

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

B. Igic^{1,2}, M. E. Hauber³, C. Moskát⁴, T. Grim⁵, M. D. Shawkey^{1,6}, P. Procházka⁷ & M. Honza⁷

1 Department of Biology and Integrated Bioscience Program, University of Akron, Akron, OH, USA

2 Research School of Biology, The Australian National University, Canberra, Australian Capital Territory, Australia

3 Department of Psychology, Hunter College and the Graduate Center of the City University of New York, New York, NY, USA

4 MTA-ELTE-MTM Ecology Research Group, a joint research group of the Hungarian Academy of Sciences, the Biological Institute of the Eötvös Loránd University and the Hungarian Natural History Museum, Budapest, Hungary

5 Department of Zoology and Laboratory of Ornithology, Palacký University, Olomouc, Czech Republic

6 Department of Biology, Terrestrial Ecology Unit, Ghent University, Ghent, Belgium

7 Institute of Vertebrate Biology AS CR, Brno, Czech Republic

Keywords

Acrocephalus arundinaceus; brood parasitism; *Cuculus canorus*; decalcification; eggshell thickness; embryonic development; common cuckoo; scanning electron microscopy.

Correspondence

Branislav Igic, Division of Evolution, Ecology and Genetics, Research School of Biology, Australian National University, Canberra 2601, Australia.

Tel: +61-04-16971049

Email: brani.igic@gmail.com

Editor: Andrew Kitchener

Received 9 February 2016; revised 28 June 2016; accepted 9 September 2016

doi:10.1111/jzo.12408

Abstract

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

Introduction

Obligate brood parasitic birds lay their eggs into nests of other bird species and use these hosts to raise parasitic offspring at the expense of the hosts' own fitness (Feeney, Welbergen & Langmore, 2014). This imposes strong selection pressures on hosts to minimize the likelihood of parasitism (Feeney, Welbergen & Langmore, 2012) or to eliminate parasitic eggs and chicks from their nests (Antonov *et al.*, 2006; Sato *et al.*, 2010). For example, hosts of brood parasites often reject foreign eggs from their nests by puncturing their eggshells and removing them from the nest (Antonov *et al.*, 2006; Rasmussen, Sealy & Underwood, 2009). In turn, brood parasites have evolved numerous counter-adaptations to improve the likelihood that hosts accept parasitic eggs. These adaptations include laying eggs that mimic the colour, patterning, and size of host eggs to evade recognition by hosts (Antonov

et al., 2010; Stoddard & Stevens, 2010, 2011; Igic *et al.*, 2012) and stronger eggshells to hinder puncture rejection when detected (Brooker & Brooker, 1991; Antonov *et al.*, 2012).

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

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

Eggshell thickness is the major contributor to eggshell breaking strength across bird species (Brooks & Hale, 1955; Ar, Rahn & Paganelli, 1979). As such, eggshells of many brood parasitic species from phylogenetically distant avian families, including cuckoos (family: Cuculidae), honeyguides (family: Indicatoridae), and cowbirds (family: Icteridae), are typically thicker than eggshells of their respective host species or non-parasitic relatives (Spaw & Rohwer, 1987; Picman, 1989; Brooker & Brooker, 1991; Spottiswoode, 2010; Igic *et al.*, 2011). The selection pressure for thick-shelled eggs imposed on brood parasites may be strong enough to produce intra-specific differences (Spottiswoode, 2010; but see Igic *et al.*, 2011 and Drobniak *et al.*, 2014). In turn, co-evolution with brood parasites may also select for thicker shelled eggs in hosts (Spottiswoode & Colebrook-Robjent, 2007). Greater breaking strength of parasitic eggs may also be achieved by rounder egg shapes, a greater density of inorganic components in the eggshell, the size or orientation of the eggshell's crystalline components, and potentially pigment composition (Picman, 1989; Picman & Pribil, 1997; Gosler, Higham & James Reynolds, 2005; Bán *et al.*, 2011). Independently of overall eggshell thickness, the innermost (mamillary) layers of common cuckoo (*Cuculus canorus*) eggshells are more resistant to compression forces than are the corresponding layers of their hosts' eggshells, potentially contributing to a greater overall breaking strength for common cuckoo eggs (Igic *et al.*, 2011).

