

1 **How much calcium to shell out? Eggshell calcium carbonate**
2 **content is greater in birds with thinner shells, larger clutches, and**
3 **longer lifespans**

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24 Abstract

25 The avian eggshell is a bio-ceramic structure that protects the embryo. It is composed
26 almost entirely of calcium carbonate and a small organic component. An optimal amount of
27 calcium carbonate in the eggshell is essential for the embryo's development, yet how the
28 ratio of calcium carbonate to organic matter varies between species has not been
29 investigated. Calcium is a limiting resource for most birds, so its investment in their eggs
30 should be optimised to a bird's life history. We measured relative calcium carbonate
31 content of eggshells in 222 bird species and tested hypotheses for how this trait has evolved
32 with these specie's life history strategies and other traits of their respective egg
33 physiologies. We found that; 1) eggshell calcium carbonate content was positively
34 correlated with species' having thinner eggshells, and smaller than expected eggs relative to
35 incubating parental mass, 2) species with small mean clutch sizes had lower calcium
36 carbonate content in their eggshells, and 3) for species with larger clutch sizes, eggshell
37 calcium carbonate content was negatively correlated with their mean lifespan. The pattern
38 of lower eggshell calcium carbonate in longer-lived, larger clutched, birds suggests that
39 calcium provision to the eggshell has long term costs for the individual.

40

41 Introduction

42

43 Life history theory explains what determines when, how, and to what extent reproduction
44 should occur for an organism to optimise its individual fitness (1). A key aspect of these
45 reproductive strategies is investment in individual reproductive bouts versus self-
46 maintenance, and the spreading of investment over multiple reproduction attempts (1,2).
47 The avian eggshell, as an extension of both a bird's phenotype and its life history, is under
48 the influence of strong selective factors, since embryonic development and reproductive
49 success are highly dependent on the optimal functionality of the eggshell (3,4). Birds'
50 eggshells have evolved many specific adaptations in their composition and structure for
51 ensuring successful embryonic development across different life histories, nest
52 environments, and climatic conditions (5,6). Egg production provides a critical example of
53 life history theory in action as the investment into an egg and/or clutch will greatly influence
54 the quality of that offspring, but conversely, will reduce the parent's resources for both

55 immediate self-maintenance and future reproductive investment (7). This trade-off has
56 been explored in the context of egg contents (8), such as androgen deposition in the yolk
57 (9,10) and pigment deposition in the shell matrix (11), yet the production of the eggshell
58 itself and its composition has not been considered within the same framework.

59

60 The avian eggshell performs multiple functions to enable and facilitate embryonic
61 development. The eggshell provides a rigid armour to protect the developing embryo from
62 mechanical damage and acts as a physical barrier to microbial infection (12). Moreover, the
63 eggshell controls the appropriate exchange of heat, water, and respiratory gases with the
64 immediate nest environment (13), while also providing a reservoir of calcium and other
65 trace minerals for absorption by the developing embryo (14). Simultaneously, pigment
66 deposited on the outer surface can play an important role in varied behaviours, such as
67 crypsis, thermoregulation and sexual signalling (15–18). The evolution and adaptations of
68 the eggshell has allowed birds to breed in almost all terrestrial environments and habitats
69 globally (19). A key component of this success has been the presence of calcium carbonate
70 in the eggshell in the form of calcite (4,20). How calcite crystals form to produce the
71 structure of the eggshell has been rigorously studied (21,22), and the detrimental impacts of
72 calcium deficiency on reproduction are well established (23,24). Despite this, the quantity of
73 calcium carbonate in the shell has rarely been considered as an evolved trait in bird species
74 (but see 25), even though broad-scale macro-ecological studies have found global patterns
75 in egg shape (26), egg size (27,28) and shell pigmentation (29–31).

76

77 Eggshells are sophisticated bio-ceramic structures consisting of a calcium-based mineral
78 structure interwoven with an organic protein matrix (12,32,33). Calcium carbonate is
79 believed to make up approximately 98% of the eggshell for most bird species (16,34),
80 though the variation across species has not been previously explored. An appropriate
81 amount of calcium carbonate deposited in the eggshell is essential for the embryo to
82 develop correctly, as incomplete calcification of the shell can lead to overly large pores and
83 desiccation, while excess calcium can lead to severely reduced gas exchange (34,35).
84 Insufficient calcium in the shell can also cause the embryo to become hypocalcemic
85 resulting in retarded growth, or in extreme cases, death (36,37).

