

1 Brood parasite and host eggshells undergo similar levels of decalcification during embryonic  
2 development

3

4 Branislav Igetic<sup>1,\*</sup>, Mark E. Hauber<sup>2</sup>, Csaba Moskát<sup>3</sup>, Tomas Grim<sup>4</sup>, Matthew D. Shawkey<sup>1+</sup>,  
5 Petr Procházka<sup>5</sup>, and Marcel Honza<sup>5</sup>

6

7 <sup>1</sup>Department of Biology and Integrated Bioscience Program, University of Akron, Akron, OH  
8 44325-3908, USA.

9

10 <sup>2</sup>Department of Psychology, Hunter College and the Graduate Center of the City University  
11 of New York, New York, NY 10065, USA.

12

13 <sup>3</sup> MTA-ELTE-MTM Ecology Research Group, Hungarian Academy of Sciences, a joint  
14 research group of the Biological Institute, Eötvös Lóránd University, Pázmány P. sétány 1/C,  
15 1117 and the Hungarian Natural History Museum, Baross u. 13., 1088, Budapest, Hungary.

16

17 <sup>4</sup>Department of Zoology and Laboratory of Ornithology, Palacky University, 17. listopadu  
18 50, 77146 Olomouc, Czech Republic.

19

20 <sup>5</sup>Institute of Vertebrate Biology AS CR, Kvetna 8, 603 65 Brno, Czech Republic.

21

22 <sup>+</sup>Current address: Department of Biology, Terrestrial Ecology Unit, University of Ghent,  
23 Ledeganckstraat 35, Ghent 9000, Belgium

24

- 25 \*Author for correspondence: Branislav Igic, [brani.igic@gmail.com](mailto:brani.igic@gmail.com), +61-04-16971049.
- 26 Current address: Division of Evolution, Ecology and Genetics, Research School of Biology,
- 27 Australian National University, Canberra 2601, Australia

28 **Abstract**

29 Common cuckoos (*Cuculus canorus*) are obligate brood parasites that lay their eggs in the  
30 nests of other (host) species. To increase the likelihood of successful parasitism, common  
31 cuckoos lay eggs with thicker and structurally stronger eggshells than those of their hosts and  
32 non-parasitic relatives. Although hatching from thicker eggshells requires greater effort and  
33 may impose physiological costs on cuckoo embryos during hatching, it is unclear whether  
34 cuckoo eggshells are indeed thicker at the time of hatching. This is because avian embryos  
35 decalcify the innermost eggshell layer (mammillary layer) for organ development during  
36 embryogenesis, reducing eggshell thickness and making hatching easier. Therefore, common  
37 cuckoo eggshells may undergo a greater degree of decalcification during embryonic  
38 development to facilitate hatching from an initially thicker-shelled egg. We used scanning  
39 electron microscopy to test this hypothesis by comparing the thickness and degree of  
40 decalcification of eggshells collected either before incubation or after hatching. We found  
41 that cuckoo eggshells undergo similar degrees of decalcification during embryonic  
42 development as the thinner eggshells of a host that lays similarly sized eggs, the great reed  
43 warbler (*Acrocephalus arundinaceus*). Cuckoo eggshells hence remain thicker than eggshells  
44 of this host throughout embryogenesis, supporting the predicted trade-off between the  
45 benefits of laying puncture resistant eggs and the physiological costs associated with  
46 hatching.

47

48 **Keywords:** *Acrocephalus arundinaceus*, brood parasitism, *Cuculus canorus*, decalcification,  
49 eggshell, embryonic development.

50 **Introduction**

51 Obligate brood parasitic birds lay their eggs into nest of other bird species and use these hosts  
52 to raise parasitic offspring at the expense of the hosts' own fitness (Davies 2000; Feeney,  
53 Welbergen & Langmore 2014). This imposes strong selection pressures on hosts to minimize  
54 the likelihood of parasitism (Feeney, Welbergen & Langmore 2012) or to eliminate parasitic  
55 eggs and chicks from their nests (Antonov *et al.* 2006; Sato *et al.* 2010). For example, hosts  
56 of brood parasites often reject foreign eggs from their nests by puncturing their eggshells and  
57 removing them from the nest (Moksnes, Røskaft & Braa 1991; Antonov *et al.* 2006;  
58 Rasmussen, Sealy & Underwood 2009). In turn, brood parasites have evolved numerous  
59 counter-adaptations to improve the likelihood that hosts accept parasitic eggs. These  
60 adaptations include laying eggs that mimic the colour, patterning, and size of host eggs to  
61 evade recognition by hosts (Antonov *et al.* 2010; Stoddard & Stevens 2010; 2011; Igic *et al.*  
62 2012) and stronger eggshells to hinder rejection when detected (Brooker & Brooker 1991;  
63 Antonov *et al.* 2012).

64

65 The eggshells of brood parasitic birds are unusually strong for their egg's size  
66 (Brooker & Brooker 1991). A stronger eggshell may prevent their hosts from rejecting  
67 parasitic eggs by piercing their eggshells (Mermoz & Ornelas 2004; Antonov *et al.* 2009) and  
68 increase the likelihood that hosts erroneously damage their own eggs in the process (Spaw &  
69 Rohwer 1987; Rohwer, Spaw & Røskaft 1989; Røskaft, Rohwer & Spaw 1993; Sealy &  
70 Neudorf 1995; Antonov *et al.* 2006). Stronger eggshells may also help prevent damage that  
71 parasitic eggs sustain when they are laid in haste and dropped into deep host nests containing  
72 weaker (host) eggs (Gaston 1976), while simultaneously ensuring that parasitic offspring  
73 experience less competition for food by damaging and destroying host eggs (Soler, Soler &  
74 Martinez 1997). Lastly, a stronger eggshell may help prevent accidental or intentional

75 damage caused by other parasitic females that subsequently parasitize the same nest (Brooker  
76 & Brooker 1991; Spottiswoode 2013; Gloag, Keller & Langmore 2014).