A potential consequence of laying thicker-shelled eggs for brood parasites is that their young may require more energy and effort to hatch (Honza *et al.*, 2001; Yoon, 2013). For example, common cuckoo hatchlings require more time and pecks to hatch than the hatchlings of a host that lays eggs of comparable size but with thinner eggshells, the great reed warbler *Acrocephalus arundinaceus* (Honza *et al.*, 2001). In turn, common cuckoo hatchlings have several morphological characteristics that may help them hatch from structurally stronger eggs, including a larger mass, longer forearms and egg teeth, and a higher density of fibres in muscles used for hatching relative to great reed warbler hatchlings (Honza *et al.*, 2001, 2015). Physiological mechanisms may also help common cuckoo hatchlings hatch from thicker-shelled eggs, including heavier egg yolks that contain greater concentrations of antioxidants (Török *et al.*, 2004; Hargitai *et al.*, 2010), but not higher concentrations of maternally derived testosterone and energy reserves (Török *et al.*, 2004; Igic *et al.*, 2015) or greater levels of gaseous exchange (Portugal *et al.*, 2014). Whether any brood parasitic species has eggshell-specific characteristics that help their young hatch from structurally stronger eggs is unknown.

Although common cuckoo eggshells are thicker than those of their hosts soon after being laid, it is unclear whether they remain thicker at the hatching stage. Avian embryos derive the calcium required for growth by decalcifying the innermost (mamillary) layer of their eggshells, reducing eggshell thickness and breaking strength, and in turn aiding hatching (Kreitzer, 1972; Freeman & Vince, 1974; Castilla *et al.*, 2007;

Chien, Hincke & McKee, 2009; Orłowski & Hałupka, 2015). Therefore, it is possible that cuckoo embryos decalcify a greater portion of their eggshells during development relative to their hosts and reduce the effort required to hatch. However, due to the shorter embryonic development of cuckoos relative to hosts (Wyllie, 1981), cuckoo embryos may also decalcify less of their eggshell during development (Karlsson & Lilja, 2008). Studies to date have only compared eggshell structure between brood parasites and their hosts or non-parasitic relatives using unincubated eggs (e.g. Spaw & Rohwer, 1987; Picman, 1989; Spottiswoode, 2010; Igic *et al.*, 2011), and very little is known about the structural changes to eggshells of brood parasites associated with embryogenesis (although see Karlsson & Lilja, 2008).

We compared the embryogenesis-related microstructural changes to eggshells of the common cuckoo (hereafter cuckoo) in relation to eggshells of its great reed warbler host (hereafter warbler). We used warbler eggs for comparison because they are comparable in size (volume) to those of cuckoos but with significantly thinner eggshells, and out of all the cuckoo's regular host species' eggs, they are one of the most similar to cuckoo eggs morphologically (Török *et al.*, 2004; Antonov *et al.*, 2006; Bán *et al.*, 2011; Igic *et al.*, 2011; Hargitai *et al.*, 2012). Moreover, this host has been used as a comparison for the cuckoo in relation to physiological and morphological adaptations associated with embryonic development and hatching (Honza *et al.*, 2001, 2015; Török *et al.*, 2004; Hargitai *et al.*, 2010; Igic *et al.*, 2015). Here, we focussed on comparing the changes in eggshell thickness between cuckoo and warbler eggs at different stages of development.

Materials and methods

Sample collection

We collected cuckoo and great reed warbler eggs from host nests across several years (Supporting Information Table S1) and two adjacent sites in the Czech Republic (Mutěnice 48°54' N 17°02' E; and Lužice 48°51' N 17°05' E) and one site in Hungary (Apaj 47°06' N 19°05' E). Such meta-replication in both space and time increases the reliability and validity of biological sampling (Johnson, 2002; Grim *et al.*, 2011), particularly as cuckoos likely adapt to their hosts at the metapopulation level rather than locally (Avilés *et al.*, 2011). Similar to findings of previous studies (Török *et al.*, 2004; Hargitai *et al.*, 2010), cuckoo and great reed warbler eggs in our study were similar in size (cuckoo vs. warbler egg volume: 3.17 cm³ (± 0.43 SD, $n = 28$) vs. 3.13 cm³ (± 0.20 SD, $n = 17$), Welch's $t_{41} = 0.47$, $P = 0.64$). We either cleaned and stored eggs in a dark dry place immediately after collection (early-stage eggs) or placed them into incubators to complete development and hatch before cleaning and storing. See Supplementary Materials for more details on sample collection and permits.

