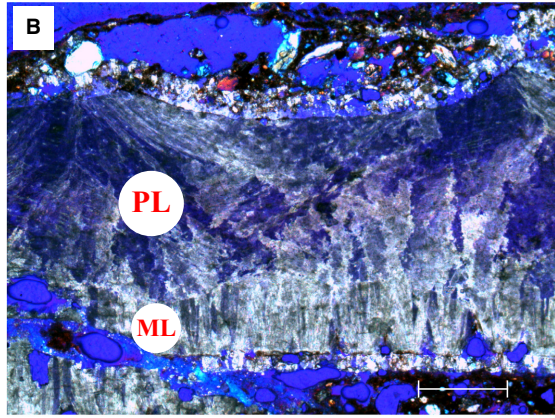
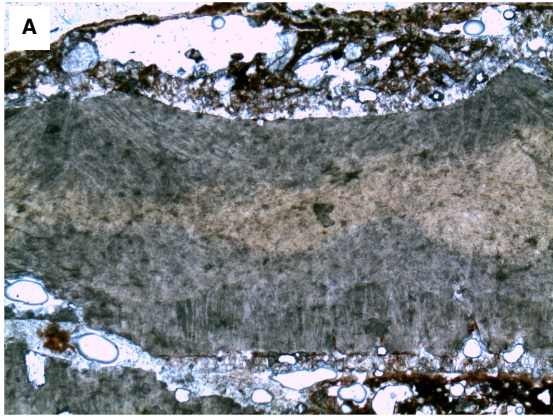
**FIG. 1.** Map showing the region of the Jiangxi Province of China (black diamond) where the elongatoolithid eggs with *in ovo* embryos have been recovered.

'Laboratoire Transferts Lithosphériques', in Saint-Etienne, France. These 30  $\mu\text{m}$ -thick slices of eggshell fragments cut perpendicular to the long axis of the shell were fixed to a glass slide with epoxy before examination with an optical microscope. Photomicrographs of the shell microstructure were taken at 40 $\times$  magnification (Fig. 2).

#### Isotopic analysis

A 3 mg aliquot of each oviraptorosaur embryo bone was ground into fine powder using a mortar and pestle. Powders from each sample were treated following the wet chemistry protocol described by Lécuyer (2004) and adapted by Bernard *et al.* (2009) for small sample weights (3 mg). This protocol involves wet chemical isolation of phosphate ions from apatite using acid dissolution followed by an anion-exchange resin separation and subsequent precipitation as  $\text{Ag}_3\text{PO}_4$  crystals. For each sample, the 3 mg of bone powder were dissolved in 1 mL of 2 M HF overnight. The  $\text{CaF}_2$  residue was separated by centrifugation and the solution neutralized by adding 1 mL of 2 M KOH. 1.5 mL of Amberlite<sup>TM</sup> anion-exchange resin

was added to the solution to separate the  $\text{PO}_4^{3-}$  ions. After 24 h, the solution was removed and the resin was eluted with 6 mL of 0.1 M  $\text{NH}_4\text{NO}_3$ . After 4 h, 0.1 mL of  $\text{NH}_4\text{OH}$  and 3 mL of an ammoniacal solution of  $\text{AgNO}_3$  were added and the samples were placed in a thermostated bath set at 70°C for 6 h allowing the precipitation of  $\text{Ag}_3\text{PO}_4$  crystals. Five aliquots of 350–400  $\mu\text{g}$  silver phosphate were mixed with 350–400  $\mu\text{g}$  pure powder graphite, loaded into silver foil capsules and pyrolysed at 1450°C using a TC-EA elemental analyser interfaced in continuous flow mode with an Isotopic Ratio Mass Spectrometer Finnigan MAT 253. Analysis was performed at the environmental isotope geochemistry laboratory of the Institute of Geology and Geophysics (Chinese Academy of Sciences, China). Measurements were calibrated against the NBS120c (natural Miocene phosphorite from Florida). The value of NBS120c was fixed at 21.7‰ (V-SMOW; Vienna Standard Mean Ocean Water) following Lécuyer *et al.* (1993) for correction of instrumental mass fractionation during CO isotopic analysis. Silver phosphate prepared from the NBS120c standard was repeatedly analysed ( $\delta^{18}\text{O}_\text{p} = 21.7 \pm 0.2\text{‰}$ ,  $n = 4$ ) along with the silver phosphate samples derived from the embryo bones to ensure



that no fractionation occurred during the wet chemistry. The average standard deviation was  $0.3 \pm 0.1\%$ .

Oviraptorosaur and extant crocodile eggshell fragments were cleaned in an ultrasonic bath with distilled water and dried at room temperature. Eggshells were then ground into fine powder in an agate mortar. For each sample, an aliquot of about 500  $\mu\text{g}$  was reacted with anhydrous oversaturated phosphoric acid at 90°C for 12 min and analysed using a MultiPrep™ automated system coupled to a dual-inlet Elementar Isoprime™ IRMS at the Laboratoire de Géologie de Lyon (LGL), France (Fourle *et al.* 2016). Carbon and oxygen isotope compositions are quoted in the  $\delta$  notation in per mil relative to V-PDB (Vienna Pee Dee Belemnite). All samples were measured in duplicate and adjusted to the international reference NIST NBS19 analysed within the same batch. External reproducibility is  $\pm 0.1\%$  for  $\delta^{18}\text{O}$  values and  $\pm 0.05\%$  for  $\delta^{13}\text{C}$  values ( $2\sigma$ ). Conversion of  $\delta^{18}\text{O}$  values of calcite from V-PDB to V-SMOW was performed using the Coplen *et al.* (1983) formula:  $\delta^{18}\text{O}$  (‰ V-SMOW) =  $\delta^{18}\text{O}$  (‰ V-PDB) \* 1.03091 + 30.91.

