Shifts in eggshell thickness are related to changes in locomotor ecology in dinosaurs

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Birds share an array of unique characteristics among extant land vertebrates. Among these, external and microstructural characteristics of extant bird eggs have been linked to changes in reproductive strategy that arose among non-avian theropod dinosaurs. More recently, differences in egg proportions recovered in crown birds relative to other dinosaurs were suggested as possibly linked to avian flight, but dense sampling close to its proposed origin was lacking. Here we assess the evolution of eggshell thickness in a targeted sample of 114 dinosaurs including birds, and test the relationship of eggshell thickness with potential life history correlates and locomotor mode using phylogenetic comparative methods. Only egg mass and flight are identified as significant predictors of eggshell thickness. While a high correlation between egg mass and eggshell thickness is expected, that relationship is much stronger in flying taxa, which show a significantly higher slope and lower residual variance than flightless species. This suggests stabilizing selection of eggshell thickness among theropods, as recovered for other traits in extant birds (e.g. genome size, metabolic rate). Within living birds, Eufalconimorphae present an apomorphic increase in relative eggshell thickness which remains unexplained, as few morphological synapomorphies of this clade have been identified.

KEY WORDS: Aves, bird, Dinosauria, eggs, flight, phylogenetic comparative methods.

The amniotic egg, a major vertebrate innovation, has been the subject of many studies investigating structural, environmental, and phylogenetic constraints on its evolution (Reisz 1997; Sander 2012). Many of these studies have focused on the eggs of extant birds, which have developed a wide variety of egg shapes and sizes compared to their extinct dinosaurian relatives (Deeming and Ruta 2014; Stoddard et al. 2017). Comparative studies have tested the influence of many life history traits on extant avian egg shape and volume, identifying several of them (e.g., flight, egg content, nest site, and type) as significant correlates (Deeming and Ruta 2014; Stoddard et al. 2017; Mytiai et al. 2017; Duursma et al. 2018; Deeming 2018; Nagy et al. 2019; Birkhead et al. 2019). In this context, various equations and variables have also been proposed to estimate egg shape and volume in birds, with no current consensus on the relative accuracy of such procedures (Biggins et al. 2018; Stoddard et al. 2019). Additionally, known correlates of egg size and shape (e.g., clutch size, clutch mass, incubation period) have shown allometric scaling with body mass (Deeming 2007a,b; Ar et al. 1974, Ar et al. 1979; Rahn et al.

1975; Deeming and Birchard 2007; Birchard and Deeming 2015), as well as a high phylogenetic signal (Deeming 2007a,b; Deeming and Birchard 2007; Birchard and Deeming 2015).

The calcified eggshell of most archosaurs is a highly variable character (Mikhailov et al. 1996; Mikhailov 1997; Zelenitsky and Therrien 2008), and its microstructure has been used as a taxonomic proxy for fossil eggs, most prominently in non-avian dinosaurs (Mikhailov 1991, 1997) – albeit not without controversy (Zelenitsky and Therrien 2008; Varricchio and Barta 2015). For this reason, the role of eggshell microstructure in water and gas exchange has been well studied in bird eggs (Ar et al. 1974; Paganelli 1980; Rahn and Paganelli 1989), and used to infer the developmental and nesting strategies of non-avian dinosaurs (Hechenleitner et al. 2016b; Seymour 1979; Deeming 2006; Jackson et al. 2008; Varricchio et al. 2013; Tanaka et al. 2015). Similarly, eggshell microstructural traits (e.g., thickness, pore, and shell unit characteristics) have been described as major regulating factors of nutrient exchange and physiological properties in avian (Ar et al. 1974; Paganelli 1980, 1991; Ar and Rahn

1985) and non-avian reptilian eggs (Ackerman et al. 1985; Deeming and Thompson 1991). However, these parameters have rarely been the subject of quantitative studies in birds and non-avian dinosaurs. Parsimony-based optimizations of discrete eggshell-related traits have been performed for dinosaurs including birds (Grellet-Tinner 2006; Grellet-Tinner et al. 2006), but the evolution of eggshell thickness clade-wide using phylogenetic comparative methods was only recently assessed in three studies, all of which focused on differences between hard- and soft-shelled eggs (Stein et al. 2019; Legendre et al. 2020; Norell et al. 2020). These studies did not test for life history or locomotor correlates for evolutionary trends of eggshell thickness among dinosaurs, and showed limited sampling for Theropoda including Aves.

Eggshell thickness has also been studied in the context of biomechanical constraints on embryonic development with smaller taxonomic samples. On the one hand, contact incubation as documented in birds has been hypothesized as a selective pressure for increased relative eggshell thickness in birds relative to that of other extant reptiles, implying a lower limit of avian eggshell thickness (Birchard and Deeming 2009; Huynen et al. 2010). On the other hand, an upper allometric constraint, linked with the need for hatchlings to manage to break out of the shell, has been proposed as a limiting factor for eggshell thickness and egg size in large dinosaurs (Schmidt-Nielsen 1984; Alexander 1989; Birchard and Deeming 2009; Deeming and Birchard 2009). These studies suggested that archosaur eggshell thickness may be a highly constrained character, with shifts potentially correlated with novel life history traits (Ar et al. 1974; Deeming 2006; Birchard and Deeming 2009; Peterson et al. 2020). Flight in extant birds has also been proposed to influence or constraint physiological and life history traits (Prange et al. 1979; Lee et al. 2014; Ji and DeWoody 2017; Benson et al. 2018), potentially explaining the acquisition of smaller genome sizes (Waltari and Edwards 2002; Organ et al. 2007; Wright et al. 2014; Kapusta et al. 2017) and higher metabolic rates (Hudson et al. 2013; Lovegrove 2017; Rezende et al. 2020), both of which tend to be much less variable in birds than in other reptiles (Gregory 2004; Kapusta et al. 2017). Stabilizing selection on an array of life history traits has also been proposed for other aerial locomotors among amniotes such as bats (Amador et al. 2020), or gliding squamates and mammals (Ord et al. 2020).

Recently, a shared allometric relationship between eggshell thickness and egg mass similar to that previously identified in Aves (Ar et al. 1974, Ar et al. 1979; Rahn et al. 1975; Birchard and Deeming 2009) was recovered for a sample of eggs from extant birds (n = 46) and non-avian reptiles (n = 9), as well as non-avian dinosaurs (n = 37). The authors performed ancestral state reconstructions for a subsample of 20 taxa, and did not recover any trend in eggshell thickness specific to birds (Stein et al. 2019). Another study, using a sample of amniotes (n = 148, including 50 birds and 39 non-avian dinosaurs) including that of Stein et al. (2019), identified another significant relationship between egg mass and eggshell thickness specific to hard-shelled eggs (Legendre et al. 2020). This study also performed ancestral state reconstructions for all species in its sample, and preliminarily identified dinosaur subclade-specific trends; sauropods and secondarily-flightless paleognath birds shared low values of relative eggshell thickness (i.e. ratio eggshell thickness/egg mass), while volant extant birds presented much higher values (Legendre et al. 2020). While not the focus of Legendre et al. (2020), these results suggested that there may be different scaling relationships between eggshell thickness and mass in different subclades of dinosaurs, and some may be marked between volant and non-volant taxa. However, these studies did not include the dense taxonomic sampling germane to specifically assessing evolutionary patterns in dinosaurian eggshell thickness: it did not densely sample eggs of non-avian dinosaurs closely related to crown birds, which could help understand if potential differences in scaling relationship were influenced by the acquisition of flight.