Examination of eggshell structure

We used a JSM-7401F scanning electron microscope (SEM, JEOL Japan) to examine the differences between early-stage

and hatched eggshells. Unlike eggshell thickness measurements collected using micrometres, measurements from SEM images more accurately capture the variation in eggshell thickness and allow the visualization of microstructural differences (Igic *et al.*, 2010, 2011). We mounted eggshell fragments from the equatorial region onto aluminium stubs to allow visualization of their cross-sections, which we sputter-coated with gold/palladium for 1 min. We viewed samples at a working distance of 7 mm, using an accelerating voltage of 7 kV, and collected images at magnifications of 450 \times and 1600 \times . Avian eggshells are divided into two visually distinct layers, an outer palisade layer and inner mammillary layer, the latter of which is decalcified and absorbed by the embryo during development (Freeman & Vince, 1974; Mikhailov, 1997). We delineated the division of these two layers by the presence of the spherical films (circular hole-like vesicles) that are characteristic of the palisade layer (Mikhailov, 1997). We used ImageJ v1.48 (National Institute of Health, USA; freely downloadable from <http://rsb.info.nih.gov/ij/>) to measure total eggshell thickness and the thickness of the two respective layers at 30–40 randomly selected areas spread evenly across eggshell cross-sections. We then calculated average values per egg for total eggshell thickness and thicknesses of the two respective eggshell layers. In total, we measured 106 eggshells; however, we calculated and used average thickness estimates for warbler eggshells from the same nest, producing a total of 100 independent samples for our analysis (49 cuckoo and 51 warbler eggshells). Both thickness measurements taken on the same image [106 images measured twice: intra-class correlation $R = 0.96$; 95% CI: (0.95, 0.98)] and taken on images of the same eggshell at different locations [12 randomly chosen eggshells imaged and measured twice: intra-class correlation $R = 0.87$; 95% CI: (0.57, 0.96)] were repeatable.

Statistical analysis

We used linear mixed models to compare structural differences between cuckoo and warbler eggshells collected at the two stages of development. We fit each model with either total

eggshell thickness, mammillary layer thickness or palisade layer thickness as a response; species (cuckoo or warbler), stage (early stage or hatched) and the interaction between species and stage as fixed effects; and an independent identifier for each site/year of collection combination as a random effect (eight total combinations; Supporting Information Table S1). We present full models without backward elimination of non-significant predictors (Forstmeier & Schielzeth, 2011). The interaction between species and stage was non-significant in all circumstances and was therefore excluded from models to allow appropriate interpretation of estimates and P -values for fixed effects (Tables 1 and 2; Engqvist, 2005); however, we present these non-significant interaction effects in the text (see Results). Excluding eggshells collected in Hungary from our analyses did not affect statistical outcomes, confirming that potential intersite differences were not responsible for the observed patterns (data not presented). We lacked collection date information for five unincubated warbler eggshells and four unincubated cuckoo eggshells; however, collection date was not a significant predictor and did not change the results when included in models fitted using data for the remaining eggs (Supporting Information Table S2), and therefore was not used in our final models with all eggs included. We used re-sampling analyses to confirm that our unbalanced dataset did not influence our results (Supporting Information Table S3). See Supplementary Materials for more details on statistical procedures.

Results

Eggshell thickness differences between early-stage and hatched eggs were similar for cuckoo and warbler eggs, such that unincubated and hatched cuckoo eggshells were thicker than unincubated and hatched warbler eggs, respectively (Fig. 1). Hatched warbler eggs were on average 4.82 μm (± 1.96 SE) thinner than early-stage warbler eggs ($P = 0.049$; Table 1; Fig. 1), whereas hatched cuckoo eggs were on average 5.69 μm (± 2.27 SE) thinner than early-stage cuckoo eggs ($P = 0.04$; Table 1; Fig. 1). This difference between eggshell

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

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

Estimates and standard errors are expressed as differences in μm . The non-significant interaction between species and stage was excluded from the model. Pair-wise comparison P -values were adjusted for multiple comparisons.