Crocodile egg albumens were carefully separated from the egg yolk after breaking the egg, then sampled with a syringe. About 3.5 mg of sodium azide ( $\text{NaN}_3$ ) per mL of albumen was added to prevent fermentation. Before analysis, albumen samples were stored in hermetic bottles in a refrigerator at 4°C. Five aliquots of 600  $\mu\text{L}$  of albumen for each egg were automatically reacted at 40°C with  $\text{CO}_2$  for 12 h to allow oxygen isotope equilibration between albumen water and  $\text{CO}_2$ , and analysed using a Multi-Prep™ automated system on line with a dual inlet Elementar Isoprime™ isotope ratio mass spectrometer (IRMS) at LGL, France (Lécuyer *et al.* 2009), along with drinking water samples. Measured oxygen isotope compositions of the five aliquots from each egg gave a standard deviation lower than 0.04%. Overall reproducibility of  $\delta^{18}\text{O}$  values is  $\pm 0.1\%$  by normalizing raw data to the isotopic ratios of V-SMOW2 (Vienna Standard Mean Ocean Water) and V-SLAP2 (Vienna Standard Light Antarctic Precipitation; Gröning *et al.* 2006)) international standards that were measured along with the samples.

### Model

A model of daily incremental deposition of oviraptorosaur embryo bone was designed based on the

following parameters, considerations and assumptions. Firstly, that after laying, the eggs were maintained at constant incubation temperature until they hatch, by active brooding. Due to embryo metabolism that increases with increasing body mass, egg temperature progressively raised during incubation (Swart & Rahn 1988; Ar 1991). The egg mass was estimated from egg dimensions using the relationship of Hoyt (1979):

$$M_{\text{egg}}(\text{g}) = 0.548 \times LB^2 \quad (1)$$

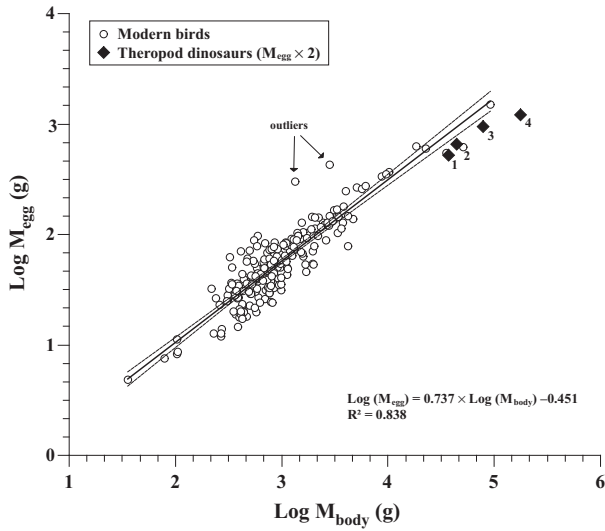
with  $L$  corresponding to the egg maximum length (in cm), and  $B$  to egg breadth (or maximum diameter, in cm). The proportions of egg contents were assumed to be similar to those of extant birds egg, thus eggshell mass corresponds to 11% of total egg mass, yolk mass corresponds to 32% and the white 57%. Water mass corresponds to 88% of the egg white and 49% of the yolk (Romanoff & Romanoff 1949). The adult body mass of the egg layer (a factor affecting the eggshell calcite – body water oxygen isotope fractionation; see below) was calculated using a relationship slightly modified from that established between modern bird egg mass and mother body mass (Werner & Griebeler 2013), and for which two outliers have been removed (Fig. 3). This relationship, established using an ordinary least square regression to fit a linear model to the data, assumes a theoretically well-constrained physical relationship between eggs and bird weight clearly linked to energetic constraints (part of the basic metabolism devoid to reproduction), anatomical constraints (pelvic dimension) and also to flight/running constraints that does not appear to show phylogenetic signal. This relationship was also adapted to theropod dinosaurs having two functional ovaries by doubling  $M_{\text{egg}}$  (see Discussion below):

$$\text{Log}(M_{\text{egg}} \times 2) = 0.737 \times \text{Log}M_{\text{body}} - 0.451 \quad (2)$$

where  $M_{\text{egg}}$  and  $M_{\text{body}}$  are measured in grams;  $R^2 = 0.84$  ( $n = 215$ ; F-value = 1100;  $p = 3.5 \times 10^{-87}$ ).

An embryo starts as a fertilized ovum with an assumed mass of 6.4 mg (Lee 2016). Hatchling mass represents about 70% of egg mass (Deeming & Birchard 2007). Total water loss during incubation is assumed to represent about 13% of egg mass (Şahan *et al.* 2003). Loss is principally from water vapour and is linear, an equal amount of water being lost each day (Smart 1991). The following fractionation equations have been used to predict the evolution of bone phosphate and egg water  $\delta^{18}\text{O}$

**FIG. 2.** Radial thin-sections of eggshell fragments from fossil specimens IVPP V14723.2 (A, B), IVPP V14723.3 (C, D), GMV2212 (E, F) and NHMG10876 (G, H) showing a good preservation of the original microstructure, in natural light (A, C, E, G), and in cross-polarized light (B, D, F, H). This would argue in favour of the pristine preservation of the stable oxygen isotope composition of eggshell calcite. Thin-sections of eggshell from fossil specimens IVPP V20182, V20183 and V20184 are shown and described in Wang *et al.* (2016). PL, prismatic layer; ML, mammilla layer. All scale bars represent 0.5 mm.