Here we estimate phenotypic trends and evolutionary rates of eggshell thickness for avian and non-avian dinosaurs, with an increased extinct theropod sample (Table S1). We again test for a significant and shared relationship between eggshell thickness and egg mass across Dinosauria. We further assess whether this relationship itself shows subclade-specific shifts. Given the recovered link between flight and egg shape recovered in extant birds (Stoddard et al. 2017) and shift in eggshell scaling exclusive to a small sample of volant extant avians (Legendre et al. 2020), we also assess whether a relationship with locomotor mode is recovered with an increased non-avian dinosaur taxonomic sample. Specifically, we ask whether the evolution of flight in dinosaurs is associated with a change in scaling relationship or in evolutionary rates of eggshell thickness. All explored models take into account other proposed explanatory variables for eggshell thickness, that is, clutch size, nest type, nesting site, and precociality (Ar and Yom-Tov 1978; Karlsson and Lilja 2008; Birchard and Deeming 2015; Nagy et al. 2019).

Material and Methods
DATASET AND PHYLOGENY
The assessed dataset includes 114 taxa – 57 extant and subfossil birds, and 57 non-avian dinosaurs (Table S1). Eggshell thickness (μm) and egg mass (g) data were first assembled from Stein et al. (2019) and Legendre et al. (2020) – see also Ar et al. (1979), Deeming (2006). Data for 27 taxa were taken from other references (Table S1; Supporting Information) to better sample the eggs of paleognaths (n = 7) and non-avian dinosaurs (n = 20). The sampling of large paleognaths increases the range of body

size among extant birds, while the additional sample of non-avian dinosaurs focuses primarily on non-avian avialans (i.e., taxa closely related to crown birds), which Legendre et al. (2020) did not sample.

Egg mass was estimated from egg length and width (both in mm) using two different allometric equations, derived from the study of eggs of extant non-avian reptiles (Deeming and Ferguson 1990) and birds (Hoyt 1979), respectively. These equations were compiled using empirical measurements of egg mass and linear dimensions, but neither of them corrects for phylogenetic non-independence of observations (Hoyt 1979; Deeming and Ferguson 1990). However, since no such equation accounting for phylogenetic non-independence is available in the literature, and because none of the studies that provide such equations (e.g., Iverson and Ewert 1991; Narushin et al. 2020) include the original data that would have allowed to replicate their results in a phylogenetic context, we used these two equations to estimate egg mass in our sample. These equations only differ in their allometric exponents – 5.60 for non-avian reptiles, and 5.48 for birds (Stein et al. 2019), resulting in both equations providing very close mass estimates for any given egg. We compiled two egg mass estimates (one for each equation) for taxa in our sample for which measurements of egg length and width were available (n = 63), and tested for a difference between them using a Wilcoxon signed-rank test (Supporting Code). Pairwise comparisons between the two estimates show a significant difference (W = 2016, $p < 0.001$). However, the two sets of estimates have identical distributions (Fig. S1) and the regressions of eggshell thickness on each of them have identical slopes ($\beta = 0.356$, with identical confidence intervals – see Supporting Code), showing that the use of two different equations has no impact on the scaling relationship of eggshell thickness and egg mass. Body mass data were not available for most fossil taxa in our sample – especially ootaxa, i.e. fossil eggs with no associated skeletal material, for which body mass cannot be estimated (Campione and Evans 2020) – and were therefore not included in subsequent analyses. We found egg mass and body mass in modern birds (n = 57) to be highly correlated ($R^2 = 0.999$, $p < 0.001$).

Traits previously proposed to affect eggshell thickness and/or egg mass were assessed as potential correlates of eggshell thickness (Table S1). Character coding for all traits was assessed through an extensive literature review (see Supporting Information for a fully referenced description of character states for all new taxa included in this analysis). Tested correlates include:

– Flight (binary: present/absent), proposed to be a strong evolutionary constraint on body mass reduction in bird evolution (Prange et al. 1979; Rayner 1988; Turner et al. 2007; Novas et al. 2012; Puttick et al. 2014; Benson et al. 2018), and a primary driver of avian egg shape variation (Stoddard et al. 2017), which is known to affect intraspecific variations of eggshell thickness in birds (Maurer et al. 2012; Birkhead et al. 2017);

– Nesting site (binary: on ground/above ground): ground-nesting has been previously associated with higher clutch size (Jetz et al. 2008), flightlessness and elongated egg shape (Nagy et al. 2019), higher egg volume and egg mass (Duursma et al. 2018; Nagy et al. 2019) – expected to result in a thicker eggshell;

– Nest type (ternary: open/semi-open/closed – Nagy et al. 2019): open nesters have been previously identified as showing higher body mass (Martin et al. 2017), egg volume, and egg mass (Nagy et al. 2019), which could be associated with a thicker eggshell, as well as lower eggshell porosity in dinosaurs (Tanaka et al. 2015);

– Precociality (binary: precocial/altricial), associated with thinner eggshell (Karlsson and Lilja 2008; Österström and Lilja 2012) and lower relative egg mass (Dyke and Kaiser 2010);

– Clutch size (continuous: mean number of eggs per clutch), correlated with lower egg mass (Williams 2001; Pellerin et al. 2016), lower body mass – albeit differentially in precocial and altricial birds (Ar and Yom-Tov 1978), and thinner eggshell in some birds (Orłowski et al. 2016).

A time-calibrated phylogenetic tree used in all comparative analyses was taken from Stein et al. (2019) and expanded using additional references with phylogenetic position and stratigraphic age data (Parham et al. 2012) for each of the 27 taxa added to the sample (Table S1; Supporting Information). These taxa were added individually to the tree in Mesquite version 3.61 (Maddison and Maddison 2019). Divergence times were based on large-scale calibrated phylogenies, and first and last occurrences of fossil taxa were based on geochronological estimates of their respective geological formations of origin (Supporting Information), with a reassessment of the phylogenetic position and age for some taxa in Stein et al.'s original sample (Supporting Information).

COMPARATIVE ANALYSES

All statistical analyses were performed in R version 4.0.4 (R Core Team 2021), using log-transformed data (natural logarithm – Sokal and Rohlf 1995; Mascaro et al. 2014 – see Supplementary Code). Ootaxa resulted in the tree including several polytomies, which were randomly resolved using 'multi2di' in ape (Paradis 2012) for each estimation of ancestral states and evolutionary rates.