86

87 Here we investigate the macro-phylogenetic patterns present in eggshell calcium carbonate
88 content across a large number of diverse avian species, and investigate the relationship
89 between eggshell calcium carbonate to organic component ratio and a species' life history
90 traits. Many life history traits can be expected to impose constraints or trade-offs in the
91 amount of calcium allocated to the eggshell. Calcite or its isoforms cannot be stored to any
92 significant amounts in most avian bodies (34,38), though cyclic osteoporosis can provide a
93 portion of the calcium for egg formation in some species (39). As such, this mineral must be
94 obtained from the mother's diet during egg formation (34). Acquiring sufficient calcium for
95 egg production for many species requires behavioural adaptations such as diet switching,
96 and/or strenuous foraging beyond their normal requirements, and outside their normal
97 ranges potentially increasing inter-territorial disputes (40,41). It is assumed that the greater
98 the number of eggs produced, the less calcium available to be provisioned to each (42).

99

100 The structure of the shell is under differing selective pressures to optimize strength, gas
101 exchange, and hatchability (3,5), among other factors, each of which might cause
102 contradicting directional selection on the eggshell calcium carbonate content. We
103 considered a number of pertinent life history traits where there is evidence of selection on
104 other aspects of egg physiology and formulated 10 key hypotheses and predictions with
105 respect to eggshell calcium content in 222 species (Table 1). These hypotheses were
106 subdivided based on the framework of Tinbergen's four questions to address variation in
107 eggshell calcium carbonate content between species from a mechanistic, proximate
108 perspective (mechanism and ontogeny) and from a broader adaptive, evolutionary
109 perspective (adaptation and phylogeny) (43,44). The goal of our novel investigation into
110 macro-evolutionary patterns of a key eggshell trait was to explore new associations
111 between eggshell content and avian life history, phylogeny and physiology.

112 Table 1. Hypotheses and predictions with supporting rationale, of how eggshell calcium carbonate content in birds relates to life history
 113 strategies and eggshell characteristics. Hypothesis are divided based on Tinbergen's four question structure (43,44)

Level of question/prediction	Hypothesis	Prediction	Rationale and / or proposed mechanism
Mechanism	1) Thicker eggshells are achieved through greater deposition of calcite but not matrix during layer formation resulting in higher relative calcium carbonate content of thicker eggshells.	Species with eggs that have thicker shells also produce shells with higher calcium carbonate content compared to species with thin-shelled eggs.	The crystalline structure of the shell is believed to be controlled primarily by the organic matrix, which modulates the deposition of calcium from the uterine fluid (45,46). Selection for thicker eggshell could increase the binding of calcite crystals to the organic matrix during shell formation.
	2) Calcium carbonate content of eggshells will be influenced by diet.	Species with diets that are normally higher in calcium invest more calcium in their eggshells.	The majority of calcium needed for egg production must be obtained from their diet during egg formation (34).
	3) Eggshell pigmentation has evolved to compensate for lower calcium carbonate content.	Pigmented eggshells contain less calcium carbonate than immaculate eggshells.	In great tits (<i>Passer major</i>) and Eurasian sparrowhawks (<i>Accipiter nisus</i>) calcium stress and eggshell thinning has been correlated with more pigmented eggshells, suggesting protoporphyrin pigment might be used to strengthen eggs in compensation for lacking calcium (47,48). However, in another species (black-headed gulls; <i>Larus ridibundus</i>) the correlation between pigmentation and shell thinning was found to be weak (49).
	4) Species eggshell calcium carbonate content will be adjusted	Species breeding at higher latitudes (further from the equator) will have a higher	Multiple egg traits are known to vary latitudinally both at an inter- and intra-species level, believed to be a response to variation in temperature and solar radiation (31,50,51). There is evidence that thicker eggshell can retain heat longer, which may benefit species breeding at colder latitudes (52),