**FIG. 3.** Log relationship between egg mass ( $M_{\text{egg}}$ ) and adult body mass ( $M_{\text{body}}$ ) of extant birds based on the dataset of Werner & Griebeler (2013). The two outliers correspond to the little spotted kiwi (*Apteryx owenii*) and the southern brown kiwi (*Apteryx australis*) which have a peculiar reproductive strategy and lay one very large egg relative to their body mass. The black diamonds plotted close to the relationship established for extant birds correspond to the four theropod dinosaurs: 1, *Troodon formosus*; 2, *Oviraptor philoceratops*; 3, *Citipati osmolskae*; 4, *Lourinhanosaurus antunesi*. The egg mass of these theropods has been doubled to account for their two functional ovaries (see text).

values during egg incubation: the phosphate–water temperature scale proposed by Lécuyer *et al.* (2013) is used to calculate the oxygen isotope composition of bone phosphate increments:

$$T(^{\circ}\text{C}) = 117.4 - 4.5 \times (\delta^{18}\text{O}_{\text{p}} - \delta^{18}\text{O}_{\text{w}}) \quad (3)$$

with  $\delta^{18}\text{O}_{\text{p}}$  and  $\delta^{18}\text{O}_{\text{w}}$  being the oxygen isotope composition of embryo bone phosphate and egg water respectively, and  $T$  ( $^{\circ}\text{C}$ ) the egg temperature. The temperature-dependent liquid–vapour fractionation of water ( $\alpha_{\text{L-v}}$ ; Horita & Wesolowski 1994) is used to calculate the oxygen isotope composition of the water vapour lost by the egg:

$$10^3 \times \ln \alpha_{\text{L-v}} = -7.685 + 6.7123 \times (10^3/T) - 1.6664 \times (10^6/T^2) + 0.35041 \times (10^9/T^3) \quad (4)$$

where  $T$  the temperature in Kelvin. The daily growth of the embryo was estimated using the relationship proposed by Lee (2016) relating the embryo body mass  $m$  (in g) at a time  $t$  (in days) during the incubation:

$$m(t) = M_{\text{body}} \left\{ 1 - \left[ 1 - \left( \frac{m_0}{M_{\text{body}}} \right)^{0.25} \right] e^{\frac{-0.25 \times p \times t}{M_{\text{body}}^{0.25}}} \right\}^{1/0.25} \quad (5)$$

with  $m_0$  being the mass of the fertilized ovum (6.4 mg) and  $p$  the metabolic mass gain parameter calculated for

precocial birds ( $=27.46 \text{ kg}^{1/4}$ ) and assumed to be applicable to theropod dinosaurs (Lee 2016). All masses ( $m$ ,  $M_{\text{body}}$  and  $m_0$ ) are expressed in kg. From this relationship, the time  $t$  at which the embryo mass reaches the expected hatchling mass is considered as the total incubation time. Because the proportion of skeleton to total body mass in the embryo is not known, the mass of embryo bone was estimated using the scaling relationship between skeleton mass (kg) and total body mass (kg) established for extant birds (Prange *et al.* 1979):

$$M_{\text{skeleton}} = 0.065 \times M_{\text{body}}^{1.071} \quad (6)$$

As a first approximation, the ossification process is started at day 15 of incubation, following the model of ostriches (Maxwell 2009), which we consider to be extant dinosaur relatives having body mass and gross morphology close to those of oviraptorosaurs. From day 15 to hatching, egg temperature is linearly raised to reach  $+2.5^{\circ}\text{C}$  at the end of incubation to account for the increase in metabolic heat production, as observed in both chickens ( $+2.3^{\circ}\text{C}$ ) and ostriches ( $+2.6^{\circ}\text{C}$ ; Swart & Rahn 1988; Ar 1991).

For each daily step, the oxygen content of skeletal growth increment is calculated from the skeletal mass assuming that 70% of the bone is bioapatite, 30% of which is phosphate, and its  $\delta^{18}\text{O}$  value is calculated using the phosphate–water temperature scale (Eqn 3) at the egg temperature. This amount of oxygen taken up for bone deposition is subtracted from the egg water oxygen content and the  $\delta^{18}\text{O}$  value of the remaining water is calculated assuming a Rayleigh distillation process:

$$\delta^{18}\text{O}_{\text{remaining water}} = (\delta^{18}\text{O}_{\text{initial water}} + 1000) \times f^{(\alpha-1)} - 1000, \quad (7)$$

where  $f$  is the fraction of remaining water oxygen and  $\alpha$  the phosphate–water fractionation factor at the given temperature ( $\delta^{18}\text{O}_{\text{w1}}$  in Amiot *et al.* 2017). Finally, the daily water oxygen loss by evaporation is subtracted from the egg water oxygen reservoir and the subsequent  $\delta^{18}\text{O}$  value of remaining water is calculated using a Rayleigh distillation process, with  $\alpha$  being the liquid–vapour fractionation factor at the given temperature ( $\delta^{18}\text{O}_{\text{w2}}$  in Amiot *et al.* 2017). The model stops when the expected body mass of the hatchling is reached.

## RESULTS

### Extant crocodylians and birds

The two Nile crocodiles have eggshell  $\delta^{18}\text{O}_{\text{c}}$  values of 22.5‰ and 25.1‰ (V-SMOW), and corresponding albumen water  $\delta^{18}\text{O}_{\text{ew}}$  values of  $-7.9$ ‰ and  $-5.5$ ‰

**TABLE 1.** Extant Nile crocodile oxygen and carbon isotope compositions of eggshell calcite ( $\delta^{18}\text{O}_c$ ) and albumen waters ( $\delta^{18}\text{O}_{ew}$ ).