Selection of variables of interest

We defined a network of possible direct and indirect correlations between eggshell thickness and all other variables (Fig. 1; Fig. S2). Phylogenetic path analysis (Hardenberg and Gonzalez-Voyer 2013; Gonzalez-Voyer and Hardenberg 2014) as
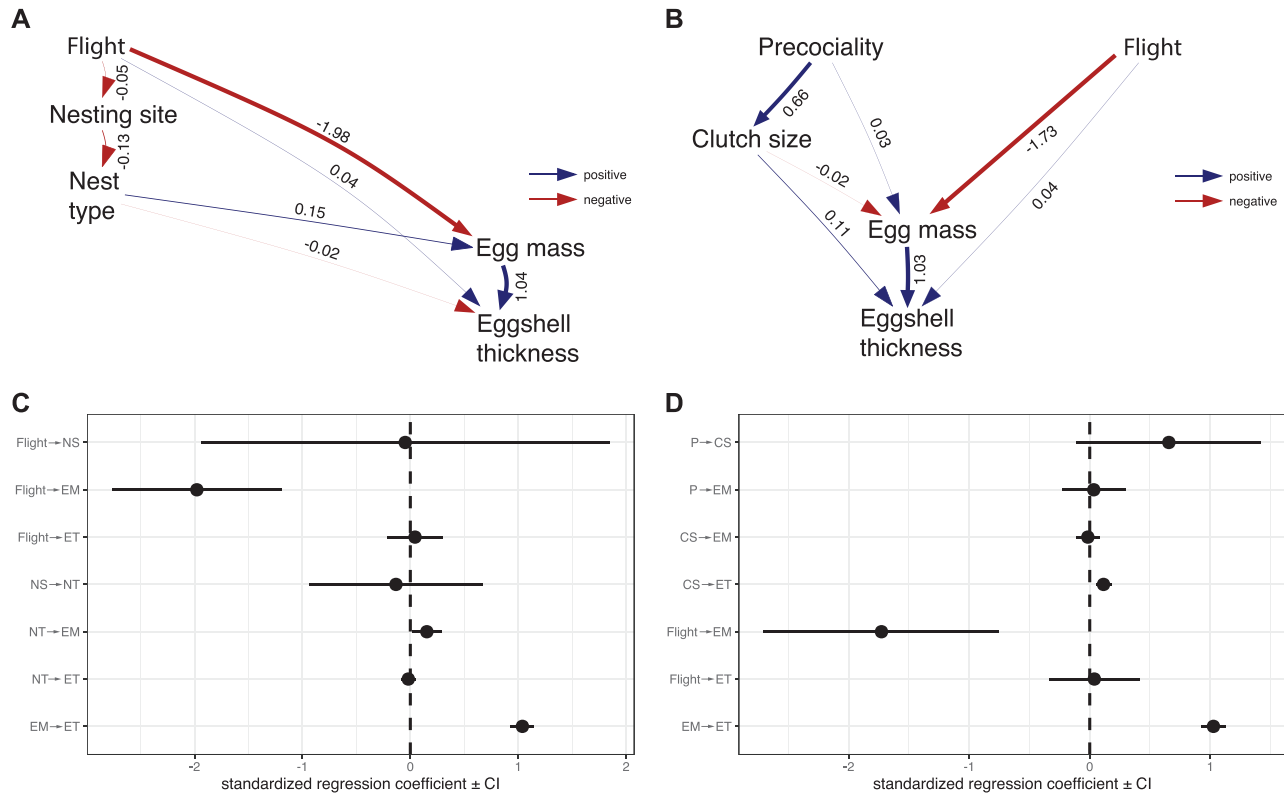
Figure 1. (A, B) Best models explaining eggshell thickness selected by phylogenetic path analysis for two sets of variables (subsets 1 and 2, respectively; see Text). (C, D) Regression coefficients with 95% confidence intervals for phylogenetic paths selected in models for subsets 1 and 2, respectively (see Text). In both subsets, only two paths are selected as significant when considering the confidence intervals of regression coefficients: the influence of egg mass on eggshell thickness (positive correlation), and that of flight on egg mass (negative correlation). The influence of nest type on egg mass in subset 1 (A) and that of clutch size on eggshell thickness in subset 2 (B) both show 95% confidence intervals that do not cross the zero threshold, but are very close (lower limit of 0.015 and 0.049, respectively) compared to those of flight on egg mass and egg mass on eggshell thickness (C, D). Abbreviations: CS, clutch size; ET, eggshell thickness; EM, egg mass; NS, nesting site; NT, nest type; P, precociality (see Text for definitions of each trait). See also Supporting Code.

implemented in phylopath (Bijl 2018) was used to identify which of these variables best explained eggshell thickness. Phylogenetic path analysis is based on structural equation modeling using d-separation (Shipley 2000): all hypothesized causal relationships (paths) between traits of interest are represented graphically using directed acyclic graphs (DAG; Fig. 1; Fig. S2). Many causal relationships, some of them contradictory, can be inferred for a given set of traits, resulting in several DAGs that can be tested against each other as hypotheses of causality. Each DAG is converted into a corresponding statistical model, with each path in it modeled as a phylogenetic generalized least squares regression (PGLS – Grafen 1989; Symonds and Blomberg 2014). Models are then tested against each other using d-separation, i.e. estimating the probabilities that nonadjacent variables in each model are statistically independent conditional on their parent variables, using Fisher's C statistic (Shipley 2000, 2016; Hardenberg and Gonzalez-Voyer 2013). The C statistic information criterion (CICc) corrected for small sample sizes (Hardenberg and Gonzalez-Voyer 2013; Gonzalez-Voyer and

Hardenberg 2014) is used to determine the model with the best fit.

Seven traits were included in the analysis, representing a high number of alternative hypotheses to consider all possible paths (Gonzalez-Voyer and Hardenberg 2014; Bijl 2018). To simplify this framework, the dataset was divided into two subsets based on previously described functional relationships among variables (Fig. 1; Fig. S2) to perform two distinct path analyses: one including eggshell thickness, egg mass, flight, nesting site, and nest type (Fig. 1A; Fig. S2a); and the second including eggshell thickness, egg mass, flight, clutch size, and precociality (Fig. 1B; Fig. S2b). We included precociality and clutch size in one model and nesting traits in another, despite the well-documented correlation of nest type and clutch size (Jetz et al. 2008; Nagy et al. 2019). This is justified by the fact that no study has so far identified a combined effect of clutch size (or precociality) and nesting parameters on egg-related traits (Nagy et al. 2019), but precociality is known to influence the correlation between clutch size and egg mass (Jetz et al. 2008; Nagy et al.

2019). Both subsets include eggshell thickness (the main variable of interest), egg mass (known correlate of eggshell thickness – Stein et al. 2019; Legendre et al. 2020), and flight (major constraint on the evolution of bird eggs – e.g., Stoddard et al. 2017); the regression of eggshell thickness on egg mass is included in the null model of both subsets (Fig. S2). The null model for the first subset also includes individual regressions of nest type on nesting site and of nesting site on flight, as these relationships have been well established in extant birds (Dial 2003; Nagy et al. 2019). Similarly, the null model for the second subset includes the regression of clutch size on precociality (Jetz et al. 2008). Other tested paths include individual regressions of eggshell thickness on all included variables, and of egg mass on all variables but eggshell thickness to test for their indirect effect on eggshell thickness through egg mass (Fig. S2). A Lambda model (i.e. a Brownian Motion model with covariances multiplied by a maximum likelihood estimate of Pagel's lambda – Freckleton et al. 2002) was compiled for each individual path, since such a model was selected as the best fit for individual phylogenetic regressions performed on the same characters (Supporting Code). Selection of the model with the best fit was then performed using CICc.