77

78 Eggshell thickness is the major contributor to eggshell breaking strength across bird  
79 species (Brooks & Hale 1955; Ar, Rahn & Paganelli 1979). As such, eggs of many brood  
80 parasitic species from phylogenetically distant avian families, including cuckoos (family:  
81 Cuculidae), honeyguides (family: Indicatoridae), and cowbirds (family: Icteridae), are  
82 typically thicker than eggshells of their respective host species or non-parasitic relatives  
83 (Spaw & Rohwer 1987; Picman 1989; Brooker & Brooker 1991; Spottiswoode 2010; Igic *et*  
84 *al.* 2011). The selection pressure for thick-shelled eggs imposed on brood parasites may be  
85 strong enough to produce intra-specific differences. For instance, the eggshell thickness of  
86 different common cuckoo (*Cuculus canorus*) and diderik cuckoo (*Chrysococcyx caprius*)  
87 *gentes* (host races) are positively correlated with the discrimination abilities or eggshell  
88 thickness of their respective host species (Spottiswoode 2010; but see Igic *et al.*, 2011 and  
89 Drobniak *et al.* 2014). Coevolution with brood parasites may also select for thicker-shelled  
90 eggs in hosts (Spottiswoode & Colebrook-Robjent 2007). In addition to eggshell thickness,  
91 other characteristics may also contribute to the greater breaking strength of parasitic eggs,  
92 including a rounder egg shape, a greater density of inorganic components in the eggshell, and  
93 the size or orientation of the eggshell's crystalline components (Picman 1989; Picman &  
94 Pribil 1997; Bán *et al.* 2011). Independently of overall eggshell thickness, the inner-most  
95 (mammillary) layers of common cuckoo eggshells are more resistant to compression forces  
96 than are the corresponding layers of their hosts' eggshells, potentially contributing to a  
97 greater overall breaking strength for common cuckoo eggs (Igic *et al.* 2011).

98

99           A potential consequence of laying thicker-shelled eggs for brood parasites is that their  
100 young may require more energy and effort to hatch (Honza *et al.* 2001; Yoon 2013). For  
101 example, common cuckoo hatchlings require more time and pecks to hatch than the  
102 hatchlings of a host that lays eggs of comparable size but with thinner eggshells, the great  
103 reed warbler (*Acrocephalus arundinaceus*; Honza *et al.* 2001). Similarly, brown-headed  
104 cowbird (*Molothrus ater*) hatchlings take longer to hatch and produce more clicks  
105 (suggesting higher pulmonary respiration) during hatching relative to hatchlings of its red-  
106 winged blackbird host (*Agelaius phoeniceus*; Yoon 2013). In turn, common cuckoo  
107 hatchlings have several morphological characteristics that may help them hatch from  
108 structurally stronger eggs, including a larger mass, longer forearms and egg teeth, and a  
109 higher density of fibres in muscles used for hatching relative to great reed warbler hatchlings  
110 (Honza *et al.* 2001; 2015). By contrast, the egg teeth of brown-headed cowbird hatchlings are  
111 smaller than those of red-wing blackbird hatchlings (Yoon 2013). Physiological mechanisms  
112 may also help common cuckoo hatchlings hatch from thicker-shelled eggs, including heavier  
113 egg yolks that contain greater concentrations of anti-oxidants (Török *et al.* 2004; Hargitai *et*  
114 *al.* 2010), but not higher concentrations of maternally derived testosterone and energy  
115 reserves (Török *et al.* 2004; Igic *et al.* 2015) or greater levels of gaseous exchange (Portugal  
116 *et al.* 2014). Whether any brood parasitic species has eggshell-specific characteristics that  
117 help their young hatch from structurally stronger eggs remains unknown.

118

119           Although common cuckoo eggshells are thicker than those of their hosts soon after  
120 being laid, it is unclear whether they remain thicker than hosts' eggshells at the hatching  
121 stage. Avian embryos derive most of the calcium required for growth by decalcifying the  
122 calcium carbonate from the inner-most (mammillary) layer of their eggshells, reducing  
123 eggshell thickness and breaking strength, and in turn aiding hatching (Kreitzer 1972;

124 Freeman & Vince 1974; Castilla *et al.* 2007; Chien, Hincke & McKee 2009). Therefore, it is  
125 possible that cuckoo embryos decalcify a greater portion of their eggshells during  
126 development relative to their hosts, which would reduce the effort required to hatch.  
127 However, due to the shorter embryonic development of cuckoos relative to hosts (Wyllie  
128 1981), cuckoo embryos may also decalcify less of their eggshell during development  
129 (Karlsson & Lilja 2008). Studies to date have only compared eggshell structure between  
130 brood parasites and their hosts or non-parasitic relatives using unincubated eggs (e.g. Spaw &  
131 Rohwer 1987; Picman 1989; Spottiswoode 2010; Igic *et al.* 2011), and very little is known  
132 regarding the structural changes to eggshells of brood parasites associated with  
133 embryogenesis (although see Karlsson & Lilja 2008).

134

135         We examined and compared the embryogenesis-related microstructural changes to  
136 eggshells of the common cuckoo (hereafter cuckoo) in relation to eggshells of its great reed  
137 warbler host (hereafter warbler). We used warblers for comparison because they lay eggs of a  
138 comparable size to those of cuckoos but with significantly thinner eggshells (Török *et al.*  
139 2004; Antonov *et al.* 2006; Bán *et al.* 2011; Igic *et al.* 2011; Hargitai *et al.* 2012). Moreover,  
140 this host has been used as a comparison for the cuckoo in relation to physiological and  
141 morphological adaptations associated with embryonic development and hatching (Honza *et*  
142 *al.* 2001; Török *et al.* 2004; Hargitai *et al.* 2010; Honza *et al.* 2015; Igic *et al.* 2015). Here,  
143 we focussed on comparing the changes in eggshell thickness between cuckoo and warbler  
144 eggs at different stages of development.

145 **Materials and Methods**

146 *Sample collection*

147 We collected cuckoo and great reed warbler eggs from host nests across several years (Table  
148 S1) and from two adjacent sites in the Czech Republic (Mutěnice 48°54′ N 17°02′ E; and  
149 Lužice 48°51′ N 17°05′ E) and one site in Hungary (Apaj 47°06′ N 19°05′ E). Such meta-  
150 replication in both space and time increases the reliability and validity of biological sampling  
151 (Johnson 2002; Grim *et al.* 2011), particularly as cuckoos likely adapt to their hosts at the  
152 metapopulation level rather than locally (Avilés *et al.* 2011). We then either cleaned, and  
153 stored in a dark dry place immediately after collection (early-stage eggs) or placed into  
154 incubators to complete development and hatch before cleaning and storing. See  
155 Supplementary Materials for more details on sample collection and permits.