Sample number	Taxon	$\delta^{13}\text{C}_c$ (‰ V-PDB)	$\delta^{18}\text{O}_c$ (‰ V-SMOW)	$\delta^{18}\text{O}_{ew}$ (‰ V-SMOW)
Croco1	<i>Crocodylus niloticus</i>	-14.8	25.1	-5.5
T0	<i>Crocodylus niloticus</i>	-15.6	22.5	-7.9

(V-SMOW), respectively (Table 1). The corresponding calcite–water fractionation factor  $\alpha_{c-ew}$  calculated for crocodiles is  $1.03067 \pm 0.00000$ . Pooling this fractionation factor with those established for five extant birds (the common quail *Coturnix coturnix*, the chicken *Gallus gallus*, the Egyptian goose *Alopochen aegyptiaca*, the mallard duck *Anas platyrhynchos* and the ostrich *Strutio camelus*; Table 2; Lazzerini *et al.* 2016), no relationship was observed between  $\alpha_{c-ew}$  and body temperature (Fig. 4A). However, a significant relationship was observed with adult body mass, and an ordinary least squares regression was used to fit a linear model to the data (Fig. 4B):

$$10^3 \times \ln(\alpha_{c-ew}) = 1.000 \times \text{Log}(M_{\text{body}}) + 25.185 \quad (8)$$

where  $M_{\text{body}}$  (in g) is the body mass of the adult egg layer;  $R^2 = 0.95$  ( $n = 6$ ; F-value = 71.68;  $p = 2.8 \times 10^{-5}$ ).

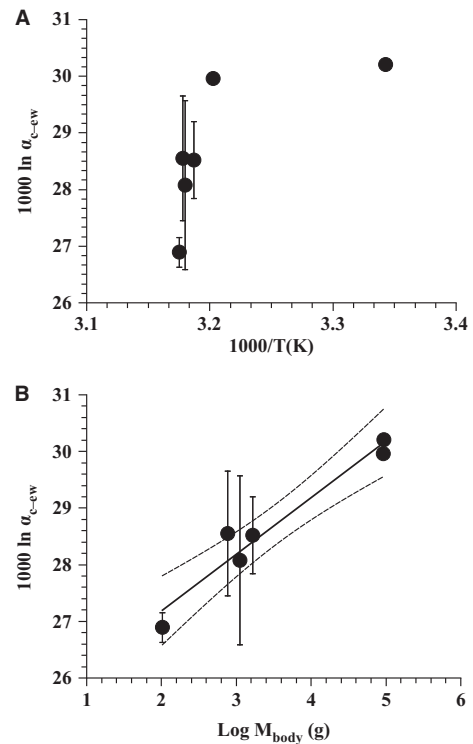
*Oviraptorosaur eggs*

Embryo bone phosphate  $\delta^{18}\text{O}_p$  values range from 11.8‰ to 16.1‰ (V-SMOW) and eggshell  $\delta^{18}\text{O}_c$  values range from 24.3‰ to 30.8‰ (V-SMOW; Table 3, Fig. 5). Egg dimensions, egg masses and adult body masses were

**TABLE 2.** Fractionation factor  $\alpha$  determined between eggshell calcite and albumen water of extant birds (Lazzerini *et al.* 2016) and the Nile crocodile (this study); body temperature, egg mass and adult body mass (Werner & Griebeler 2013).

Taxon	$\alpha_{c-ew}$		$T_{\text{body}}$ (°C)	Adult $M_{\text{body}}$ (g)	$M_{\text{egg}}$ (g)
	Mean	SD			
<i>Coturnix coturnix</i>	1.0273	0.00027	41.8	103	8.2
<i>Gallus gallus</i>	1.029	0.00113	41.5	767.5	29.6
<i>Anas platyrhynchos</i>	1.0285	0.00153	41.3	1123	49.9
<i>Alopochen aegyptiaca</i>	1.0289	0.00070	40.6	1650	95.7
<i>Struthio camelus</i>	1.0304	n.a.	39.1	91 750	1500
<i>Crocodylus niloticus</i>	1.0307	n.a.	26.0	94 200	107.1

estimated and allowed the  $\delta^{18}\text{O}_{ew}$  values of egg water to be calculated (Table 3). Values ranged from  $-5.7\text{‰}$  to  $+0.2\text{‰}$  and should correspond to the initial oxygen isotope composition of egg water when the egg was produced. Using these  $\delta^{18}\text{O}_{ew}$  values, the model was run for incubation temperatures of 30, 32, 34, 36, 38 and 40°C and allowed us to reproduce the embryo  $\delta^{18}\text{O}_p$  value for two of the seven specimens (IVPP V14723.2 and V14723.3) and also for specimen NHMG10876 by lowering the amount of egg water loss (Fig. 6; see Discussion below). According to the model, these embryos would have been incubated at temperatures ranging from 35°C to 40°C (Fig. 6; Amiot *et al.* 2017). The remaining specimens (GMV2212, IVPP V20182, V20183 and V20184) had embryo  $\delta^{18}\text{O}_p$  values completely out of the expected range, corresponding to unlikely incubation temperatures far above 45°C (Fig. 7). This hints at a possible alteration of the original isotope compositions of either (or both) eggshell calcite or embryo bone, despite apparently good preservation of egg or bone microstructure (Wang *et al.* 2016).

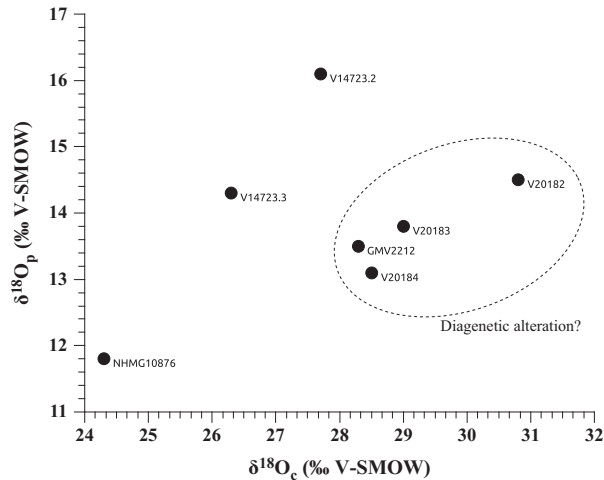


**FIG. 4.** A, body temperature expressed as  $1000/T$  (K) is plotted against the oxygen isotope fractionation factor between eggshell calcite and egg water. B, log adult body mass plotted against the oxygen isotope fractionation factor between eggshell calcite and egg water; note the significant relationship between the two variables described in the text as Equation 8:  $10^3 \times \ln(\alpha_{c-ew}) = 1.000 \times \text{log}(M_{\text{body}}) + 25.185$  ( $R^2 = 0.95$ ).