	to their breeding latitude as a result of calcium availability and selection for thicker shells in colder climates.	calcium carbonate content in their eggs.	which lead to greater calcium carbonate content in these eggs. Additionally, calcium availability in the environment is known to increase in higher latitudes (42).
Ontogeny/Proximate	5) Precocial species deposit more calcium overall into their eggshell in order to supply the higher demand for embryonic growth without compromising the integrity of the eggshell through excessive thinning.	Eggshell calcium carbonate content is higher in species with precocial modes of development.	Nestlings of precocial species hatch in a more developed state than those of altricial species, in particular they have a more ossified skeleton and muscles, and larger brains (53). This requires greater sequestration of calcium during development, which is supplied by a greater number of mammillary tips of the eggshell (15,53).
	6) Incubation period influences calcium carbonate content.	Species with longer incubation periods will have more calcium carbonate in their eggshell.	Longer incubation period requires less porous eggshells to prevent excessive water loss, and as a result may have denser eggshell produced through greater calcite crystal deposition (54). (55) show an evolutionary relationship between eggshell porosity and incubation length in Alcidae species.
Adaptation/Ultimate	7) Calcium carbonate content will be influenced by reproductive investment (clutch size).	Calcium carbonate content decreases with increasing clutch size	(42) suggested that the evolution of clutch size is influenced by the availability of calcium in the breeding habitat. This would suggest a strong correlation between clutch size and eggshell calcium content
	8) A species lifespan influences calcium carbonate content per	Lifespan is negatively correlated with calcium carbonate content.	If calcium foraging is an expensive activity, longer lived species might invest less calcium in eggs per clutch in order to conserve energy for future reproductive attempts compared to species which only have the opportunity to breed few times over their short lifespan. There is evidence that lifespan

	egg.		influences egg size and clutch size in birds (56).
	9) Eggshell calcium carbonate content is higher in species with eggs that are smaller than predicted for the weight of the incubating parents.	Calcium carbonate content will be predicted by the residual difference between fresh egg weight (as a proxy for egg size) and adult body mass.	Egg traits such as the size, shape, and thickness of eggs has evolved in tight concert with adult body mass, as the egg needs to be able to support the weight of the parent during incubation, yet remain thin enough to allow the chick to hatch (3,57). Smaller eggs experience a greater force per unit area of the shell from the weight of the incubating parent and as such could require a higher calcium carbonate content to compensate.
Phylogeny/Ultime	10) A large component of variation in eggshell calcium carbonate content is correlated with species phylogenetic position.	Calcium carbonate content has a phylogenetic signal close to, but less than, 1 (Pagel's λ) (58).	Many eggshell characteristics have been shown to strongly covary with phylogenetic relatedness in birds (5,30,59), as such we expect eggshell calcium carbonate content to be similarly correlated to phylogeny.

114 **Methods**

115

116 **Calcium carbonate content (ash) of eggs**

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118 All eggshells were obtained from the Destructible Collection at The Natural History Museum
119 Tring, a unique resource containing blown eggs mainly of European breeding birds,
120 identified to species levels but otherwise too data-poor to allow admission to the museum's
121 main collection (5,30). Due to limitations of the information available for this (destructible)
122 subset of the collection, we did not have specific details about where eggs were collected or
123 the clutch size they were taken from. Eggs were assumed to be freshly laid at collection, due
124 to the small size of blow holes. A small blow hole suggests no substantial embryo was
125 present, as the egg content were extruded through this opening. All eggshells were cut in
126 half vertically (from sharp to blunt pole) using a diamond-tipped dentist drill (Milnes Bros.,
127 Surrey, UK). One half of each egg was weighed on a precision electronic balance (Sartorius,
128 Göttingen, Germany), before being put in an oven at 60 °C to dry to a constant mass. To
129 assess this, all shell halves were weighed individually twice daily, between 09:00-10:00 and
130 16:00-17:00, until no change in mass was detected for four consecutive weighing sessions,
131 at which point they were considered 'dry'. Following this, each shell half was placed in a
132 small ceramic crucible and weighed with this container. The crucibles with the dry shell
133 were then placed into a muffle furnace (AAF 1100; Carbolite, Hope, UK) for 30 hours at 650
134 °C to burn off the organic component of the shell. Immediately after removal from the
135 furnace, each crucible with the shell ash was placed in a desiccator to cool down without
136 absorbing moisture from the air before being weighed again. Calcium carbonate content
137 was calculated as the ash mass of the shell half, as a percentage of the dry mass of the shell
138 half. Other inorganic minerals that occur in trace amounts alongside calcium carbonate in
139 the eggshell (e.g., phosphorous and magnesium) were not considered separately as they
140 occur in extremely small quantities (<0.1% of the eggshell) (60,61)