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

Predictor	Mammillary layer					Palisade layer				
	Estimate (SE)	95% CI	Wald <i>t</i>	d.f.	<i>P</i>	Estimate (SE)	95% CI	Wald <i>t</i>	d.f.	<i>P</i>
Year/location	Random					Random				
Intercept	12.67 (1.15)	10.38, 14.95	11.02	89	<0.0001	66.29 (1.48)	63.34, 69.23	44.70	89	<0.0001
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	<0.0001
Stage (early stage – hatched)	3.93 (0.77)	2.39, 5.46	5.08	89	<0.0001	0.83 (1.38)	–1.91, 3.56	0.60	89	0.55

Estimates and standard errors are expressed as differences in μm . Non-significant interactions between species and stage were excluded from each of the models.

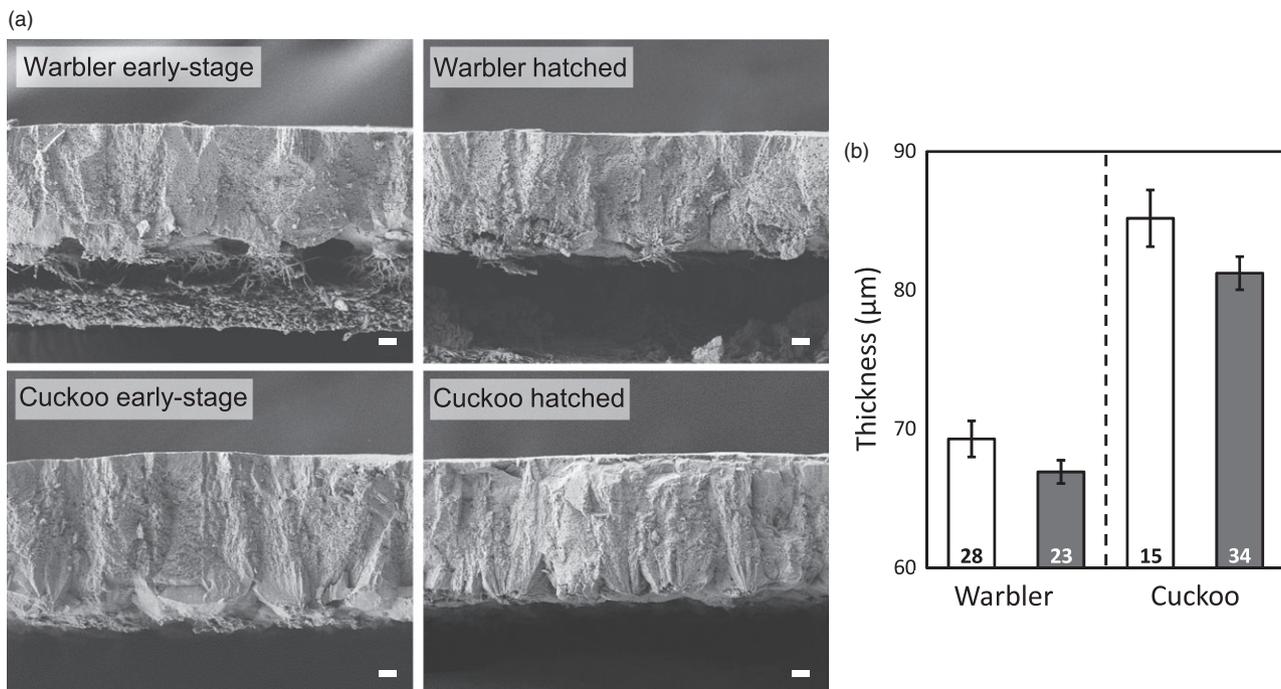


Figure 1 (a) Scanning electron microscope images of eggshell cross-sections of early-stage and hatched great reed warbler and common cuckoo eggshells. Scale bar: $10 \mu\text{m}$. (b) Mean eggshell thickness ($\pm\text{SE}$) of great reed warbler and common cuckoo eggshells collected either early-stage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