**TABLE 3.** Oxygen and carbon isotope compositions of oviraptorosaur eggshells,  $\delta^{18}\text{O}_p$  values of embryo bones, egg dimensions, and estimated egg mass, hatching and adult body masses, incubation time and the  $\delta^{18}\text{O}_{\text{ew}}$  value of egg water.

Sample number	Taxon	Measured				Estimated					
		Egg length (cm)	Egg width (cm)	$\delta^{13}\text{C}_c$ (‰ V-PDB)	$\delta^{18}\text{O}_c$ (‰ V-SMOW)	$\delta^{18}\text{O}_p$ (‰ V-SMOW)	$M_{\text{egg}}$ (g)	Adult $M_{\text{body}}$ (g)	Hatching mass (g)	Incubation time (days)	$\delta^{18}\text{O}_{\text{ew}}$ (‰ V-SMOW)
IVPP V14723.2	Oviraptorosauria indet.	19–20	8–9	–10.3	27.7	16.1	888	105 224	622	53	–2.8
IVPP V14723.3	Oviraptorosauria indet.	19–20	9–10	–10.2	26.3	14.3	1096	140 005	767	56	–4.4
GMV2212	Oviraptorosauria indet.	18–19	8–9	–9.6	28.3	13.5	843	98 055	622	53	–2.3
NHMG10876	Oviraptorosauria indet.	14–15	6–7	–9.3	24.3	11.8	403	36 017	282	44	–5.7
IVPP V20182	Oviraptorosauria indet.	19.83	8.8	–9.9	30.8	14.5	842	97 897	589	52	0.2
IVPP V20183	Oviraptorosauria indet.	16.35	7.48	–11.0	29.0	13.8	501	48 394	351	46	–1.3
IVPP V20184	Oviraptorosauria indet.	17.95	9.21	–10.7	28.5	13.1	834	96 637	584	52	–2.0



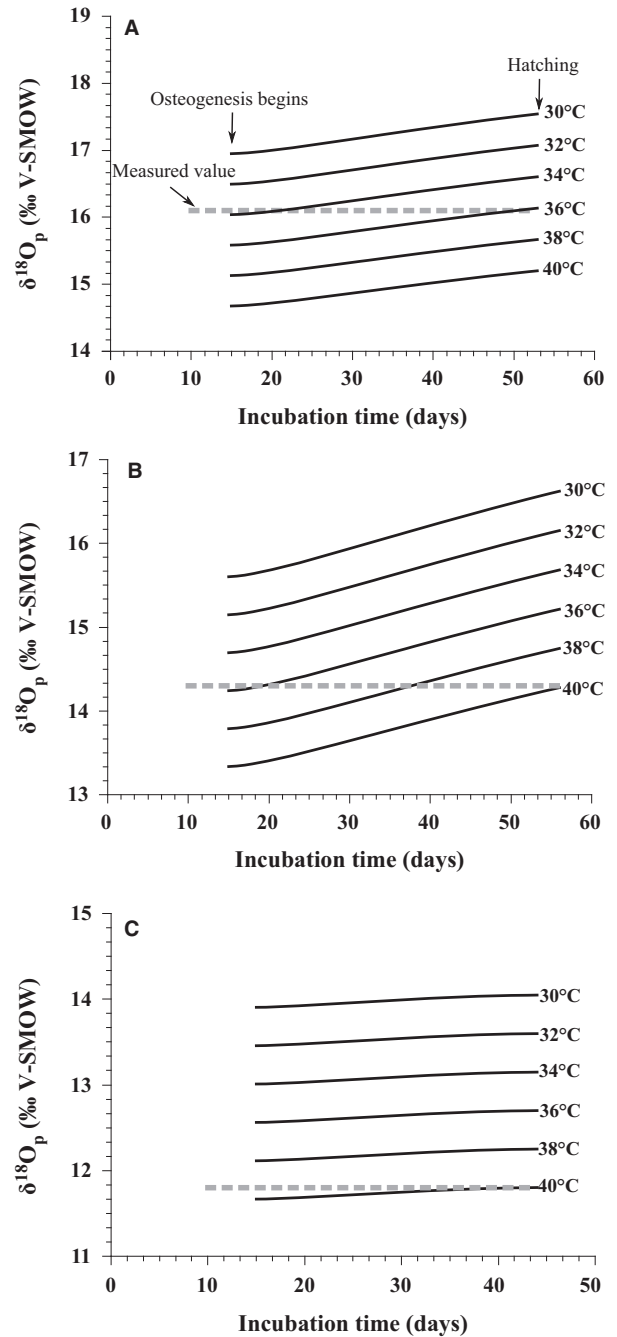


**FIG. 5.** Oxygen isotope compositions of eggshell calcite plotted against their corresponding oxygen isotope composition of embryo bone phosphate. Data points circled with a dotted line represent anomalous  $\delta^{18}\text{O}_p - \delta^{18}\text{O}_c$  pairs that result in an overestimated incubation temperature and hint at a possible diagenetic alteration of either the oxygen isotope composition of phosphate (values too low) or carbonate (values too high).