156

157 *Examination of eggshell structure*

158 We used a JSM-7401F scanning electron microscope (SEM, JEOL Japan) to examine the  
159 differences between early-stage and hatched eggshells. Unlike eggshell thickness  
160 measurements collected using micrometres, measurements from SEM images more  
161 accurately capture the variation in eggshell thickness and allow the visualization of  
162 microstructural differences (Igic *et al.* 2010; 2011). We mounted eggshell fragments from the  
163 equatorial region onto aluminium stubs to allow visualization of their cross-sections, which  
164 we sputter-coated with gold/palladium for 1 min. We viewed samples at a working distance  
165 of 7 mm, using an accelerating voltage of 7 kV, and collected images at magnifications of  
166 450x and 1600x. Avian eggshells are divided into two visually distinct layers, an outer  
167 palisade layer and inner mammillary layer, the latter of which is decalcified and absorbed by  
168 the embryo during development (Freeman & Vince 1974; Mikhailov 1997). We delineated  
169 the division of these two layers by the presence of the spherical films (circular hole-like

170 vesicles) that are characteristic of the palisade layer (Mikhailov 1997). We used ImageJ v1.48  
171 (National Institute of Health, USA; freely downloadable from <http://rsb.info.nih.gov/ij/>) to  
172 measure total eggshell thickness and the thickness of the two respective layers at 30-40  
173 randomly selected areas spread evenly across eggshell cross-sections. We then calculated  
174 average values per egg for total eggshell thickness and thicknesses of the two respective  
175 eggshell layers. In total we measured 106 eggshells; however, we calculated and used  
176 average thickness estimates for warbler eggshells from the same nest, producing a total of  
177 100 independent samples for our analysis (49 cuckoo and 51 warbler eggshells). Both  
178 thickness measurements taken on the same image (106 images measured twice:  $R = 0.96$ ;  
179 95% C.I: [0.95, 0.98]) and taken on images of the same eggshell at different locations (12  
180 randomly chosen eggshells imaged and measured twice:  $R = 0.87$ ; 95% C.I: [0.57, 0.96])  
181 were repeatable.

182

### 183 *Statistical analysis*

184 We used linear mixed models to compare structural differences between cuckoo and warbler  
185 eggshells collected at the two stages of development. We fit each model with either total  
186 eggshell thickness, mammillary layer thickness, or palisade layer thickness as a response;  
187 species (cuckoo or warbler), stage (early stage or hatched), and the interaction between  
188 species and stage as fixed effects; and an independent identifier for each site/year of  
189 collection combination as a random effect (8 total combinations; Table S1). We present full  
190 models without backward elimination of non-significant predictors (Forstmeier & Schielzeth  
191 2011). The interaction between species and stage was non-significant in all circumstances  
192 and was therefore excluded from models to allow appropriate interpretation of estimates and  
193  $P$ -values for fixed effects (Tables 1 & 2; Engqvist 2005); however, we present these non-  
194 significant interaction effects in the text (see Results). Excluding eggshells collected in

195 Hungary from our analyses did not affect statistical outcomes, confirming that potential trans-  
196 site differences were not responsible for the observed patterns (data not presented). We  
197 lacked collection date information for five unincubated warbler eggshells and four  
198 unincubated cuckoo eggshells; however, collection date was not a significant predictor and  
199 did not change the results when included in models fitted using data for the remaining eggs  
200 (Table S2), and therefore was not used in our final models. We used re-sampling analyses to  
201 confirm that our unbalanced dataset did not influence our results (Supplementary Materials;  
202 Table S3). See Supplementary Materials for more details on statistical procedures.

203 **Results**

204 Eggshell thickness differences between early-stage and hatched eggs were similar for cuckoo  
205 and warbler eggs, such that unincubated and hatched cuckoo eggshells were thicker than  
206 unincubated and hatched warbler eggs, respectively (Figure 1). Hatched warbler eggs were on  
207 average  $4.82 \mu\text{m}$  ( $\pm 1.96$  s.e.) thinner than early-stage warbler eggs ( $P = 0.049$ ; Table 1;  
208 Figure 1), whereas hatched cuckoo eggs were on average  $5.69 \mu\text{m}$  ( $\pm 2.27$  s.e.) thinner than  
209 early-stage cuckoo eggs ( $P = 0.04$ ; Table 1; Figure 1). This difference between eggshell  
210 thickness of early-stage and hatched cuckoo eggs was not significantly greater than that for  
211 warbler eggs (interaction between species and developmental stage:  $-0.87 \mu\text{m} \pm 2.72$  s.e.;  
212 95% C.I:  $[-6.29, 4.54]$ ;  $t_{88} = -0.32$ ;  $P = 0.75$ ). Early-stage cuckoo eggshells were  $16.21 \mu\text{m}$   
213 ( $\pm 1.78$  s.e.) thicker than early-stage warbler eggshells, whereas hatched cuckoo eggshells  
214 were  $17.09 \mu\text{m}$  ( $\pm 2.12$  s.e.) thicker than hatched warbler eggshells (both  $P < 0.001$ ; Table 1).  
215 Hatched eggshells of both species were thinner than their early-stage counterparts because of  
216 thinner mammillary layers ( $P < 0.0001$ ; Table 2; Figure 2) and not because of differences in  
217 the thicknesses of their palisade layers ( $P = 0.55$ ; Table 2; Figure 2). This difference between  
218 mammillary layer thickness of early-stage and hatched eggs did not differ for cuckoo  
219 eggshells relative to warbler eggshells (interaction between species and developmental stage:  
220  $-1.61 \mu\text{m} \pm 1.29$  s.e; 95% C.I:  $[-4.17, 0.96]$ ;  $t_{88} = -1.25$ ;  $P = 0.22$ ).

221 **Discussion**

222 We found that cuckoo eggshells were thicker than eggshells of their great reed warbler hosts  
223 at all stages of development. As eggshell thickness is the strongest contributor to eggshell  
224 breaking strength (Brooks & Hale 1955; Ar, Rahn & Paganelli 1979), our findings imply that  
225 cuckoo eggshells maintain a greater breaking strength than warbler eggshells throughout  
226 embryonic development and support the hypothesis that cuckoos require a greater effort to  
227 hatch than warblers (Honza *et al.* 2001).

228

229 The eggshell thinning of both cuckoo and warbler eggshells during embryonic  
230 development was associated with similar degrees of decalcification of the innermost  
231 mammillary layer. This contrasts with expectation that the faster developing cuckoo embryo  
232 should decalcify the eggshell less than the slower developing great reed warbler embryo  
233 (Blom & Lilja 2004; Karlsson & Lilja 2008). The average incubation period of cuckoo eggs  
234 is 11.63 days versus 12.85 days for great reed warbler eggs, as measured from the onset of  
235 incubation, at our Hungarian site (Geltsch *et al.* 2016). However, the difference between  
236 incubation periods of the two species may be due to internal incubation of eggs by cuckoos  
237 prior to laying, rather than faster overall embryonic development (Birkhead *et al.* 2011). The  
238 eggshell thickness differences between early-stage and hatched eggs were comparable for  
239 cuckoos and warblers, and were similar to those found for other altricial, mostly non-  
240 passerine, species (Table 3). Therefore, the small (0.87  $\mu\text{m}$ ) differences between cuckoo and  
241 warbler eggs detected here are likely not due to the cuckoo's brood parasitic reproductive  
242 strategy. Through visual examination, a previous study suggested that common cuckoo  
243 eggshells undergo similar degrees of mammillary layer erosion as other altricial species'  
244 eggshells (Karlsson & Lilja 2008). Similarly, we could not visually ascertain any obvious

245 structural differences that would suggest cuckoo eggshells underwent greater degrees of  
246 decalcification relative to warbler eggshells (Figures 1 & 2).