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142 **Life history and physical egg traits**

143

144 Life history and ecological data were gathered primarily from the Handbook of the Birds of
145 the World Volumes 1–13 (62), and cross-referenced with Birds of the Western Palearctic

146 (63). Body mass of adult birds was taken as a mean of the mass of both sexes, primarily
147 from the Handbook of Avian Body Masses (64). Residual variation in egg size was calculated
148 as the residual variance of each species from the predicted values of a linear correlation
149 between \log_{10} corrected body mass and \log_{10} corrected fresh egg mass. Lifespan was
150 extracted from (65), and mean breeding latitude was calculated from (66). Clutch size data
151 were collected as mean number of eggs but subsequently divided into two categories, with
152 species producing either a single egg or two eggs per clutch categorised as 'small', and all
153 other species categorised as 'large'. This is due to an unequal distribution of clutch sizes in
154 the data (Supplementary Figure 1) and preliminary results supporting a categorical rather
155 than a continuous effect of clutch size. Species mean eggshell thickness values were
156 extracted from (67).

157

158 Statistical analyses

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160 All statistical analyses were conducted in R statistical software Rv3.3.2 (68) through the
161 Integrated Development Environment 'R Studio' (69). A phylogenetic tree was constructed
162 for the 222 species included in this study from the Open Tree of Life project, using the R
163 package 'rotl' (70), which constructs a tree using multiple taxonomies as a backbone. The
164 strength of the phylogenetic signal (Pagel's λ) in calcium percentage of the eggshells, was
165 estimated on the mean values for each species, using the 'phylosig' function in the R
166 package 'Phytools' (71,72). The R package 'caper' (73) was used to construct
167 phylogenetically informed least squares models (PGLS) using the constructed phylogenetic
168 tree. In these models we were able to include phylogeny and Pagel's λ as a covariance
169 matrix, thereby accounting for phylogenetic non-independence of the residual error in the
170 response variable (calcium content). Pagel's λ was assigned by maximum likelihood in all
171 models (74).

172

173 Calcium carbonate percentage was first arcsine transformed to account for the proportional
174 nature of the data, and then \log_{10} transformed to account for a non-normal distribution
175 (Supplementary Figure 2). This was our response value in the subsequent models and tested
176 against life history and physiological traits as predictors. PGLS models require a single
177 response value per species, as such mean calcium carbonate content was determined for

178 each species. To test our hypotheses, candidate PGLS models (72) were constructed with
179 combinations of the following predictors: log eggshell thickness (mm), residual egg size
180 variance relative to adult body mass (g), precociality – assigned categorically by whether or
181 not eyes are open at hatching (precocial/altricial), mean clutch size (small (< 2) or large (2.5
182 to 16)), mean incubation period (days), species mean breeding latitude (degrees), species
183 diet (omnivore or carnivore, no herbivores were available in the dataset), mean lifespan
184 (years), and whether eggs are pigmented or immaculate (yes/no). Several two-way
185 interactions were also included in PGLS models, listed here (*denotes interaction): log
186 eggshell thickness*calcium diet, log eggshell thickness*precociality, log eggshell
187 thickness*clutch size, lifespan*clutch size, clutch size*precociality, mean incubation
188 period*clutch size, mean incubation period*precociality, and lifespan*precociality.

189

190 These candidate models were ranked based on Akaike Information Criterion values
191 corrected for small sample sizes (AICc), and model averaging was applied to all models (n=3)
192 which could not be rejected based on having an AICc score within 2 points of the lowest
193 AICc valued model (75). The R software package ‘MuMIn’ was used for model selection and
194 averaging (76). The averaged model produced contained only the predictors: log eggshell
195 thickness, residual egg mass, lifespan, clutch size, latitude, and the interaction between
196 lifespan and clutch size (Supplementary table S1).

197

198 PGLS models can only compare mean calcium content value (77) and do not account for
199 intraspecies variability; to account for this we further constructed a phylogenetically
200 informed multivariate mixed model (PMM) (78), which included all measurements per
201 species (samples per species varied between N = 1 and 5, see Supplementary Table S3),
202 tested against the predictors of the averaged PGLS model list above. The PMM was fitted
203 with the package ‘sommer’ R v. 4.0 (79), using the same phylogenetic tree described above.
204 The phylogenetic tree (Figure 1) was visualised using ‘ggtree’ package (80).