## DISCUSSION

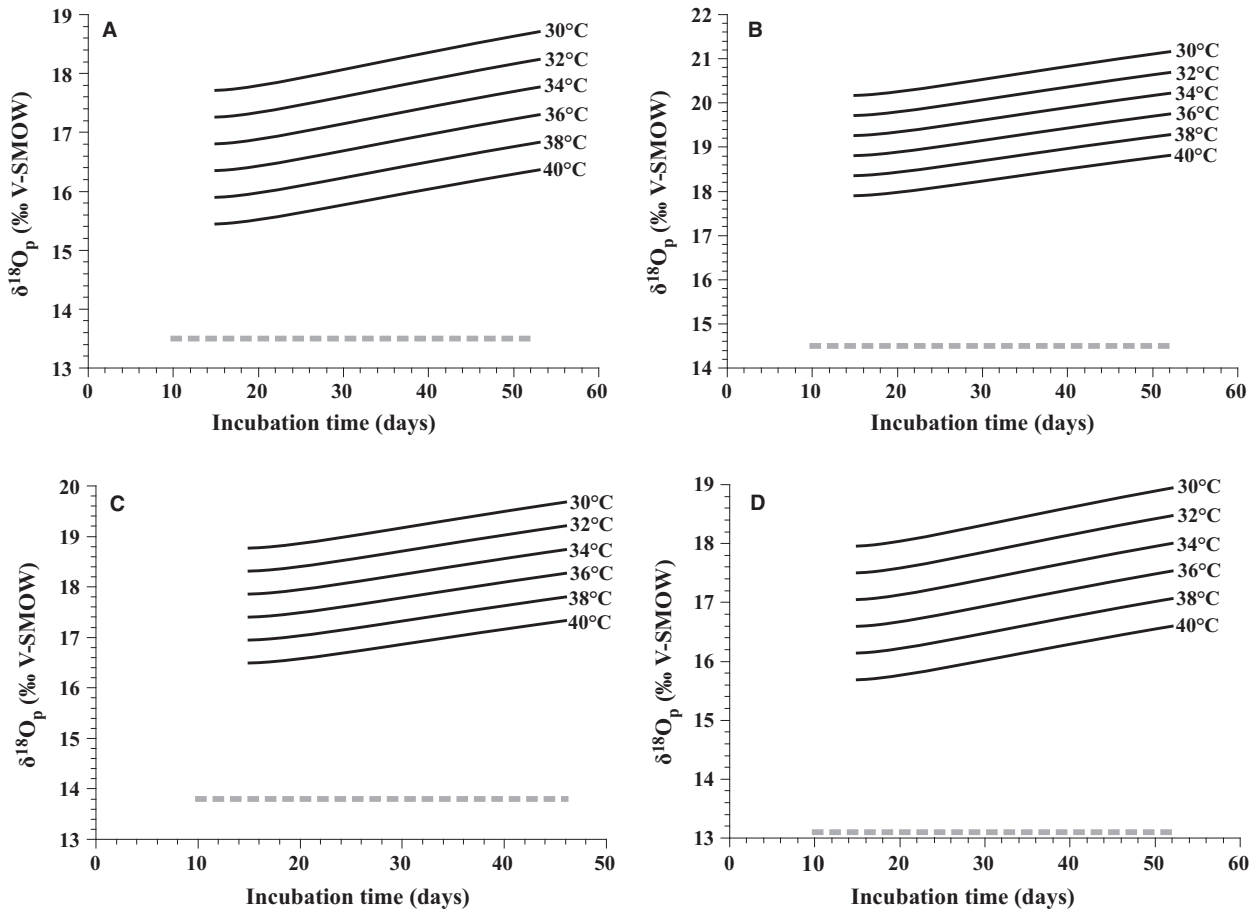
*On the allometric relationship established between birds egg mass and adult body mass*

It is well known that body mass and reproductive investment in terms of egg mass, clutch mass or annual clutch mass, are highly correlated in extant reptiles and birds (Rahn *et al.* 1975; Blueweiss *et al.* 1978). In a study devoted to the relationship between body mass and reproductive traits, Werner & Griebeler (2013) observed that the egg masses of dinosaurs, including theropods, match neither the egg masses of similar-sized or scaled-up birds nor those of reptiles, but were in between. However, when considering the clutch size or annual clutch size, the relationship for theropod dinosaur fits the bird model. Whereas Werner & Griebeler (2013) invoked peculiar reproductive strategies in theropod dinosaurs to account for this conflicting pattern, another explanation might reconcile this apparent discrepancy. As evidenced by the fossil record, oviraptorids, and most likely all non-avian dinosaurs had two functioning ovaries that allowed them to produce paired eggs in a monoautochronous sequence (Sato *et al.* 2005). Most birds, on the other hand, have one functional ovary and oviduct (the left one, as the right one regresses during development and is nonfunctional in the adult bird) and therefore produce one egg at a time. In terms of reproductive cost, the egg as a 'unit' cannot be considered equivalent in modern birds and dinosaurs, but two eggs in dinosaurs would



**FIG. 6.** Outputs of the model predicting the temporal evolution of the  $\delta^{18}\text{O}_p$  value of oviraptorosaur embryo bone phosphate as a function of the embryo body temperature. Total embryo bone  $\delta^{18}\text{O}_p$  value is plotted against incubation time for temperatures ranging from 30 to 40°C (black lines) and compared to the measured  $\delta^{18}\text{O}_p$  value of the embryo bone of unknown growth stage (dashed grey line). A, IVPP V14723.2. B, IVPP V14723.3. C, NHGM10876.

correspond to one egg in modern birds. By multiplying theropod egg mass by two, theropods match the egg mass to body mass relationship of similar-sized or scaled-up



**FIG. 7.** Outputs of the model as in Figure 6 for specimens that probably do not preserve their initial oxygen isotope compositions of eggshell calcite. A, GMV2212. B, IVPP V20182. C, IVPP V20183. D, IVPP V20184.

birds (Fig. 3). The relevance of this consideration is supported by the fact that theropod clutch mass (and annual clutch mass) also matches that of similar size or scaled-up birds, because in a clutch or annual clutch, all eggs are accounted for in both birds and non-avian theropods, regardless of their timing of laying. Consequently, the egg mass to body mass allometry established for birds should be applied to dinosaurs considering ( $M_{\text{egg}} \times 2$ ), as proposed in Equation 2.