247

248         The potential physiological consequences of greater eggshell decalcification during  
249 embryogenesis or a greater risk of eggshell breakage may outweigh the potential benefits of  
250 greater embryonic eggshell decalcification for cuckoos. Decalcification and calcium  
251 absorption by avian embryos is an active metabolic process, requiring both production and  
252 secretion of acidic substances to dissolve the eggshell and energy expenditure for cellular  
253 transportation of calcium (Terepka, Stewart & Merkel 1969; Garrison & Terepka 1972).  
254 Although greater decalcification enables growth of skeletally larger or more ossified embryos  
255 (Honza *et al.* 2001; Blom & Lilja 2004) and reduces eggshell breaking strength to facilitate  
256 hatching (Freeman & Vince 1974; Castilla *et al.* 2007), cuckoo embryos may lack the energy  
257 reserves required to accomplish greater levels of decalcification (Igic *et al.* 2015). Calcium  
258 ions are important for a number of physiological functions during embryogenesis, including  
259 cell-cell signalling, cell division, and organ development (Romanoff 1967; Berridge 1995).  
260 The perturbation of calcium homeostasis or hypercalcemia can cause embryonic mortality  
261 (Packard & Packard 1993), which in turn may limit the degree of eggshell decalcification that  
262 cuckoo embryos can safely achieve. Greater eggshell decalcification at later stages of  
263 development may also allow hosts to postpone eggshell puncture rejection behaviour to a  
264 period where parasitic eggshells are sufficiently thin to be punctured (Antonov *et al.* 2008).  
265 Therefore, selection may have favoured the evolution of developmental adaptations, such as a  
266 greater hatchling size, to facilitate hatching from a thicker-shelled egg (Honza *et al.* 2001;  
267 2015), rather than greater embryonic eggshell decalcification.

268

269           Despite similar changes in eggshell thickness, it is still possible that cuckoo eggshells  
270 undergo a greater reduction in overall breaking strength compared with warbler eggshells  
271 following embryogenesis. The mammillary layer of warbler eggshells is structurally weaker  
272 than their palisade layer, whereas the mammillary and palisade layers of cuckoo eggshells  
273 can withstand similar levels of compression force (Igic *et al.* 2011). Therefore, cuckoo  
274 eggshells could theoretically experience a greater reduction in overall hardness compared to  
275 warblers even if both experience the same degree of decalcification due to the reduction of a  
276 structurally stronger layer. This requires further investigation through comparisons of  
277 breaking strength between early-stage and hatched cuckoo and warbler eggshells. Given the  
278 16  $\mu\text{m}$  difference between hatched cuckoo and warbler eggshells, cuckoo eggs likely retain a  
279 structurally stronger eggshell compared to warblers throughout development. To elucidate  
280 whether any potential differences are due to brood parasite specific adaptations, future work  
281 should also include comparisons with non-parasitic relatives (e.g. Krüger & Davies 2002).  
282 Indeed, other than differences in egg size and eggshell thickness, little is known regarding  
283 eggshell-specific differences between eggs of parasitic and non-parasitic cuckoos (Payne  
284 1974; Krüger & Davies 2004; although see Mikhailov, 1997; Picman and Pribil, 1997), and  
285 particularly so in relation to changes associated with embryonic development. A particularly  
286 fruitful area for future work is testing whether parasitic species' eggshells contain specific  
287 structural characteristics that facilitate breakage initiated from inside the egg while  
288 preventing breakage caused by external forces (Entwistle, Silyn-Roberts & Abuodha 1995;  
289 Nedomová, Buchar & Křivánek 2014).

290 **Acknowledgements:** We thank the Shawkey lab for comments on the manuscript. We are  
291 thankful to Miroslav Čapek, Alena Dvorská, Kateřina Feikusová, Václav Jelínek, Jaroslav  
292 Koleček, Beata Matysioková, Milica Požgayová, Peter Samaš, Kateřina Sosnovcová, and  
293 Michal Šulc for their field assistance in the Czech Republic; and to István Zsoldos, Anikó  
294 Zölei, and Nikoletta Geltsch for their help with nest searching in Hungary. For funding we  
295 thank the Human Frontier Science Program (RGY 69/07 to TG and MEH, and RGY83/2012  
296 to MEH, TG, and MDS) and the Grant Agency of the Czech Republic (grant no.  
297 P506/12/2404 to MH and TG). The study was also supported by the Hungarian National  
298 Research Fund (OTKA, grant no. 83217 to CM) and the National Research, Development  
299 and Innovation Office (NKFIH, NN118194 to CM).