thickness of early-stage and hatched cuckoo eggs was not significantly greater than that for warbler eggs [interaction between species and developmental stage: $-0.87 \mu\text{m} \pm 2.72 \text{ SE}$; 95% CI: $(-6.29, 4.54)$; $t_{88} = -0.32$; $P = 0.75$]. Early-stage cuckoo eggshells were $16.21 \mu\text{m}$ ($\pm 1.78 \text{ SE}$) thicker than early-stage warbler eggshells, whereas hatched cuckoo eggshells were $17.09 \mu\text{m}$ ($\pm 2.12 \text{ SE}$) thicker than hatched warbler eggshells (both $P < 0.001$; Table 1). Hatched eggshells of both species were thinner than their early-stage counterparts because of thinner mammillary layers ($P < 0.0001$; Table 2; Fig. 2) and not because of differences in the thicknesses of their palisade layers ($P = 0.55$; Table 2; Fig. 2). This difference between mammillary layer thickness of early-stage and hatched

eggs did not differ for cuckoo eggshells relative to warbler eggshells [interaction between species and developmental stage: $-1.61 \mu\text{m} \pm 1.29 \text{ SE}$; 95% CI: $(-4.17, 0.96)$; $t_{88} = -1.25$; $P = 0.22$].

Discussion

We found that cuckoo eggshells were thicker than eggshells of their great reed warbler hosts at both stages of development. As eggshell thickness is the strongest contributor to eggshell breaking strength (Brooks & Hale, 1955; Ar *et al.*, 1979), our findings imply that cuckoo eggshells maintain a greater breaking strength than warbler eggshells throughout embryonic