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212 **Results**

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214 Our final PMM (containing predictors identified by model averaging of PGLS candidate
215 models, Supplementary Table 1) contained the predictors log shell thickness and residual
216 egg size variance, as well as key life history traits of clutch size, lifespan, the interaction
217 between clutch size and lifespan, and mean breeding latitude. All other predictors and
218 interactions were not retained in the averaged model set of PGLS models, indicating these
219 variables neither improved the fit of the model nor were significant predictors of eggshell
220 calcium content, and as such were not included in the PMM. There was an effect of
221 phylogeny on mean eggshell calcium carbonate content with an intermediate Pagel's λ
222 value of 0.82, which was significantly different from both zero and one ($p > 0.005$, 95 % CI:
223 (0.686, 0.906), suggesting close relatives were correlated in the values of eggshell calcium
224 content, though less than would be seen under a strict Brownian motion model of
225 evolution.

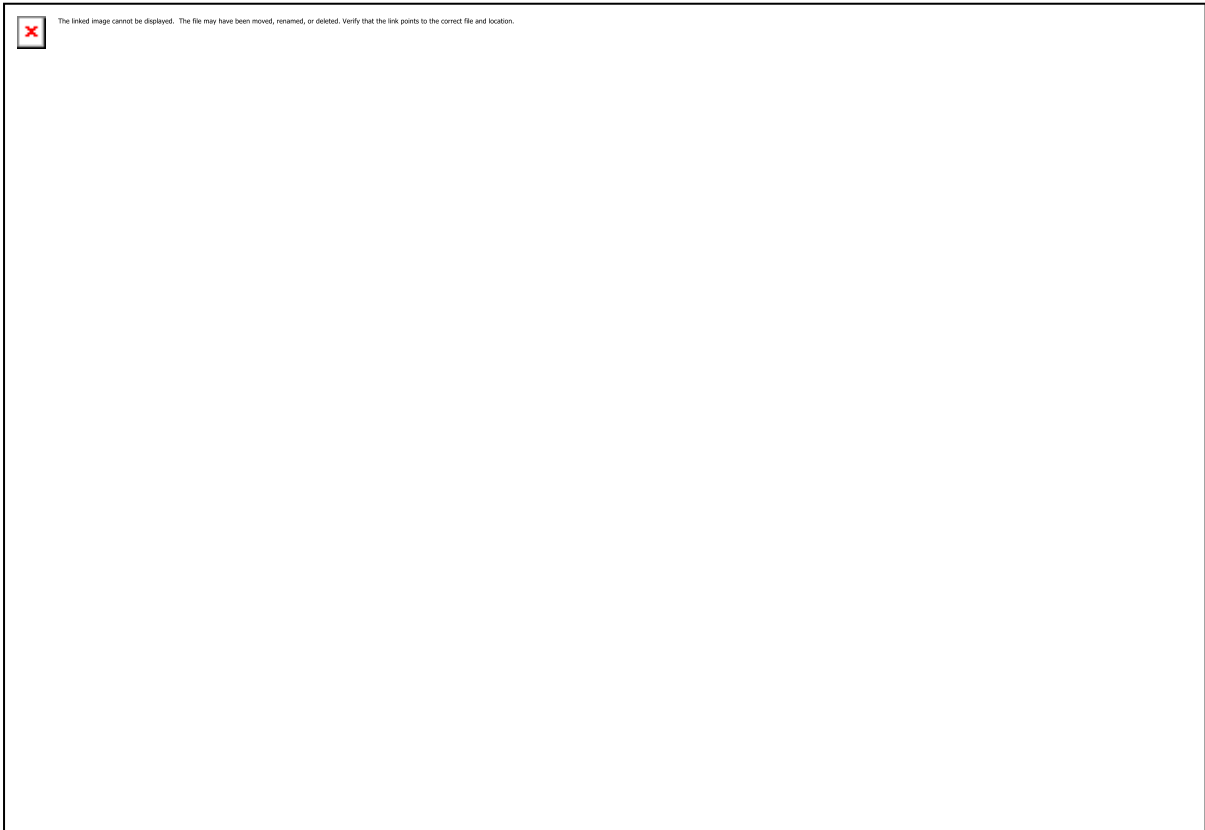
226

227 Calcium carbonate content was negatively correlated with eggshell thickness (estimate = -
228 0.04, SE \pm 0.006, $t = 6.86$, $p < 0.005$), after accounting for phylogeny, with thicker eggshells
229 having a lower calcium carbonate content (as a percentage of dried shell mass) than thinner
230 eggshells (Figure 1). There was a significant effect of residual variation in adult body mass
231 relative to egg mass on eggshell calcium carbonate content (estimate = -0.01, SE \pm 0.005, $t =$
232 2.46, $p = 0.01$), indicating that species with eggs that were larger than expected for their
233 adult body mass had a higher eggshell calcium carbonate content. Calcium carbonate
234 content was also predicted by clutch size, with species with smaller clutches having lower
235 eggshell calcium carbonate content (estimate = -0.02, SE \pm 0.007, $t = 2.85$, $p = 0.004$).
236 Additionally, there was an interaction between clutch size and lifespan on calcium
237 carbonate content (Figure 2): among species with a clutch size over two eggs, calcium
238 carbonate content of eggs decreased with increased lifespan, however, this effect was not
239 evident in species with less than an average of 2.5 eggs per clutch (interaction, estimate =
240 0.0007, SE \pm 0.0002, $t = 3.13$, $p = 0.002$). There was also a pattern of lower eggshell calcium
241 carbonate content at higher breeding latitudes (estimate = 0.0001, SE \pm 0.00005, $t = 2.63$, p