300 **Literature Cited**

- 301 Abarca, B., Luis, D., Quintana, L., Jose, A., Casaubon, H. & Rosales, S. G. (2011)  
 302 Assessment of Eggshell Quality Before and After Incubation from 29 and 46 Weeks-  
 303 old Leghorn Hens. *Int. J. Poult. Sci.* **10**, 977-982.
- 304 Antonov, A., Stokke, B., Vikan, J., Fossøy, F., Ranke, P., Røskaft, E., Moksnes, A., Møller,  
 305 A. & Shykoff, J. (2010) Egg phenotype differentiation in sympatric cuckoo *Cuculus*  
 306 *canorus* gentes. *J. Evol. Biol.* **23**, 1170-1182.
- 307 Antonov, A., Stokke, B. G., Fossøy, F., Liang, W., Moksnes, A., Røskaft, E., Yang, C. &  
 308 Møller, A. P. (2012) Why do brood parasitic birds lay strong-shelled eggs? *Chin.*  
 309 *Birds* **3**, 245-258.
- 310 Antonov, A., Stokke, B. G., Moksnes, A., Kleven, O., Honza, M. & Røskaft, E. (2006)  
 311 Eggshell strength of an obligate brood parasite: a test of the puncture resistance  
 312 hypothesis. *Behav. Ecol. Sociobiol.* **60**, 11-18.
- 313 Antonov, A., Stokke, B. G., Moksnes, A. & Røskaft, E. (2008) Getting rid of the cuckoo  
 314 *Cuculus canorus* egg: why do hosts delay rejection? *Behav. Ecol.* **19**, 100-107.
- 315 Antonov, A., Stokke, B. G., Moksnes, A. & Røskaft, E. (2009) Evidence for egg  
 316 discrimination preceding failed rejection attempts in a small cuckoo host. *Biol. Lett.* **5**,  
 317 169-171.
- 318 Ar, A., Rahn, H. & Paganelli, C. V. (1979) The avian egg: mass and strength. *Condor* **81**,  
 319 331-337.
- 320 Avilés, J., Vikan, J., Fossøy, F., Antonov, A., Moksnes, A., Røskaft, E., Shykoff, J., Møller,  
 321 A., Jensen, H., Procházka, P. & Stokke, B. G. (2011) The common cuckoo *Cuculus*  
 322 *canorus* is not locally adapted to its reed warbler *Acrocephalus scirpaceus* host. *J.*  
 323 *Evol. Biol.* **24**, 314-325.
- 324 Balkan, M., Karakaş, R. & Biricik, M. (2006) Changes in eggshell thickness, shell  
 325 conductance and pore density during incubation in the Peking Duck (*Anas*  
 326 *platyrhynchos* f. *dom.*). *Ornis. Fenn.* **83**, 117-123.
- 327 Bán, M., Barta, Z., Munoz, A., Takasu, F., Nakamura, H. & Moskát, C. (2011) The analysis  
 328 of Common Cuckoo's egg shape in relation to its hosts' in two geographically distant  
 329 areas. *J. Zool.* **284**, 77-83.
- 330 Berridge, M. J. (1995) Calcium signalling and cell proliferation. *Bioessays* **17**, 491-500.
- 331 Birkhead, T., Hemmings, N., Spottiswoode, C., Mikulica, O., Moskát, C., Bán, M. &  
 332 Schulze-Hagen, K. (2011) Internal incubation and early hatching in brood parasitic  
 333 birds. *Proc. Roy. Soc. B* **278**, 1019-1024.
- 334 Blom, J. & Lilja, C. (2004) A comparative study of growth, skeletal development and  
 335 eggshell composition in some species of birds. *J. Zool.* **262**, 361-369.
- 336 Booth, D. T. (1989) Regional changes in shell thickness, shell conductance, and pore  
 337 structure during Incubation in eggs of the mute swan. *Physiol. Zool.* **62**, 607-620.
- 338 Booth, D. T. & Seymour, R. S. (1987) Effect of eggshell thinning on water vapor  
 339 conductance of malleefowl eggs. *Condor* **89**, 453-459.
- 340 Brooker, M. G. & Brooker, L. (1991) Eggshell strength in cuckoos and cowbirds. *Ibis* **133**,  
 341 406-413.
- 342 Brooks, J. & Hale, H. P. (1955) Strength of the shell of the hen's egg. *Nature* **175**, 848-849.
- 343 Bunck, C. M., Spann, J. W., Pattee, O. H. & Fleming, W. J. (1985) Changes in eggshell  
 344 thickness during incubation: implications for evaluating the impact of organochlorine  
 345 contaminants on productivity. *Bull. Environ. Contam. Toxicol.* **35**, 173-182.
- 346 Capen, D. E. (1977) Eggshell thickness variability in the white-faced ibis. *Wilson Bull.* **89**,  
 347 99-106.

- 348 Castilla, A. M., Herrel, A., Díaz, G. & Francesch, A. (2007) Developmental stage affects  
349 eggshell-breaking strength in two ground-nesting birds: the partridge (*Alectoris rufa*)  
350 and the quail (*Coturnix japonica*). *J. Exp. Zool. A: Ecol. Genet. Physiol.* **307**, 471-  
351 477.
- 352 Castilla, A. M., Herrel, A., Robles, H., Malone, J. & Negro, J. J. (2010) The effect of  
353 developmental stage on eggshell thickness variation in endangered falcons. *Zoology*  
354 **113**, 184-188.
- 355 Chien, Y. C., Hincke, M. T. & McKee, M. D. (2009) Ultrastructure of avian eggshell during  
356 resorption following egg fertilization. *J. Struct. Biol* **168**, 527-538.
- 357 Dahlgren, R. B. & Linder, R. L. (1971) Effects of polychlorinated biphenyls on pheasant  
358 reproduction, behavior, and survival. *J. Wildl. Manag.* **35**, 315-319.
- 359 Davies, N. B. (2000) *Cuckoos, cowbirds and other cheats*. London: T. & A.D. Poyser.
- 360 Drobniak, S. M., Dyrz, A., Sudyka, J. & Cichoń, M. (2014) Continuous variation rather than  
361 specialization in the egg phenotypes of cuckoos (*Cuculus canorus*) parasitizing two  
362 sympatric reed warbler species. *PLoS ONE* **9**, e106650.
- 363 Engqvist, L. (2005) The mistreatment of covariate interaction terms in linear model analyses  
364 of behavioural and evolutionary ecology studies. *Anim. Behav.* **70**, 967-971.
- 365 Entwistle, K., Silyn-Roberts, H. & Abuodha, S. O. (1995) The relative fracture strengths of  
366 the inner and outer surfaces of the eggshell of the domestic fowl. *Proc. Roy. Soc. B*  
367 **262**, 169-174.
- 368 Feeney, W. E., Welbergen, J. A. & Langmore, N. E. (2012) The frontline of avian brood  
369 parasite–host coevolution. *Anim. Behav.* **84**, 3-12.
- 370 Feeney, W. E., Welbergen, J. A. & Langmore, N. E. (2014) Advances in the study of  
371 coevolution between avian brood parasites and their hosts. *Annu. Rev. Ecol. Evol.*  
372 *Syst.* **45**, 227-246.
- 373 Finnlund, M., Hissa, R., Koivusaari, J., Merilä, E. & Nuuja, I. (1985) Eggshells of arctic terns  
374 from Finland: effects of incubation and geography. *Condor* **87**, 79-86.
- 375 Forstmeier, W. & Schielzeth, H. (2011) Cryptic multiple hypotheses testing in linear models:  
376 overestimated effect sizes and the winner's curse. *Behav. Ecol. Sociobiol.* **65**, 47-55.
- 377 Freeman, B. M. & Vince, M. A. (1974) *Development of the avian embryo. A behavioural and*  
378 *physiological study*. London: Chapman and Hall.
- 379 Garrison, J. C. & Terepka, A. R. (1972) Calcium-stimulated respiration and active calcium  
380 transport in the isolated chick chorioallantoic membrane. *J. Membr. Biol.* **7**, 128-145.
- 381 Gaston, A. J. (1976) Brood Parasitism by the pied crested cuckoo *Clamator jacobinus*. *J.*  
382 *Anim. Ecol.* **45**, 331-348.
- 383 Geltsch, N., Bán, M., Hauber, M. E. & Moskát, C. (2016) When should common cuckoos  
384 *Cuculus canorus* lay their eggs in host nests? . *Bird Study* **63**, 45-51.
- 385 Gloag, R., Keller, L.-A. & Langmore, N. E. (2014) Cryptic cuckoo eggs hide from competing  
386 cuckoos. *Proc. Roy. Soc. B* **281**, 20141014.
- 387 Grim, T., Samaš, P., Moskát, C., Kleven, O., Honza, M., Moksnes, A., Røskft, E. & Stokke,  
388 B. G. (2011) Constraints on host choice: why do parasitic birds rarely exploit some  
389 common potential hosts? *J. Anim. Ecol.* **80**, 508-518.
- 390 Handrich, Y. (1989) Incubation water loss in king penguin egg. I. Change in egg and brood  
391 pouch parameters. *Physiol. Zool.*, 96-118.
- 392 Hargitai, R., Costantini, D., Moskát, C., Bán, M., Muriel, J. & Hauber, M. E. (2012)  
393 Variation in plasma oxidative status and testosterone level in relation to egg-ejection  
394 effort and age of brood-parasitic common cuckoo nestlings. *Condor* **114**, 782-791.
- 395 Hargitai, R., Moskát, C., Bán, M., Gil, D., López-Rull, I. & Solymos, E. (2010) Eggshell  
396 characteristics and yolk composition in the common cuckoo *Cuculus canorus*: are  
397 they adapted to brood parasitism? *J. Avian Biol.* **41**, 177-185.