Phylogenetic linear models

PGLS were used to assess individual relationships identified by path analysis as significant. Variables identified by path analysis as having an indirect influence on eggshell thickness (i.e., influence through another predictor) were directly included as predictors in multiple regressions, rather than in a regression of their residuals (Freckleton 2002). PGLS were performed using function 'gls' in nlme (Pinheiro et al. 2021) and evolutionary correlation structures in ape (Paradis and Schliep 2019), using the argument 'weights' to correct for residual variance heterogeneity associated with a non-ultrametric tree (Paradis 2012). For each regression analysis, the model with the best fit was selected using AICc (corrected Akaike information criterion – Burnham and Anderson 2004) in AICcmodavg (Mazerolle 2020) from five candidate models: Brownian Motion (Felsenstein 1985), Ornstein-Uhlenbeck (Butler and King 2004), Early Burst (Blomberg et al. 2003), Lambda (Pagel 1999), and White Noise (i.e., non-phylogenetic correlation structure). Normality of the residuals was assessed with a Shapiro-Wilk normality test and a Q-Q plot, and homoscedasticity with a residuals versus fitted plot; identified outliers (i.e. *Massospondylus*, *Mussaurus*, *Lufengosaurus*, and *Triprismatoolithus*) were subsequently removed, following standard guidelines for PGLS (Mundry 2014). A pseudo R^2 and p-value based on a likelihood-ratio test between the tested model and a null model (Paradis 2012) was compiled for each regression. The effect of discrete predictors (i.e. flight, nesting site, nest type, precociality) on eggshell thickness was further assessed with a phylogenetic analysis of variance (phyANOVA – Garland et al. 1993), using 'phyANOVA' in phytools (Revell 2012) with False Discovery Rate post hoc corrections (Benjamini and Hochberg 1995). Because egg mass is a known correlate of eggshell thickness, we expect discrete predictors to have an effect on this correlation, which also needs to be tested independently of their effect on eggshell thickness alone. This was assessed by performing phylogenetic analyses of covariance (phyANCOVA) within a PGLS framework (Lavin et al. 2008; Smaers and Rohlf 2016; Smaers et al. 2017), using a likelihood-ratio test to test for the significance of the interaction term – i.e. test if the effect of egg mass on eggshell thickness differs for each group of the discrete predictor.

An inherent issue of phylogenetic comparative methods is the difficulty to infer causation from correlation due to the effect of shared ancestry on the occurrence of a given trait value. A high similarity in evolutionary patterns between two traits can be due to a functional link (causal relationship), but it can also reflect a single evolutionary event that affected both traits independently, which implies an absence of causal relationship and cannot be detected by phylogenetic generalized linear models such as PGLS or phyANOVA (Maddison and FitzJohn 2015; Uyeda et al. 2018). Such an event is a potential source of bias in our sample: a significant difference between, for example, flying and non-flying taxa for a given trait could be due not to functional constraints of flight itself, but to sampling, given that most non-avian dinosaurs are flightless and most birds are flighted.

Path analysis performs causal inference through the use of DAGs that represent functional relationships between traits (Uyeda et al. 2018), but does not allow a test of whether other uninvestigated apomorphies of a particular clade may explain these relationships. New variables representing clade membership alone were added to the phyANCOVAs performed with other discrete traits and egg mass as predictors. Since the acquisition of powered flight homologous with that in Aves, is inferred to have occurred within paravians (Pei et al. 2020), a variable "paravian/non-paravian" was included and tested for a significant relationship with eggshell thickness. In addition, we tested a six-state variable including paleognaths, neognaths, and four paraphyletic groups progressively closer to modern birds: non-maniraptoran dinosaurs, non-paravian maniraptorans, non-avialan paravians, and non-avian avialans (Fig. 2B). PhyANCOVAs were performed for all possible combinations of discrete predictors and tested evolutionary models (Supporting Code), and ranked using AICc selection procedure to identify the best fitting model. For discrete predictors with a significant effect, a new regression was compiled for each group (Fig. 2A,B). Differences in slopes between groups were estimated by performing post hoc comparisons of their estimated marginal means using 'lstrends' in emmeans (Lenth et al. 2021), and differences in residual variance were tested using Levene's tests (Garland et al. 1993).
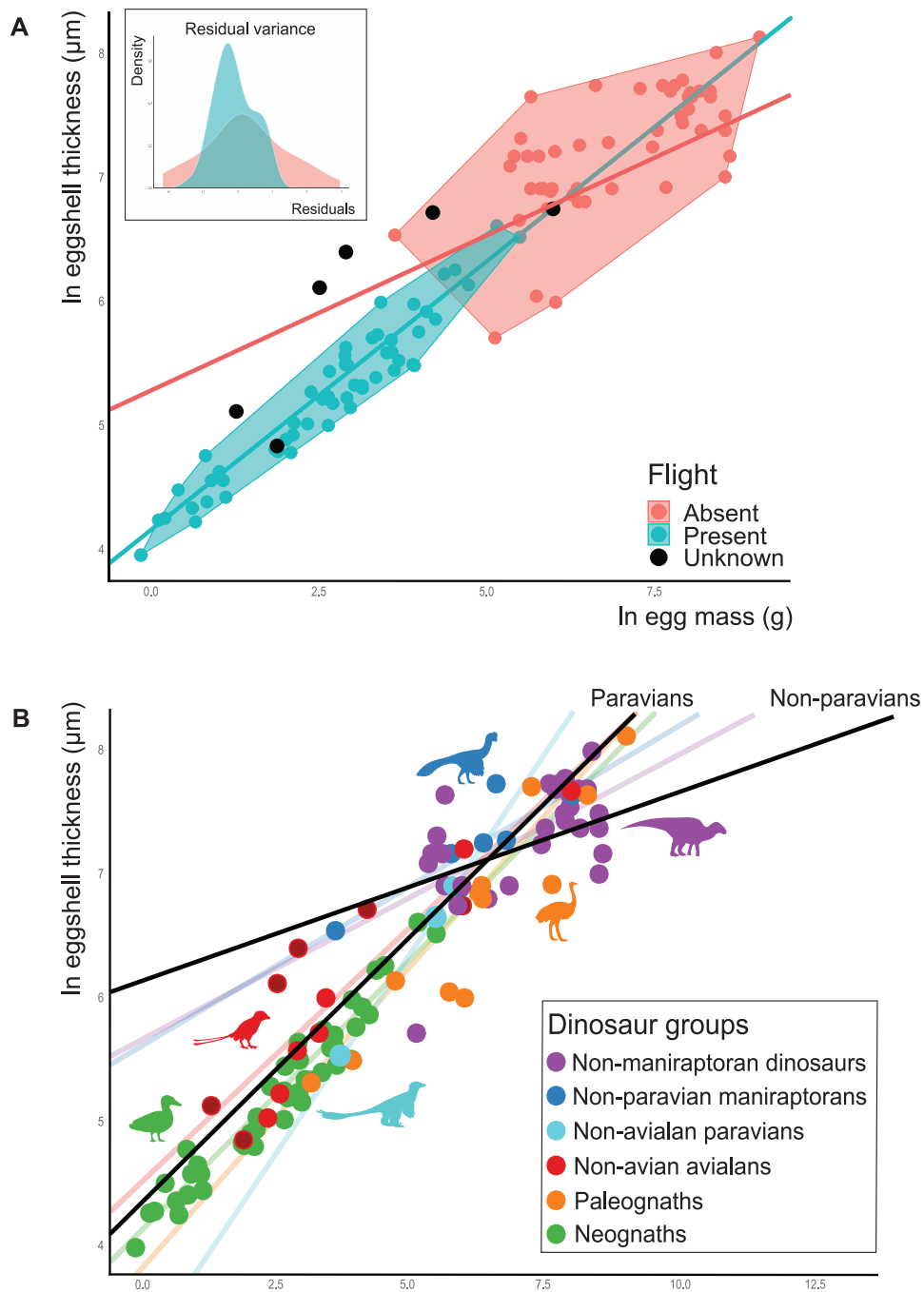
Figure 2. Regressions of eggshell thickness on egg mass, showing different allometric scaling relationships depending on (A) presence/absence of flight, and (B) group of interest. In (A), an upper left insert shows the distribution of residuals for regressions performed on flyers and non-flyers, respectively, showing a difference in residual variance between the two groups (see Text). An alternate version of this panel, figuring regressions for paravians and non-paravians, is available as Fig. S3a; a version featuring regressions for presence/absence of flight, but with both flight and paravians/non-paravians color schemes for the dots, is available as Fig. S3b. In (B), the main two fitted lines (in black) represent regressions performed on paravian and non-paravian dinosaurs, respectively. The other six fitted lines, partly transparent, follow the same color scheme as the dots (see 'Dinosaur groups' insert), and correspond to regressions for each of the six considered groups of interest, respectively. Not all of these six groups present scaling relationships significantly different from each other (see Main Text), and these fitted lines are only included for visual comparison. The six taxa in A for which presence/absence of flight is scored as unknown (all of them non-avian avialans) are indicated in (B) by a darker red circle inside each red dot representing one of them.
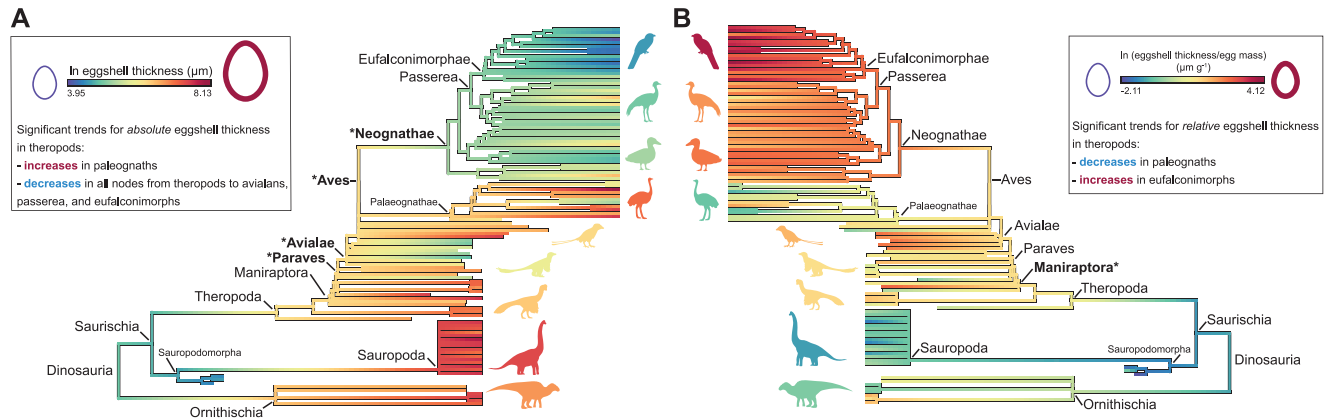
Figure 3. Phenotypic trends for absolute (A) and relative (B) eggshell thickness, estimated using 'search.trend' in RRphylo (Castiglione et al. 2019) and plotted using 'contMap' in phytools (Revell 2013 – see text). Relative eggshell thickness corresponds to the ratio eggshell thickness/egg mass (see text). Major dinosaur clades discussed in the text are labeled; nodes for which a shift toward a lower evolutionary rate was detected are labeled in bold and with an asterisk. In each panel, an insert lists the nodes for which a significant trend (i.e., change in value) was detected for the trait of interest.