242 = 0.012). Lifespan alone was not a not significant predictor of eggshell calcium carbonate
243 content ($p = 0.99$) outside of the interaction with clutch size. The high value of phylogenetic
244 signal ($H^2=0.80 \pm 0.04$) of the PMM (accounting for intraspecific variation) was consistent
245 with the high Pagels λ value found for mean calcium carbonate content.



246
247 **Figure 1. Phylogenetic tree of mean eggshell calcium carbonate content (ash % of dry**
248 **eggshell mass) of species eggs.** Phylogenetic tree of all included species (n=222) generated
249 from the open tree of life (70). Branch colour represents ancestral reconstruction of
250 eggshell calcium content (log Arcsine of eggshell calcium %) with green representing higher
251 calcium carbonate content and orange representing a lower content. Purple bars display log
252 eggshell thickness (mm) of each species. Inset graph: calcium carbonate content (ash % of
253 dry eggshell mass) predicted by (log) eggshell thickness.
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Figure 2. Mean carbonate calcium content (ash % of dry eggshell mass) of species eggs in relation to lifespan and clutch size (eggs/nest). Mean eggshell calcium carbonate content of 222 species (Log – Arcsine transformed) calculated from 817 eggs, showing ash % decreases with increasing lifespan in species with large sized clutches, but not species with small clutches ($t = 3.13$, $p = 0.002$). The regression lines are representative of linear regression, not corrected for phylogenetic relatedness.

265

266 **Discussion**

267

268 Our results support several of the proposed hypotheses, such as a species lifespan and
269 clutch size dictating its eggshell calcium investment, while also showing an interesting
270 negative correlation with eggshell thickness, which was opposite to our predictions
271 (Supplementary Table 2). We found that differences observed in eggshell calcium carbonate
272 content covary with a combination of physiological traits (eggshell thickness, and egg mass)
273 and life history traits (lifespan, clutch size and breeding latitude). We found a phylogenetic
274 signal in the variation in eggshell calcium carbonate content between species that was
275 stronger than would be expected if this trait was evolving neutrally (Brownian-motion
276 model of evolution) (58), meaning that closely related species were more similar to one
277 another than distantly related species, as a result of shared ancestry (74). This would
278 suggest that calcium carbonate content is under strong genetic control, as is the case for
279 other known calcium-related eggshells properties such as calcite crystal size and
280 organisation (81).