- 398 Honza, M., Feikusová, K., Procházka, P. & Picman, J. (2015) How to hatch from the  
399 common cuckoo (*Cuculus canorus*) egg: implications of strong eggshells for the  
400 hatching muscle (*musculus complexus*). *J. Ornithol.* **156**, 679-685.
- 401 Honza, M., Picman, J., Grim, T., Novák, V., Čapek Jr M. & Mrlík, V. (2001) How to hatch  
402 from an egg of great structural strength. A study of the common cuckoo. *J. Avian*  
403 *Biol.* **32**, 249-255.
- 404 Igic, B., Braganza, K., Hyland, M. M., Silyn-Roberts, H., Cassey, P., Grim, T., Rutila, J.,  
405 Moskát, C. & Hauber, M. E. (2011) Alternative mechanisms of increased eggshell  
406 hardness of avian brood parasites relative to host species. *J. R. Soc. Interface* **8**, 1654-  
407 1664.
- 408 Igic, B., Cassey, P., Grim, T., Greenwood, D. R., Moskát, C., Rutila, J. & Hauber, M. E.  
409 (2012) A shared chemical basis of avian host–parasite egg colour mimicry. *Proc. Roy.*  
410 *Soc. B* **279**, 1068-1076.
- 411 Igic, B., Hauber, M. E., Galbraith, J. A., Grim, T., Dearborn, D. C., Brennan, P. L., Moskát,  
412 C., Choudhary, P. K. & Cassey, P. (2010) Comparison of micrometer- and scanning  
413 electron microscope-based measurements of avian eggshell thickness. *J. Field*  
414 *Ornithol.* **81**, 402-410.
- 415 Igic, B., Zarate, E., Sewell, M. A., Moskát, C., Cassey, P., Rutila, J., Grim, T., Shawkey, M.  
416 D. & Hauber, M. E. (2015) A comparison of egg yolk lipid constituents between  
417 parasitic Common Cuckoos and their hosts. *Auk* **132**, 817-825.
- 418 Johnson, D. H. (2002) The Importance of replication in wildlife research. *J. Wildl. Manag.*  
419 **66**, 919-932.
- 420 Karlsson, O. & Lilja, C. (2008) Eggshell structure, mode of development and growth rate in  
421 birds. *Zoology* **111**, 494-502.
- 422 Kern, M. D., Cowie, R. J. & Yeager, M. (1992) Water loss, conductance, and structure of  
423 eggs of pied flycatchers during egg laying and incubation. *Physiol. Zool.*, 1162-1187.
- 424 Kreitzer, J. F. (1972) The effect of embryonic development on the thickness of the egg shells  
425 of coturnix quail. *Poult. Sci.* **51**, 1764-1765.
- 426 Krüger, O. & Davies, N. (2002) The evolution of cuckoo parasitism: a comparative analysis.  
427 *Proc. Roy. Soc. B* **269**, 375-381.
- 428 Krüger, O. & Davies, N. B. (2004) The evolution of egg size in the brood parasitic cuckoos.  
429 *Behav. Ecol.* **15**, 210-218.
- 430 Mermoz, M. E. & Ornelas, J. F. (2004) Phylogenetic analysis of life-history adaptations in  
431 parasitic cowbirds. *Behav. Ecol.* **15**, 109-119.
- 432 Mikhailov, K. E. (1997) *Avian eggshells: an atlas of scanning electron micrographs*.  
433 Newbury: British Ornithologists' Club Occasional Publications, No. 3. The Nature  
434 Conservancy Bureau Limited.
- 435 Moksnes, A., Røskaft, E. & Braa, A. T. (1991) Rejection behavior by common cuckoo hosts  
436 towards artificial brood parasite eggs. *Auk* **108**, 348-354.
- 437 Nedomová, Š., Buchar, J. & Křivánek, I. (2014) The effect of the egg's shape on the stress  
438 distribution in the eggshell at internal pressure loading. *Acta Univ. Agric. Silvic.*  
439 *Mendel. Brun.* **55**, 129-142.
- 440 Packard, M. J. & Packard, G. C. (1993) Water loss from eggs of domestic fowl and calcium  
441 status of hatchlings. *J. Comp. Physiol. B* **163**, 327-331.
- 442 Payne, R. B. (1974) The evolution of clutch size and reproductive rates in parasitic cuckoos.  
443 *Evolution* **28**, 169-181.
- 444 Picman, J. (1989) Mechanism of increased puncture resistance of eggs of brown-headed  
445 cowbirds. *Auk* **106**, 577-583.
- 446 Picman, J. & Pribil, S. (1997) Is greater eggshell density an alternative mechanism by which  
447 parasitic cuckoos increase the strength of their eggs? *J. Ornithol.* **138**, 531-541.