Evolutionary patterns

Evolutionary rates and phenotypic trends for eggshell thickness were estimated using functions in the package RRphylo (Castiglione et al. 2018). RRphylo uses phylogenetic ridge regressions (Kratsch and McHardy 2014), which estimate phenotypic change along the branches of a tree as a sum of individual vectors of phenotypic transformations, each corresponding to a different predictor in the regression (Kratsch and McHardy 2014; Castiglione et al. 2018). Such regressions were compiled for eggshell thickness, expressed either as absolute eggshell thickness (with egg mass as a co-predictor; Fig. 3A) or as relative eggshell thickness, that is, using the ratio eggshell thickness/egg mass (with flight as a co-predictor, to account for difference in egg mass between flyers and non-flyers in the sample – see Results, Fig. 3B). Additionally, we performed similar regressions on egg mass (with flight as co-predictor), to compare its evolutionary pattern with that of eggshell thickness. We searched for significant rate shifts via randomization using "search.shift" (Castiglione et al. 2018), and for significant trends in both phenotype and rates via comparison with Brownian Motion model simulations, using "search.trend" (Castiglione et al. 2019; Serio et al. 2019). We tested for a difference in both value and rate of eggshell thickness (absolute and relative) and egg mass for nine individual nodes in the tree (Fig. 3), corresponding to major clades: Theropoda, Maniraptora, Paraves, Avialae, Aves, Palaeognathae, Neognathae, Passerea, and Eufalconimorphae (Jarvis et al. 2014). We did not test for such differences for other major dinosaur clades (Sauropodomorpha, Ornithischia) due to the low sample size and high number of ootaxa (thus of uncertain phylogenetic position) attributed to these clades (15 out of 19 sauropodomorphs, six out of eight ornithischians).

To account for phylogenetic uncertainty and sampling error, we tested for the significance of shifts and trends against 100 simulations under Brownian Motion using 'overfitRR' (Serio et al. 2019; Melchionna et al. 2020), using random permutations for 20% of terminal taxa and node ages. We used a threshold of 75 (i.e., *p*-values significant in three-fourths of simulations) to assess significance. Estimates of phenotypic change and evolutionary rates were then vectorized and mapped on the tree using "contMap" in phytools (Revell 2013; argument: method = "user"). Three outliers – *Spheroolithus albertensis*, *Spheroolithus sp.*, and an indeterminate theropod egg ('N° 1' – Stein et al. 2019) – were removed from the resulting tree for absolute eggshell thickness, as their extreme values prevented the visualization of the evolutionary pattern for other taxa in the tree.

Results

Phylogenetic path analyses selected a model with egg mass as a direct predictor of eggshell thickness across Dinosauria, and flight as the only significant indirect predictor of eggshell thickness (Fig. 1A,B). Other variables were also selected as either direct or indirect predictors of eggshell thickness (Fig. 1A,B) in the models selected by CICc; however, the effect of these other variables was negligible, as each of them explained 15% or less of the variance in eggshell thickness. Furthermore, the standardized path coefficient for each of these variables was either not significantly different from zero or very close to that threshold (<0.05 using 95% confidence intervals; Fig. 1C,D). Thus, only egg mass and flight were included as predictors of eggshell thickness in model selection for PGLS.

The best PGLS model (phyANCOVA) selected by AICc was identified as a Lambda model with egg mass, flight, and

their interaction term as predictors (n = 108; $R^2 = 0.998$; $p < 0.001$). Presence/absence of flight was selected as a better discrete predictor (ΔAICc: 0.3; Supporting Code) than our clade membership variables – the six-state clade variable, the variable "paravian/non-paravian," and combinations of these (see also the high overlap of convex hulls for paravians and non-paravians in Fig. S3a, and the clear separation of flying and non-flying paravians in Fig. S3b). This suggests that the difference in scaling relationships between flyers and non-flyers is indeed due to their locomotor strategy rather than explained by phylogenetic relatedness alone – volant birds have significantly lower egg mass and thinner eggshells (Fig. 1).