#### *Primary preservation of the stable isotope compositions*

The methodology proposed in this study requires that both the eggshell calcite and embryo bone apatite phosphate preserve their pristine oxygen isotope compositions. To assess the structural preservation of these biominerals, eggshell thin-sections were inspected under a light microscope. The eggshell is composed of a mammillary layer and a columnar layer separated by a clear boundary. The columnar layer is characterized by undulating growth

lines lying parallel to the outer surface of eggshell and visible in radial sections (Fig. 2). These features are diagnostic for the oofamily Elongatoolithidae (Zhao 1975) and indicate apparently good preservation of the original calcitic structure of the eggshell. Published thin-sections have also been cut in embryo long bones of specimens IVPP V20182, V20183 and V20184, and show a typical cortex formed by fibrolamellar bone containing numerous elongate to circular vascular canals. This cortex encircles the medullary cavity, a feature observed in all known embryonic dinosaurs (Wang *et al.* 2016). This in turn would support the preservation of the original oxygen isotope composition of phosphate, as these structures cannot result from any diagenetic processes.

One possible way to assess the state of isotopic preservation of the eggshell and embryo  $\delta^{18}\text{O}_p$ – $\delta^{18}\text{O}_c$  pairs is to compare variation among the seven specimens. As endothermic animals, oviraptorosaurs maintained a constant body temperature, so the eggshell precipitated at constant body temperature should only reflect the oxygen isotope composition of the mother's body fluids. These

body fluids are also transmitted to the egg via swelling or plumping, a process taking place at the beginning of and during eggshell construction (Fertuck & Newstead 1970). During incubation, embryo bone precipitates from these fluids. Considering a constant incubation temperature, it should therefore be expected that the bone phosphate  $\delta^{18}\text{O}_p$  value of the embryo should also reflect the oxygen isotope composition of the mother's body fluids. Consequently, it should be expected that  $\delta^{18}\text{O}_p$  and  $\delta^{18}\text{O}_c$  values follow a close to linear relation, keeping in mind that water loss of individual eggs during incubation might add some scattering. Interestingly, Figure 5 shows that the samples IVPP V14723.2, V14723.3 and NHMG10876 have  $\delta^{18}\text{O}_p$ – $\delta^{18}\text{O}_c$  pairs that seem to follow a straight line, whereas GMV2212, IVPP V20182, V20183 and V20184, having the highest  $\delta^{18}\text{O}_c$  values, seem to be clustered. The modeled incubation temperature for these specimens that would fit both  $\delta^{18}\text{O}_p$  and  $\delta^{18}\text{O}_c$  values that are out of the known range for living animals ( $\gg 45^\circ\text{C}$ ), the estimated  $\delta^{18}\text{O}_{ew}$  values being too high relative to the embryo  $\delta^{18}\text{O}_p$  values. Because embryo phosphate  $\delta^{18}\text{O}_p$  values of GMV2212, IVPP V20182, V20183 and V20184 are within the range of other specimens, but the eggshell carbonate  $\delta^{18}\text{O}_c$  values are more positive, we suspect that some exogenous carbonate may have affected the measured eggshell  $\delta^{18}\text{O}_c$  values, despite the careful cleaning of the eggshells and the microscope observations showing no apparent signs of extensive recrystallization. Pending additional analyses, specimens GMV2212, IVPP V20182, V20183 and V20184 are considered to have been affected by diagenetic alteration, and will not be considered further in the subsequent discussion.

#### *Ecological specificities of the oviraptorosaur theropods from Jiangxi*

The Nanxiong Formation has yielded at least eight oviraptorid taxa, including *Banji long* (Xu & Han 2010), *Ganzhousaurus nankangensis* (Wang *et al.* 2013), *Jiangxisaurus ganzhouensis* (Wei *et al.* 2013), *Heyuannia huangi* (Lü 2002), *Shixinggia oblita* (Lü & Zhang 2005), *Huanansaurus ganzhouensis* (Lü *et al.* 2015), *Nankangia jiangxiensis* (Lü *et al.* 2013, 2016) and *Tongtianlong limosus* (Lü *et al.* 2016). According to Equation 2, the body mass of the oviraptorosaurs that laid the Jiangxi eggs ranged from about 36 kg to 140 kg (Table 3), which is comparable to other estimates ranging from 20 kg to 75 kg for some of the Jiangxi taxa (Paul 2016).

Estimated incubation duration from Equation 5 ranges from about 44 to 56 days, close to those estimated using the same equation for *Citipati osmolskai* (44 days) and *Oviraptor philoceratops* (40 days; Lee 2016). It is noteworthy that these durations are significantly shorter than the

3- to 6-month period recently estimated for ornithischian dinosaurs using daily incremental growth line count of embryo teeth (Erickson *et al.* 2017). It is possible that the model developed by Lee (2016), based on modern bird physiological characteristics, gives too short estimates of incubation duration for theropods (for example, the metabolic mass gain parameter  $p$  of precocial birds is used to infer theropod embryo mass gain (Lee 2016)). However, the study performed by Erickson *et al.* (2017) only includes ornithischian dinosaurs that may have retained reptile-grade reproductive physiology whereas theropods close to the avian lineage (like oviraptorosaurs) may already have acquired bird-like reproductive physiology traits.