- 448 Portugal, S. J., Hauber, M. E., Maurer, G., Stokke, B. G., Grim, T. & Cassey, P. (2014) Rapid  
449 development of brood-parasitic cuckoo embryos cannot be explained by increased gas  
450 exchange through the eggshell. *J. Zool.* **293**, 219-226.
- 451 Rasmussen, J. L., Sealy, S. G. & Underwood, T. J. (2009) Video recording reveals the  
452 method of ejection of brown-headed cowbird eggs and no cost in American robins  
453 and gray catbirds. *Condor* **111**, 570-574.
- 454 Rohwer, S., Spaw, C. D. & Røskaft, E. (1989) Costs to northern orioles of puncture-ejecting  
455 parasitic cowbird eggs from their nests. *Auk* **106**, 734-738.
- 456 Romanoff, A. L. (1967) *Biochemistry of the avian embryo*. New York: Wiley.
- 457 Røskaft, E., Rohwer, S. & Spaw, C. D. (1993) Cost of puncture ejection compared with costs  
458 of rearing cowbird chicks for northern orioles. *Ornis Scand.* **24**, 28-32.
- 459 Şahan, Ü., Altan, Ö., Ipek, A. & Yilmaz, B. (2003) Effects of some egg characteristics on the  
460 mass loss and hatchability of ostrich (*Struthio camelus*) eggs. *British poultry science*  
461 **44**, 380-385.
- 462 Sato, N. J., Tokue, K., Noske, R. A., Mikami, O. K. & Ueda, K. (2010) Evicting cuckoo  
463 nestlings from the nest: a new anti-parasitism behaviour. *Biol. Lett.* **6**, 67-69.
- 464 Sealy, S. G. & Neudorf, D. L. (1995) Male northern orioles eject cowbird eggs: implications  
465 for the evolution of rejection behavior. *Condor* **97**, 369-375.
- 466 Soler, M., Soler, J. J. & Martinez, J. G. (1997) Great spotted cuckoos improve their  
467 reproductive success by damaging magpie host eggs. *Anim. Behav.* **54**, 1227-1233.
- 468 Sotherland, P. R., Packard, G. C., Taigen, T. L. & Boardman, T. J. (1980) An altitudinal cline  
469 in conductance of cliff swallow (*Petrochelidon pyrrhonota*) eggs to water vapor. *Auk*  
470 **97**, 177-185.
- 471 Spaw, C. D. & Rohwer, S. (1987) A comparative study of eggshell thickness in cowbirds and  
472 other passerines. *Condor* **89**, 307-318.
- 473 Spottiswoode, C. (2010) The evolution of host-specific variation in cuckoo eggshell strength.  
474 *J. Evol. Biol.* **23**, 1792-1799.
- 475 Spottiswoode, C. N. (2013) A brood parasite selects for its own egg traits. *Biol. Lett.* **9**,  
476 20130573.
- 477 Spottiswoode, C. N. & Colebrook-Robjent, J. F. (2007) Egg puncturing by the brood parasitic  
478 greater honeyguide and potential host counteradaptations. *Behav. Ecol.* **18**, 792-799.
- 479 Stoddard, M. C. & Stevens, M. (2010) Pattern mimicry of host eggs by the common cuckoo,  
480 as seen through a bird's eye. *Proc. Roy. Soc. B* **277**, 1387-1393.
- 481 Stoddard, M. C. & Stevens, M. (2011) Avian vision and the evolution of egg color mimicry  
482 in the common cuckoo. *Evolution* **65**, 2004-2013.
- 483 Terepka, A., Stewart, M. E. & Merkel, N. (1969) Transport functions of the chick chorio-  
484 allantoic membrane: II. Active calcium transport, in vitro. *Exp. Cell Res.* **58**, 107-117.
- 485 Török, J., Moskát, C., Michl, G. & Péczely, P. (2004) Common cuckoos (*Cuculus canorus*)  
486 lay eggs with larger yolk but not more testosterone than their great reed warbler  
487 (*Acrocephalus arundinaceus*) hosts. *Ethol. Ecol. Evol.* **16**, 271-277.
- 488 Wyllie, I. (1981) *The cuckoo*. London: BT Batsford.
- 489 Yoon, J. (2013) Comparative hatching characteristics of nonparasitic and parasitic icterids: is  
490 the hatching of cowbird young constrained by an unusually thick eggshell? *J. Ethol.*  
491 **31**, 35-40.

492

493 Table 1. Linear mixed model and associated post-hoc analysis comparing total eggshell thickness among cuckoo and great reed warbler  
 494 eggshells collected either soon after laying (early-stage) or after eggs have hatched.

<b>Total eggshell thickness</b>					
<b>Predictor</b>	<b>Estimate (s.e.)</b>	<b>95% C.I.</b>	<b>Wald <i>t</i></b>	<b>df</b>	<b><i>P</i></b>
Year/Location	Random				
Intercept	79.02 (1.94)	[75.17, 82.87]	40.78	89	< <b>0.001</b>
Species (host – cuckoo)	-16.57 (1.36)	[-19.28, -16.57]	-12.14	89	< <b>0.001</b>
Stage (early stage – hatched)	5.18 (1.60)	[2.01, 8.35]	3.25	89	<b>0.002</b>
<b>Pair-wise comparison</b>	<b>Estimate (s.e.)</b>	<b>95% C.I.</b>	<b>Wald <i>Z</i></b>		<b><i>P</i></b>
Early stage cuckoo – hatched cuckoo	5.69 (2.27)	[0.13, 11.26]	2.51		<b>0.04</b>
Early stage host – hatched host	4.82 (1.96)	[0.01, 9.63]	2.52		<b>0.05</b>
Hatched host – hatched cuckoo	-16.21 (1.78)	[-20.53, -11.90]	-9.22		< <b>0.001</b>
Early stage host – early stage cuckoo	-17.09 (2.12)	[-22.30, -11.87]	-2.04		< <b>0.001</b>

495 Estimates and standard errors are expressed as differences in  $\mu\text{m}$ . The non-significant interaction between species and stage was excluded from  
 496 the model.

497 Table 2. Linear mixed models comparing thicknesses of mammillary and palisade layers of cuckoo and great reed warbler eggshells collected  
 498 either soon after laying (early-stage) or after eggs have hatched.

Predictor	Mammillary layer					Palisade layer				
	Estimate (s.e.)	95% C.I.	Wald <i>t</i>	df	<i>P</i>	Estimate (s.e.)	95% C.I.	Wald <i>t</i>	df	<i>P</i>
Year/Location	Random					Random				
Intercept	12.67 (1.15)	[10.38, 14.95]	11.02	89	< <b>0.0001</b>	66.29 (1.48)	[63.34, 69.23]	44.70	89	< <b>0.0001</b>
Species (host – cuckoo)	-1.20 (0.65)	[-2.49, 0.10]	-1.84	89	0.07	-15.08 (1.19)	[-17.46, -12.71]	-12.63	89	< <b>0.0001</b>
Stage (early stage – hatched)	3.93 (0.77)	[2.39, 5.46]	5.08	89	< <b>0.0001</b>	0.83 (1.38)	[-1.91, 3.56]	0.60	89	0.55

499 Estimates and standard errors are expressed as differences in  $\mu\text{m}$ . Non-significant interactions between species and stage were excluded from  
 500 each of the models.

501 Table 3. Percentage of eggshell thickness change associated with embryonic development for eggs of several avian species.