Separate PGLS regressions for flying (n = 54) and non-flying taxa (n = 54) show that both groups retain a highly significant relationship between eggshell thickness and egg mass (flyers: $R^2 = 0.902$, $p < 0.001$; non-flyers: $R^2 = 0.868$, $p = 0.004$; Fig. 2A), but flyers have a significantly higher slope (phyANCOVA: likelihood-ratio: 51.7; $p < 0.001$). This difference between flyers and non-flyers is also true for extant birds (phyANCOVA: likelihood-ratio: 30.84; $p < 0.001$). PhyANOVAs identify a difference between flyers and non-flyers for both eggshell thickness (F = 175.1; $p = 0.001$) and egg mass (F = 286.5; $p = 0.001$), but not for residuals of the regression of eggshell thickness on egg mass (F = 0.011; $p = 0.981$). However, a Levene's test on these residuals identifies a significant difference (F = 16.61; $p < 0.001$), showing that for a given egg mass, eggshell thickness is much less variable in flyers than in non-flyers (Fig. 2A).

Pairwise comparisons between groups identify the regression slope for non-avian maniraptorans as different from that of non-avian avialans (contrast: 0.227; $p < 0.001$), paleognaths (contrast: 0.300; $p < 0.001$), and neognaths (contrast: -0.281; $p < 0.001$). When plotting individual regressions for each group, the regression lines appear to be clustered in two categories corresponding to non-paravian and paravian dinosaurs, respectively (Fig. 2B). The individual regression of eggshell thickness on egg mass is significant for paravians (n = 74; $R^2 = 0.849$; $p < 0.001$), but the one for non-paravians is not (n = 36; $R^2 = 0.026$; $p = 0.328$), and the two regressions have significantly different slopes (contrast: -0.21; $p < 0.001$). This difference in slope is also visible in smaller subgroups: non-maniraptoran dinosaurs and non-paravian maniraptorans have slopes below 0.3, whereas neognaths, paleognaths, and non-avian paravian groups all have slopes above 0.4 (Fig. 2B).

Phylogenetic ridge regressions identified significant trends in both absolute eggshell thickness values, egg mass, and evolutionary rates for these traits in theropods. Values of absolute eggshell thickness tend to decrease significantly across all nodes from Theropoda to Avialae, as well as in Passerea and Eufalconimorphae (Fig. 3A), resulting in a general trend toward a decrease in these values across dinosaur evolution. An increase in absolute thickness, however, was detected at the base of Palaeognathae. Evolutionary rates for absolute eggshell thickness also decrease significantly for all nodes from Paraves to Neognathae (Fig. 3A). Values of egg mass show a significant decrease for Theropoda, Maniraptora, and Eufalconimorphae, and an increase for Palaeognathae, but no significant change in evolutionary rates.

There is no general trend in relative eggshell thickness (i.e., ratio of eggshell thickness to egg mass) across most of Dinosauria. However, a significant increase in values of relative eggshell thickness is detected for Eufalconimorphae, while a decrease is found at the base of Palaeognathae (Fig. 3B), consistent with the results of Legendre et al. (2020). A decrease in evolutionary rate is detected only for Maniraptora (Fig. 3B).

Discussion

FLIGHT AND THE SCALING OF EGGSHELL THICKNESS

Our analyses identify a significant correlation between eggshell thickness and egg mass, but also that the evolution of powered flight had a significant effect on the scaling relationship between the two. In extant birds, egg mass is tightly linked to body mass ($R^2 = 0.999$; see Supporting Code), and shifts in egg mass recovered in Theropoda follow proposed general trends toward miniaturization (Turner et al. 2007; Lee et al. 2014; Benson et al. 2014, Benson et al. 2018). Absolute eggshell thickness progressively decreases in the theropod lineage leading to neognaths, coincidental with a decrease in body mass, while it increases in large non-volant paleognaths and sauropods (Fig. 3A). Conversely, relative eggshell thickness does not present a conspicuous pattern, and varies significantly only in eufalconimorphs and paleognaths (Fig. 3B). The evolutionary pattern of absolute eggshell thickness in our sample is thus proposed to generally follow that of body mass. However, that correlation between egg mass and body mass does not necessarily imply that the relationship between the two cannot differ between dinosaur clades, including among birds (Birchard and Deeming 2009).

Flight is identified as having a significant effect on allometric scaling between eggshell thickness and egg mass. When corrected for egg mass, eggshell thickness is not significantly different between flying and flightless taxa in our sample (also reflected by overlapping convex hulls in Fig. 2A), suggesting that their difference in absolute eggshell thickness is entirely driven by their difference in egg mass, which may be correlated to changes in body mass. However, the significant difference in residual variance and in slope between the two groups shows that the allometric constraint of egg mass on eggshell thickness is much stronger in flying birds than in flightless ones (Fig. 2A). This shift in slope and variance in flyers matches the similar shift

in slope identified in paravians (Fig. 2B), as well as the rate shifts identified in paravians and less inclusive clades (i.e., Avialae, Aves, Neognathae) for eggshell thickness (Fig. 3A). These shifts suggest that flight is not only a predictor of egg mass (which in turn affects eggshell thickness), but also a major limiting factor on eggshell thickness variation for a given egg mass.

Flying paravians in our sample present smaller eggs with an absolutely thinner, but relatively thicker eggshell (Legendre et al. 2020), with a highly limited range of values (Fig. 2) and low evolutionary rates (Fig. 3). The earliest shift in egg mass is in maniraptorans, while the earliest one in eggshell thickness is in paravians, which acquired powered flight early in their evolutionary history (Dececchi et al. 2016; Pei et al. 2020). This pattern fits with a previously proposed evolutionary sequence of body mass and locomotor transitions in birds: the rapid initial decrease in body mass in maniraptorans (Turner et al. 2007; Lee et al. 2014; Puttick et al. 2014; Benson et al. 2014, Benson et al. 2018) prior to the acquisition of aerial locomotion in paravians (Turner et al. 2007; Brusatte et al. 2014; Puttick et al. 2014; Benson et al. 2014, Benson et al. 2018), which in turn produced high physiological and biomechanical constraints on avian reproduction (Dyke and Kaiser 2010; Zheng et al. 2013; Deeming and Ruta 2014; Birchard and Deeming 2015; Varricchio and Jackson 2016; Stoddard et al. 2017). Although these constraints are not well understood outside of crown birds (Varricchio and Jackson 2016), this may explain why paravians in our sample present a more significant relationship between eggshell thickness and egg mass, and a decrease in variance for relative eggshell thickness (Fig. 2). Such a pattern is consistent with stabilizing selection (Wainwright and Price 2016), which would also explain the lower evolutionary rates for eggshell thickness in paravians after the initial egg mass decrease in maniraptorans. The addition of body mass estimates will be important to decipher the specific evolutionary mechanisms behind this selective pressure; such estimates require skeletal material associated with eggs, which is currently unavailable for most fossil taxa in our sample.