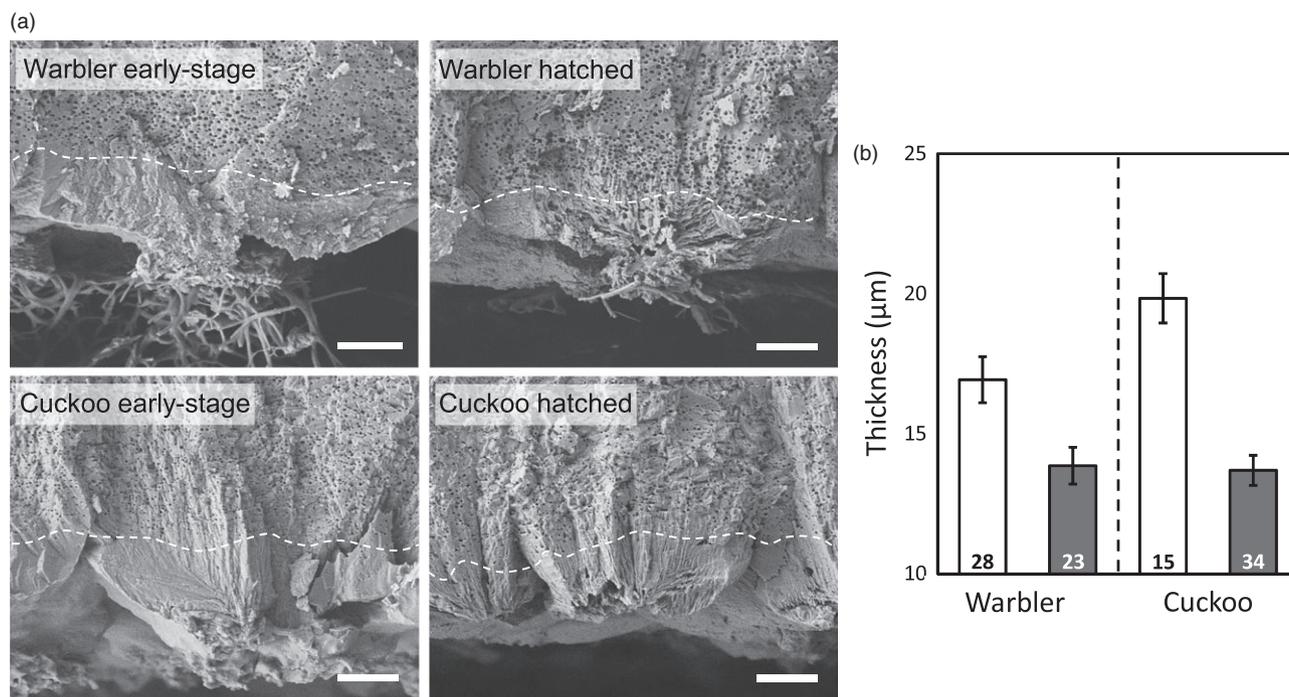


Figure 2 (a) Scanning electron microscope images of the innermost mammillary eggshell layers of early-stage and hatched great reed warbler and common cuckoo eggshells. Dashed lines delineate the outer palisade (above) and inner mammillary (below) layers identified by the presence of spherical vesicles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

development and support the hypothesis that cuckoos require a greater effort to hatch than warblers (Honza *et al.*, 2001).

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

cuckoo eggshells underwent greater degrees of decalcification relative to warbler eggshells.

The potential physiological consequences of greater eggshell decalcification during embryogenesis or a greater risk of eggshell breakage may outweigh the potential benefits of greater embryonic eggshell decalcification for cuckoos. Decalcification and calcium absorption by avian embryos is an active metabolic process, requiring both production and secretion of acidic substances to dissolve the eggshell and energy expenditure for cellular transportation of calcium (Terepka, Stewart & Merkel, 1969; Garrison & Terepka, 1972). Although greater decalcification enables growth of skeletally larger or more ossified embryos (Honza *et al.*, 2001; Blom & Lilja, 2004) and reduces eggshell breaking strength to facilitate hatching (Freeman & Vince, 1974; Castilla *et al.*, 2007), cuckoo embryos may lack the energy reserves required to accomplish greater levels of decalcification (Igic *et al.*, 2015). Calcium ions are important for a number of physiological functions during embryogenesis, including cell-cell signalling, cell division and organ development (Romanoff, 1967; Berridge, 1995). The perturbation of calcium homeostasis or hypocalcaemia can cause embryonic mortality (Packard & Packard, 1993), which in turn may limit the degree of eggshell decalcification that cuckoo embryos can safely achieve. Greater eggshell decalcification at later stages of development may also allow hosts to postpone eggshell puncture rejection behaviour to a period where parasitic eggshells are sufficiently thin to be

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

Development	Common name	Scientific 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. domestica</i>	0.445	-7.9	No	Balkan, Karakaş & 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 & Seymour (1987)
Precocial	Common pheasant	<i>Phasianus colchicus</i>	0.320	-25.0	Yes/no ^a	Dahlgren & 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 & 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 ^b	No	Sotherland <i>et al.</i> (1980)
Altricial	White-faced ibis	<i>Plegadis chihi</i>	0.324	-4.3 ^c	Unspecified	Capen (1977)
Altricial	Arctic tern	<i>Sterna paradisaea</i>	0.146	-7.6 ^d	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)

The only brood parasitic species studied to date is underlined.

^aUnincubated measurement taken with membrane, hatched measurement taken without membrane.

^bUndeveloped eggs without chorioallantois versus developed eggs with chorioallantois.

^cSix-day-old eggs versus 17-day-old eggs (4 days prior to hatching).

^dEarly incubation (little to no embryo development) versus late incubation (shortly before hatching).

punctured successfully (Antonov *et al.*, 2008; Hanley *et al.* 2016). Therefore, selection may have favoured the evolution of developmental adaptations, such as a greater hatchling size, to facilitate hatching from a thicker shelled egg (Honza *et al.*, 2001, 2015), rather than greater embryonic eggshell decalcification.