Development	Common name	Latin name	Initial eggshell thickness (mm)	% change	Eggshell membranes included in measurements	Source
Precocial	Mallard	<i>Anas platyrhynchos</i>	0.386	-5.6	Unspecified	Bunck <i>et al.</i> (1985)
Precocial	Peking duck	<i>A. p. domesticus</i>	0.445	-7.9	No	Balkan, Karakaş and Biricik (2006)
Precocial	King penguin	<i>Aptenodytes patagonica</i>	0.734	-4.2	No	Handrich (1989)
Precocial	Japanese quail	<i>Coturnix japonica</i>	0.193	-7.3	Unspecified	Kreitzer (1972)
Precocial	Mute swan	<i>Cygnus olor</i>	0.657	-4.4	No	Booth (1989)
Precocial	White leghorn chicken	<i>Gallus gallus domesticus</i>	0.350	-5.1	No	Abarca <i>et al.</i> (2011)
Precocial	Malleefowl	<i>Leipoa ocellata</i>	0.279	-20.8	Unspecified	Booth and Seymour (1987)
Precocial	Common pheasant	<i>Phasianus colchicus</i>	0.320	-25.0	Yes/no <sup>a</sup>	Dahlgren and Linder (1971)
Precocial	Ostrich	<i>Struthio camelus</i>	19.2	-1.0	No	Şahan <i>et al.</i> (2003)
Altricial	Great reed warbler	<i>Acrocephalus arundinaceus</i>	0.069	-3.4	No	This study
<u>Altricial</u>	<u>Common cuckoo</u>	<u><i>Cuculus canorus</i></u>	<u>0.085</u>	<u>-4.6</u>	<u>No</u>	<u>This study</u>
Altricial	Saker falcon	<i>Falco cherrug</i>	0.321	-4.4	No	Castilla <i>et al.</i> (2010)
Altricial	Peregrine falcon	<i>F. peregrinus peregrinus</i>	0.284	-4.8	No	Castilla <i>et al.</i> (2010)
Altricial	Red shaheen falcon	<i>F. p. babylonicus</i>	0.255	-1.6	No	Castilla <i>et al.</i> (2010)
Altricial	American kestrel	<i>F. sparverius</i>	0.193	+3.0	Unspecified	Bunck <i>et al.</i> (1985)
Altricial	Pied flycatcher	<i>Ficedula hypoleuca</i>	0.038	-26.3	No	Kern, Cowie and Yeager (1992)
Altricial	Screech owl	<i>Megascops asio</i>	0.231	+0.8	Unspecified	Bunck <i>et al.</i> (1985)
Altricial	Black-crowned night heron	<i>Nycticorax nycticorax</i>	0.295	+2.9	Unspecified	Bunck <i>et al.</i> (1985)
Altricial	American cliff swallow	<i>Petrochelidon pyrrhonota</i>	0.071	-5.6 <sup>b</sup>	No	Sotherland <i>et al.</i> (1980)
Altricial	White-faced ibis	<i>Plegadis chihi</i>	0.324	-4.3 <sup>c</sup>	Unspecified	Capen (1977)
Altricial	Arctic tern	<i>Sterna paradisaea</i>	0.146	-7.6 <sup>d</sup>	No	Finnlund <i>et al.</i> (1985)
Altricial	Barn owl	<i>Tyto alba</i>	0.310	-2.4	Unspecified	Bunck <i>et al.</i> (1985)

502 <sup>a</sup>Unincubated measurement taken with membrane, hatched measurement taken without membrane.

503 <sup>b</sup>Undeveloped eggs without chorioallantois versus developed eggs with chorioallantois.

504 <sup>c</sup>6 day-old eggs versus 17 day-old eggs (4 days prior to hatching).

505 <sup>d</sup>Early incubation (little to no embryo development) versus late incubation (shortly before hatching).

506 The only brood parasitic species studied to date is underlined.

507 **Figure Captions:**

508

509 Figure 1. (a) Scanning electron microscope images of eggshell cross-sections of early-stage  
510 and hatched great reed warbler and common cuckoo eggshells. Scale bar: 10 $\mu$ m. (b) Mean  
511 eggshell thickness ( $\pm$  standard error) of great reed warbler and common cuckoo eggshells  
512 collected either early-stage (white bars) or after hatching (grey bars). Numbers within bars  
513 represent the number of eggs used in analysis.

514

515 Figure 2. (a) Scanning electron microscope images of the inner-most mammillary eggshell  
516 layers of early-stage and hatched great reed warbler and eggshells common cuckoo. Dashed  
517 lines delineate the outer palisade (above) and inner mammillary (below) eggshell layers  
518 identified by the presence of spherical vesicles in the palisade. Scale bar: 10 $\mu$ m. (b) Mean  
519 mammillary layer thickness ( $\pm$  standard error) of great reed warbler and common cuckoo  
520 eggshells collected either early-stage (white bars) or after hatching (grey bars). Numbers  
521 within bars represent the number of eggs used in analysis.

522

523 Fig. 1

524

525

526

527

528

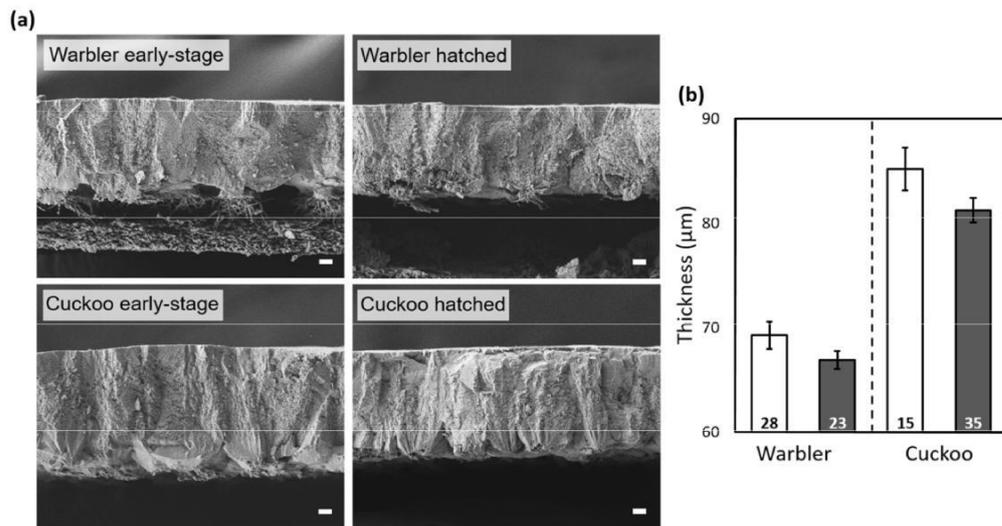
529

530

531

532

533



534 Fig. 2

535

536

537

538

539

540

541

542

543