The observed impact of flight is likely to reflect the indirect effect of associated physiological or ecological shifts not accounted for in this study. Since flight in Aves is associated with many novel traits (e.g., Wright et al. 2014; Ji and DeWoody 2017), it is currently difficult to decipher the mechanism or mechanisms through which its acquisition may have impacted eggshell thickness. A possible factor is the evolution of egg shape in theropods, constrained by the long, regionalized oviduct of archosaurs (Palmer and Guillette 1992) and the fused pubic symphysis of stem birds, which limited egg width and increased egg elongation (Dyke and Kaiser 2010; Deeming and Ruta 2014; Deeming and Mayr 2018). Among extant birds, the presence of powered flight further increases the ellipticity and asymmetry of eggs, likely through the acquisition of a streamlined body

plan (Stoddard et al. 2017). These elongated and asymmetric eggs present a higher shell mass for a given egg mass (Deeming 2018), which may explain why flying birds present a high relative eggshell thickness. Furthermore, highly elongated and asymmetric eggs tend to present a regionalization of their shell thickness – the equator being thicker and the poles thinner (Birkhead et al. 2017; Peterson et al. 2020), which might limit the overall variation of mean eggshell thickness for a given egg mass.

Another possible explanation for the limited variation of eggshell thickness among flying birds is the increased amount of calcium associated with high growth rate and non-shivering thermogenesis in endotherms (Walter and Seebacher 2009; Huttenlocker and Farmer 2017; Bal and Periasamy 2020). Calcium metabolism in birds in highly specialized – the absorption of calcium from the blood during eggshell formation is at least five times as fast as that of mammals (Simkiss 1961b). This specialization is linked with the ability to store calcium absorbed from the eggshell in the yolk (Packard and Packard 1984). Vitamin D_3, present in the yolk, is absorbed by the embryo and controls its calcium intake – first from the yolk, then from the eggshell (Matos 2008), which provides about 80% of the total calcium content of the hatchling (Simkiss 1961a). Since a large yolk is correlated with an elongated egg shape in birds (Deeming 2018), a high calcium intake may have originated among non-avian theropods that laid elongated eggs (i.e. maniraptorans, paravians), and further increased with the acquisition of flight. Additionally, in birds, the proportion of calcium removed from the eggshell during ontogeny is correlated with density of shell units (Blom and Lilja 2004; Karlsson and Lilja 2008), which is negatively correlated with eggshell thickness (Tyler and Fowler 1978). This suggests that shell erosion due to calcium removal is proportionally higher in thinner eggshells, which would make calcium intake an important constraint on the lower limit of eggshell thickness. Since both egg shape parameters and embryonic calcium absorption are highly variable among birds (Orłowski and Hałupka 2015; Stoddard et al. 2017), their respective allometric relationships with eggshell thickness may differ between individual avian subclades. Investigating the effect of these variables on eggshell thickness and other microstructural traits would require additional data and analyses.

Our results identified flight, rather than clade membership (or a combination of both), as the best predictor of eggshell thickness scaling. This result, however, is conditional on our sample, which only includes some of the many lineages of non-avian paravians described in the literature, due to the lack of egg remains for most of them (e.g., Deeming 2006; Jackson et al. 2013; Varricchio and Jackson 2016; Stein et al. 2019). Our sample includes only four non-avialan paravians, of which only one – *Styloolithus sabathi*, the phylogenetic position of which might be located inside crown avialans (Varricchio and Barta

2015) – was assessed to have been flighted (Supporting Information). All non-avian avialans in our sample are ootaxa, among which only oospecies attributed to enantiornithines have been identified as flighted based on skeletal remains (Supporting Information). Additionally, some of the ootaxa assigned to non-avian ornithuromorphs have also been proposed to be the eggs of crown birds (Supporting Information).

Anatomical requirements for powered flight are estimated to have been acquired among non-avialan paravians (Xu et al. 2014). However, a recent study estimating wing loading for a wide range of non-avian paravians proposed that only two of them – dromaeosaurid *Microraptor* and unenlagiid *Rahonavis* – crossed the threshold for potential powered flight, based on the size of potential airfoils (Pei et al. 2020). Several other species, however, were recovered as close to that threshold among dromaeosaurids, troodontids, and non-avian avialans. Some of these taxa, along with other early paravians (e.g., scansoriopterygids) not sampled by Pei et al. (2020), might represent anatomical adaptations compatible with an evolutionary origin of flight distinct from that of crown birds (Pei et al. 2020). If flight evolved multiple times among paravians, and since early flying paravians are not represented in our sample, our binary character 'presence/absence of flight' might not reflect the diversity of paravian flight strategies. In this context, quantitative proxies for avian flight such as the commonly used hand-wing index (Wright et al. 2014; Stoddard et al. 2017; Sheard et al. 2020) may provide a clearer pattern of the influence of powered flight on allometric scaling of eggshell thickness during the early diversification of paravians. The lack of skeletal remains for almost all available non-avian paravian eggs, however, prevents such proxies to be included as predictors in our analyses. The discovery of new paravian specimens associated with egg material, as well as the quantification of traits representative of wing loading, are thus likely to improve our understanding of flight evolution and eggshell thickness scaling in that clade.

The effect of flight acquisition on eggshell thickness among dinosaurs does not necessarily imply an opposite effect of subsequent losses of flight among living birds. We observe such a discrepancy for eggshell thickness among flightless paleognaths, which have larger eggs than neognaths (Fig. 3B), but show no significant change in scaling relationship of eggshell thickness with egg mass (Fig. 2B). Accordingly, paleognaths do not show a shift in absolute eggshell thickness (Fig. 3A), suggesting their ancestral eggshell thickness did not vary significantly from that of their last common ancestor with neognaths. This decoupling might be linked with the fact that paleognath lineages lost flight independently (three to six independent losses – Sackton et al. 2019) and possibly recently in the evolutionary history of modern birds (Eocene-Oligocene boundary or later – Crouch and Clarke 2019). Such independent losses may have resulted in more than

one shift in allometric scaling of egg mass among paleognaths, which our small sample for that clade (n = 12, including three volant taxa) would not reflect adequately, or that might have occurred too recently to uniformly affect eggshell thickness for the whole clade.

Contact incubation may also have reduced the potential for strong variations of eggshell thickness in large paleognath taxa with increased egg mass (Birchard and Deeming 2009; Deeming and Birchard 2009), which may explain why their eggshell thickness scaling is not significantly different from that of neognaths. In extant birds, the shell needs to be thick enough for open-nesting contact incubation (Birchard and Deeming 2009; Huynen et al. 2010). Such constraints may not have been present in non-avian dinosaurs, the eggs of which have been described as either partially or completely buried, and could likely not sustain direct incubation (Hechenleitner et al. 2016a; Seymour 1979; Deeming 2006; Tanaka et al. 2015, 2018; Varricchio and Jackson 2016; Yang et al. 2019). Because of this, contact incubation has been proposed to have evolved relatively late in birds, well after the evolution of flight (Fernández et al. 2013; Deeming and Mayr 2018).