281

282 Some of our results – lower eggshell calcium carbonate content in longer lived, large
283 clutched species – indicate that the allocation of calcium in avian eggshell production is
284 likely to be a feature of life history evolution to maximize lifetime fitness. These findings
285 complement current understandings of life history evolution (1), assuming calcium
286 deposition in eggshells is costly to the female (34,82). Species with shorter lifespans are
287 likely to have fewer opportunities to reproduce and, as such, are more likely to invest
288 heavily in the few broods that they do produce (1). In contrast, long-lived species may
289 reserve energy and resources for future reproduction at the expense of their current
290 reproductive effort (7). Excess calcium is not known to be stored in the body long-term in
291 most birds, meaning that current investment of calcium into a brood is unlikely to
292 significantly impact future calcium availability (34). However, the investment of calcium into
293 a clutch of eggs may have other costs to future reproduction. The calcium needed for egg
294 production must be acquired from the environment within a brief window prior to egg
295 laying, in order to increase circulating calcium (34,38,82). This requires strenuous foraging,
296 often for food sources that differ from the usual diet or requiring extraterritorial excursions,

297 which increases energy expenditure and predation risk for the female (40,41,83). The extent
298 of calcium-targeted foraging can have an impact on body condition and, therefore,
299 probability of survival to the next breeding season (84). Females of many bird species are
300 believed to be osteoporotic during egg laying as a result of calcium sequestration from
301 medullary bones (34), especially where dietary calcium is limited (39), resulting in higher
302 susceptibility to skeletal fractures (85). Reducing the calcium carbonate content of eggshells
303 might, therefore, present a trade-off between producing eggs with a strong shell and
304 bountiful calcium supplies for the embryo, or optimizing lifetime reproductive output by
305 producing many clutches of eggs with sufficient but less than ideal eggshell calcium
306 carbonate content.

307

308 For bird species with small clutches (one or two eggs), there was no statistical effect of
309 lifespan on eggshell calcium carbonate content. Overall, species with small clutch sizes had
310 lower calcium carbonate content per eggshell than other birds. Investment strategies of
311 species producing such 'micro-clutches' might differ from the investment strategies
312 predicted in larger clutched birds (86,87). One theory of clutch size evolution is that greater
313 risk of predation selects for smaller clutches (28,88). As small clutches are associated with
314 species under high predation risk (28,89), it would be strategic to reduce the calcium
315 carbonate content of these eggs, in addition to reducing clutch size, in favour of survival and
316 conserving body condition for future reproductive attempts by the female. This would
317 especially be the case if calcium foraging increases the risk of adult mortality by increasing
318 predation risk, as has been proposed but not tested (41).

319

320 There is a global gradient of increasing environmental calcium availability with higher
321 latitude which is thought to have influenced the evolution of bigger clutches at higher
322 latitudes (42). We expected to see higher calcium carbonate content in eggs of birds
323 breeding at higher latitudes due to this greater availability, and potential selection for
324 denser shells in colder climates. However, contrary to this, we found a decrease in
325 proportional calcium carbonate content in eggshells of species breeding at higher latitudes.
326 As this study composed primarily species breeding in the northern hemisphere, increasing
327 latitude corresponded to greater distance from the equator. Although this does not
328 correspond with global calcium availability patterns, or our rationale regarding temperature,

329 there are many other factors that vary latitudinal such as climate and food availability
330 (90,91), and as such it is difficult to identify the root cause of latitudinal variation.
331 Additionally, the present study relied on mean breeding latitudes of these species, as
332 detailed information on collection location did not exist for these eggs. As such, we were
333 unable to account for intraspecific variation in latitude. Future studies should consider
334 intraspecific variation and compare high latitude, temperate, species to those endemic to
335 the tropics where environmental calcium availability is dramatically lower (42).