The clutches of oviraptorosaurs are arranged to form a spiral ring of two to three tiers of subhorizontally-oriented eggs while their centre is devoid of eggs. The adult oviraptorosaur sat in the center of the clutch with their arms surrounding the eggs, in a position comparable to that of brooding birds, as evidenced by at least four specimens fossilized in brooding position (Dong & Currie 1996; Clark *et al.* 1999). Active brooding behaviour seems to be well supported by the paired  $\delta^{18}\text{O}_p$ – $\delta^{18}\text{O}_c$  values of embryo phosphate and eggshell carbonate with predicted temperature of embryo bone formation within the 35–40°C range. As mentioned in the Results above, sample NHGM10876 fits the range of possible embryo bone formation temperatures if water loss is lowered by two thirds relative to predicted daily loss (Fig. 6). This specimen has the lowest eggshell  $\delta^{18}\text{O}_c$  value, which in turn implies a low value of mother body water as well as of environmental drinking water. According to Lazzarini *et al.* (2016), terrestrial birds display a drinking water-body water  $^{18}\text{O}$ -enrichment of about +4‰ (at least in ostrich and chicken). Based on the egg water  $\delta^{18}\text{O}_{ew}$  value of –5.7‰ calculated for NHGM10876, a  $\delta^{18}\text{O}_w$  value of local surface waters of about –9.7‰ can be estimated. During the deposition of the Nanxiong Formation, the Jiangxi Province was located at a low palaeolatitude of about 20°N (Cogné *et al.* 2013). Such a low  $\delta^{18}\text{O}_w$  value in subtropical to tropical latitudes could be the signature of local rainwater characterized by high amount of seasonal precipitation, such as monsoon rains experienced today, or the influence of high altitude precipitations from nearby mountain ranges supplied to local rivers. Whereas the latter hypothesis is difficult to test due to the lack of regional palaeoenvironmental studies, the low eggshell  $\delta^{13}\text{C}$  values, ranging from –11.0‰ to –9.3‰ (V-PDB), seems to support a regime of elevated amounts of precipitation. Indeed, the carbon isotope composition of eggshell calcite reflects the  $\delta^{13}\text{C}$  value of the mother's diet with a fractionation controlled by its digestive physiology (Johnson *et al.* 1998; Angst *et al.* 2014). For Mesozoic terrestrial vertebrates, the  $\delta^{13}\text{C}$  value of their

eggshells ultimately reflects that of local C<sub>3</sub> vegetation consumed either directly (plant eater) or indirectly (predator of a plant eater), the carbon isotope composition of which is controlled by the amount of local precipitation and aridity (Diefendorf *et al.* 2010; Kohn 2010). Montanari *et al.* (2013) analysed the  $\delta^{13}\text{C}$  values of oviraptorosaur eggshells (oofamily Elongatoolithidae) from the Upper Cretaceous Djadokhta ( $-5.2\text{‰}$  to  $-4.6\text{‰}$ ) and Nemegt ( $-5.6\text{‰}$ ) formations of Mongolia, contemporaneous to the Nanxiong Formation and located at mid palaeolatitudes ( $\sim 45^\circ\text{N}$ ). They interpreted these high values as reflecting local vegetation growing in a semi-arid desert most likely dominated by gymnosperms (Montanari *et al.* 2013). With significantly lower carbon isotope composition of carbonate, Jiangxi eggshells and especially the specimen NHGM10876 strongly suggest that these oviraptorosaurs had consumed directly or indirectly local plants growing under wet conditions before laying their eggs. This wet period that could be seasonal was probably characterized by elevated amounts of precipitation as suggested by the low  $\delta^{18}\text{O}$  value of the eggshell. The atmosphere would in turn be saturated in vapour pressure, thus reducing egg water loss during incubation.

The estimated temperature of embryo bone formation range of 35–40°C for oviraptorosaurs falls within the known range for extant birds (Rahn 1991). It implies a slightly lower incubation temperature (due to heat loss during brooding) and an adult body temperature within the range of modern endotherms. According to some stable oxygen isotope analyses and clumped isotope thermometry of bone and teeth phosphate, dinosaurs were warm blooded (Barrick & Showers 1994; Fricke & Rogers 2000) their body temperature being raised and kept at a constant level within the 36–38°C range (Amiot *et al.* 2006; Eagle *et al.* 2011). However, the clumped isotope analysis of oviraptorid eggshells of Mongolia gave a formation temperature estimate of  $31.9 \pm 2.9^\circ\text{C}$  (Eagle *et al.* 2015). This one exception is quite surprising, oviraptorosaurs being closely related to the avian lineage (Brusatte *et al.* 2014) and sometimes having even been considered to be derived birds (Maryanska *et al.* 2002). Further investigation is needed to determine whether these oviraptorosaur eggshell samples constitute an isolated case or if large variation in body temperature existed within the Dinosauria.

## CONCLUSION

The combined analysis of the stable oxygen isotope compositions of eggshell carbonate and embryo bone phosphate from oviraptorosaur eggs preserved with *in ovo* embryos has allowed their incubation temperatures to be estimated. This work acts as a methodological study to test whether an incubation temperature could be

extracted, given that active brooding behaviour among oviraptorosaurs is already demonstrated by the discoveries of adult skeletons fossilized sitting on their egg clutches. From the seven available specimens, three yielded consistent temperature of embryo bone formation within the 35–40°C range, comparable to that of extant birds. Applying this method to other non-avian dinosaurs as well as to other egg-laying amniotes may provide new insight into the environmental constraints that influenced the geographical distribution of biodiversity in terms of reproductive strategies and capabilities.

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## DATA ARCHIVING STATEMENT

Data for this study are available in the Dryad Digital Repository: <https://doi.org/10.5061/dryad.k14tt>

*Editor.* Roger Benson

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