OTHER POTENTIAL CORRELATES AND SPECIFIC TRENDS WITHIN CROWN BIRDS

Previous studies have identified altriciality and a small clutch size as correlates of flight and body mass reduction in modern birds (Ar and Yom-Tov 1978; Jetz et al. 2008; Dyke and Kaiser 2010). Our results, however, did not identify precociality and clutch size as having a significant effect on eggshell thickness, egg mass, or flight in our sample of dinosaurs. This might be due to limited taxon sampling, since many bird groups with extreme values in clutch size or hyper-altriciality are not represented, e.g. among Passeriformes (Ar and Yom-Tov 1978; Birchard and Deeming 2015; Nagy et al. 2019). It might also be linked with the low number of discrete character states used to represent nesting strategies. Clutch size and degree of altriciality are correlated with specialized nesting strategies that have only been documented in Aves (Nagy et al. 2019). The incredible diversity of these life history traits in extant birds (Jetz et al. 2008; Dyke and Kaiser 2010; Brusatte et al. 2015; Mayr 2017; Nagy et al. 2019) might not be well represented by the binary or multistate discrete characters used in our analysis, which might be overly simplistic in this context (Mainwaring et al. 2014). A larger sample with more altricial species and higher variation in clutch size, as well as a reassessment of character states for specific nesting strategies beyond open/closed types and on/off-ground nesting, are needed to better assess the influence of these traits on eggshell thickness.

The clade Eufalconimorphae, which comprises falcons and caracaras, parrots, and passerines (Jarvis et al. 2014), is the only avian subclade consistently recovered as showing a distinct

evolutionary pattern (Fig. 3; Legendre et al. 2020, Fig. 3): a significant decrease in egg mass and increase in relative eggshell thickness. It is the only theropod clade to show such a trend. Eufalconimorphs is a recently-defined clade of birds, supported by whole-genome phylogenetic analyses (Jarvis et al. 2014; Prum et al. 2015; Kimball et al. 2019), and for which both molecular and morphological synapomorphies have been identified (Suh et al. 2011; Mayr 2014). This result, however, is unexpected, since eufalconimorphs have never been proposed as showing a shared derived reproductive strategy distinct from those of other birds. While an eggshell microstructure or egg morphology specific to eufalconimorphs has yet to be discovered, it is interesting to note that many myological similarities have been described in the forelimb of parrots and falcons (and passerines to a lesser extent), and might be associated with a flight strategy ancestral to the whole clade (Razmadze et al. 2018). Further functional studies on the flight and reproduction of eufalconimorphs are required to establish a potential link between these hypothetical flight-related traits and an ancestral increase in eggshell thickness.

Significant differences in pore and shell microstructure have been reported in Theropoda (Mikhailov 1997; Deeming and Ruta 2014), especially in Palaeognathae among extant Aves (Tyler and Simkiss 1959; Board 1982). Investigating the relationship of these shifts with eggshell scaling, potentially distinct from those observed in eggshell thickness, demands denser sampling among those groups. In paleognaths, for example, the eggs of some moa likely received little to no incubation (Huynen et al. 2010), as found in closed-nesting birds, which present higher egg porosity than open nesters (Tanaka et al. 2015). This would explain why moa eggs have simple pores (Tyler and Simkiss 1959) and a higher porosity than expected for open nesters (Tanaka et al. 2015), and were likely protected by some form of nest covering (Wood 2008; Huynen et al. 2010). Conversely, ostriches, which have fully open nests and long pre-incubation periods during which the eggs are exposed to the sun (Bertram and Burger 1981), present other adaptations to ensure reproductive success, including brown or white pigmentation to limit predation or overheating, respectively (Deeming and Ar 1999; Magige et al. 2008), as well as pores with a complex branching structure, hypothesized to increase gas exchange and limit water loss (Christensen et al. 1996; Willoughby et al. 2016). This variety in pore structure and nesting behavior among paleognaths is likely independent of eggshell thickness (Tyler and Simkiss 1959), and further analyses of paleognath eggshell microstructure will be required to understand how these individual constraints may have affected their egg characteristics and reproductive strategies.

IMPLICATIONS FOR FOSSIL OOTAXA

While this paper focuses on the influence of egg and lifestyle parameters on eggshell thickness in dinosaurs, the dataset built for this study may also have interesting implications for the use of eggshell thickness as a proxy for lifestyle parameters in non-avian dinosaurs. Among the six ootaxa in the sample for which presence or absence of flight is unknown, three are unambiguously grouped with flyers and two with non-flyers in our phylogenetic regressions of eggshell thickness on egg mass (Fig. 2A). The remaining ootaxon, *Medioolithus geiseltalensis*, has the highest egg mass out of the six and falls on the rightmost part of the plot, where the two regression lines (for flyers and non-flyers, respectively) become less distinguishable from one another (Fig. 2A). Such preliminary results cannot be clearly interpreted in a phylogenetic context without a larger sample, but this suggests that eggshell thickness is a good predictor of powered flight for small dinosaur species (egg mass $\leq$ 100 g), while its residual variance might be too high to achieve such predictions above that threshold. With many other lifestyle traits to include in future studies testing their correlation with dinosaurian eggshell microstructure, a phylogenetic comparative framework could potentially help further solve the many issues described for classic eggshell parataxonomy over the past two decades (Zelenitsky and Modesto 2003; Zelenitsky and Therrien 2008; Choi et al. 2020). The definition of additional eggshell traits and further use of phylogenetic comparative methods will be a crucial aspect of defining a clearer picture of the evolution of dinosaur eggs, and help understanding the role of eggshell microstructure in the radiation of modern birds.

DATA ARCHIVING
All data are available as Supplementary Table 1, with additional information and references provided as Supplementary Information. Supplementary Code with corresponding data and tree files is available on GitHub at https://github.com/LucasLegendre/dinosaureggshellproject

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Supplementary Material

Supplementary Figure 1. a, **b**, Scatterplots of eggshell thickness vs egg mass, with egg mass estimated using the allometric equation for birds (**a** – Hoyt 1979) and non-avian reptiles (**b** – Deeming and Ferguson 1990), respectively. **c**, Boxplots with individual data points for egg mass, estimated using each allometric equation. The distribution of the data appears identical in all three panels, but pairwise comparisons between the two egg mass estimates show a significant difference (see Main Text). Abbreviations: L, egg length (mm); M, egg mass (g); W, egg width (mm).

Supplementary Figure 2. Directed acyclic graphs representing candidate models tested in phylogenetic path analysis. Due to the high number of variables, the dataset was divided into two sets of five variables each. Each set includes a null model containing relationships included in all other models for this set, and twenty-six other models that each includes different combinations of inferred relationships between variables in that set. **a**, First set, including eggshell thickness (ET), egg mass (EM), flight (F), nesting site (NS), and nest type (NT) as variables. **b**, Second set, including eggshell thickness, egg mass, flight, clutch size (CS) and precociality (P) as variables. See Main Text for more information on individual tested relationships in each model set.

Supplementary Figure 3. a, Regression of eggshell thickness on egg mass for paravians and non-paravians, respectively, with a color scheme identical to that of Fig. 2a for presence/absence of flight. Paravians and non-paravians present a much higher overlap than flyers and non-flyers. **b**, Regression of eggshell thickness on egg mass for flying and non-flying taxa in our sample, respectively. Non-paravians (red) and flightless paravians (pink) overlap with each other, while flying paravians (blue) are clearly distinct from them. Since the color scheme represents the interaction of two variables (presence/absence of flight and paravian/non-paravian), the six taxa for which presence/absence of flight is unknown (see Fig. 2a) are not depicted.

Supplementary Table 1. Dataset used in this study. Measurements of eggshell thickness and potential correlates (egg mass, flight, nesting site, nest type, mean clutch size, and precociality) for 114 species of dinosaurs including birds, with corresponding references (see full list of references in Supplementary Information).