336

337 In addition to correlations with life history traits, there was a strong negative pattern
338 between species eggshell thickness and eggshell calcium carbonate content. This is likely to
339 be linked to the strength requirements of the eggshell, which needs to be finely balanced
340 between being strong enough to support the body mass of the incubating parent while also
341 remaining breakable from the inside for the chick to hatch (3,92). Eggshell strength
342 increases with eggshell thickness (92), although other factors such as egg shape or calcite
343 crystal size and orientation also influence strength (57,93,94). However, our results indicate
344 that the increased strength with increasing thickness may not be achieved through greater
345 calcium carbonate deposition, but rather a thicker eggshell may achieve this greater
346 strength via alternative mechanisms. The eggshell is formed by the precipitation of calcium
347 carbonate from the uterine fluid to form calcite crystals on the surface of the egg
348 membrane (12). The formation of these crystals, particularly the unit size of each crystal and
349 how they orientate to and interlock with each other, is controlled by the organic component
350 of the eggshell (12,93). Moreover, this is highly heritable (81) and largely determines the
351 strength of the shell (94,95). An increase in osteopontin, a major component of the organic
352 portion of the shell, leads to smaller crystal units in the nanostructure of the shell which
353 increases the overall hardness of the material (93,96). Moreover, binding of osteopontin to
354 calcite crystals during formation increases fracture resistance (97). The observed lower
355 calcium carbonate content in thicker shelled eggs indicates a greater organic component
356 which could strengthen the shell in such a manner (93). Further investigation into how
357 calcium carbonate content directly correlates with fracture resistance would be useful to
358 elucidate this. Lower calcium carbonate in thicker eggshells may be a constraint of other
359 required properties of the shell, such as flexibility and stiffness, which will vary with
360 allometric scaling and thickness (57). Conversely, thinner eggshell might require more

361 calcium carbonate formed into denser calcite crystals to be strong enough to protect the
362 egg. Eggshell thickness and egg size are strongly and positively correlated (3,98); as a result
363 it is feasible that in smaller eggs, an increase in thickness would increase the required
364 interior breaking force (difficulty for the chick to hatch) to a greater extent than for larger
365 eggs, due to shape and allometry (57,92,98). As such, smaller eggs may achieve strength
366 through denser calcium carbonate deposition while remaining thin enough for the
367 developed chick to hatch. Further investigation into the role of calcium carbonate content
368 on the structural properties of eggshells would be beneficial to our understanding of how
369 this trait has evolved. Potentially, this association between eggshell thickness, calcium
370 carbonate and size could explain the low eggshell calcium carbonate content seen in small
371 clutches, since eggshells of single egg clutches tend to be larger and hence thicker shelled
372 (3,13,92,98).

373

374 There is a consistent scaling relationship between egg size, eggshell thickness and the body
375 mass of the incubating parents (3,92). We found that eggshell calcium carbonate content
376 decreases as species' residual body mass (body mass relative to egg mass) increases. As
377 such, species with eggs that are small relative to the size of the incubating parent have a
378 lower calcium carbonate content in their eggshells, which would suggest that the shell's
379 ability to support the mass of the incubating parent is not increased with calcium carbonate
380 content. This agrees with the above discussion that a greater organic component could
381 imbue greater strength to eggshells by regulating the organisation of calcite crystals (93,96).
382 Additionally, high calcium carbonate content in eggs that are larger than predicted for a
383 species body size would likely represent a substantial investment. Body mass is tightly
384 correlated with skeletal mass in birds (99) and will likewise affect the potential quantity of
385 circulating blood calcium that can be maintained during egg production, thereby increasing
386 the rate at which calcium must be obtained during the period of shell formation (34,83,100).
387 This is relevant to our understanding of the costs of egg production and how it effects
388 investment strategies across avian families.

389

390 Our findings change the understanding of how avian species allocate mineral resources to
391 their eggs and how this connects with their life history investment strategies. Calcium in
392 eggs has long been acknowledged as an important factor for reproductive success, however,

393 the association with lifespan should make us reconsider the investment costs involved.
394 Along with the strong phylogenetic signal, this suggest that species are under selection to
395 optimise individual per egg calcium allocation for maximum lifetime reproductive success.
396 This contradicts previous suggestions that calcium allocation to eggshells does not apply a
397 long term cost to breeding females (101). These findings highlight how little we know about
398 the costs associated with calcium acquisition, and what the benefits are to eggshell's
399 structural integrity of a higher or lower calcium carbonate content. Additionally, it is not yet
400 known what genetic factors control calcium allocation during eggshell formation and how
401 flexible this trait is within a species under different conditions. Eggshell thinning as a result
402 of environmental pollution (102,103), but also deduced environmental calcium availability
403 (104,105), has had severe detrimental effects on bird populations. Greater understanding of
404 the optimal eggshell composition for a species' reproductive biology and life-history would
405 enable us to better assist breeding programs for endangered birds. The specialisation in
406 shape and microstructure of eggshells has evolved these vessels to be highly optimised for
407 embryo development given a species specificities (5,26), and these results show how
408 eggshell calcium content has likewise evolved to complement avian life histories.

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410

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