Despite similar changes in eggshell thickness, it is still possible that cuckoo eggshells underwent a greater reduction in overall breaking strength compared with warbler eggshells following embryogenesis. The mammillary layer of warbler eggshells is structurally weaker than their palisade layer, whereas the mammillary and palisade layers of cuckoo eggshells can withstand similar levels of compression force (Igic *et al.*, 2011). Therefore, cuckoo eggshells could theoretically experience a greater reduction in overall hardness compared to warblers even if both experience the same degree of decalcification due to the reduction of a structurally stronger layer. This requires further investigation through comparisons of breaking strength between early-stage and hatched cuckoo and warbler eggshells. Given the 16 µm difference between hatched cuckoo and warbler eggshells, cuckoo eggs likely retain a structurally stronger eggshell compared to warblers throughout development. To elucidate whether any potential

differences are due to brood parasite specific adaptations, future work should also include comparisons with non-parasitic cuckoos (e.g. Krüger & Davies, 2002). Indeed, other than differences in egg size and eggshell thickness, little is known regarding eggshell-specific differences between eggs of parasitic and non-parasitic cuckoos (Payne, 1974; Krüger & Davies, 2004; although see Mikhailov, 1997; Picman & Pribil, 1997), and particularly so in relation to changes associated with embryonic development. A particularly fruitful area for future work is testing whether parasitic species' eggshells contain specific structural characteristics that facilitate breakage initiated from inside the egg while preventing breakage caused by external forces (Entwistle, Silyn-Roberts & Abuodha, 1995; Nedomová, Buchar & Krivánek, 2014).

Acknowledgements

We thank the Shawkey lab for comments on the paper; Miroslav Čapek, Alena Dvorská, Kateřina Feikusová, Nikoleta Geltsch, Václav Jelínek, Jaroslav Koleček, Beata Matysioková, Mílca Požgayová, Peter Samaš, Kateřina Sosnovcová, Michal Šulc, Anikó Zölei, and István Zsoldos for their field assistance and help with nest searching. For funding, we thank the

Human Frontier Science Program (RGY 69/07 to TG and MEH, and RGY83/2012 to MEH, TG and MDS) and the Grant Agency of the Czech Republic (grant no. P506/12/2404 to MH and TG). The study was also supported by the Hungarian National Research Fund (OTKA, grant no. 83217 to CM) and the National Research, Development and Innovation Office (NKFIH, NN118194 to CM).

References

- Abarca, B., Luis, D., Quintana, L., Jose, A., Casaubon, H. & Rosales, S.G. (2011). Assessment of eggshell quality before and after incubation from 29 and 46 Weeks-old Leghorn Hens. *Int. J. Poult. Sci.* **10**, 977–982.
- Antonov, A., Stokke, B.G., Moksnes, A., Kleven, O., Honza, M. & Røskft, E. (2006). Eggshell strength of an obligate brood parasite: a test of the puncture resistance hypothesis. *Behav. Ecol. Sociobiol.* **60**, 11–18.
- Antonov, A., Stokke, B.G., Moksnes, A. & Røskft, E. (2008). Getting rid of the cuckoo *Cuculus canorus* egg: why do hosts delay rejection? *Behav. Ecol.* **19**, 100–107.
- Antonov, A., Stokke, B.G., Moksnes, A. & Røskft, E. (2009). Evidence for egg discrimination preceding failed rejection attempts in a small cuckoo host. *Biol. Lett.* **5**, 169–171.
- Antonov, A., Stokke, B., Vikan, J., Fossøy, F., Ranke, P., Røskft, E., Moksnes, A., Møller, A. & Shykoff, J. (2010). Egg phenotype differentiation in sympatric cuckoo *Cuculus canorus* gentes. *J. Evol. Biol.* **23**, 1170–1182.
- Antonov, A., Stokke, B.G., Fossøy, F., Liang, W., Moksnes, A., Røskft, E., Yang, C. & Møller, A.P. (2012). Why do brood parasitic birds lay strong-shelled eggs?. *Chin. Birds* **3**, 245–258.
- Ar, A., Rahn, H. & Paganelli, C.V. (1979). The avian egg: mass and strength. *Condor* **81**, 331–337.
- Avilés, J., Vikan, J., Fossøy, F., Antonov, A., Moksnes, A., Røskft, E., Shykoff, J., Møller, A., Jensen, H., Procházka, P. & Stokke, B.G. (2011). The common cuckoo *Cuculus canorus* is not locally adapted to its reed warbler *Acrocephalus scirpaceus* host. *J. Evol. Biol.* **24**, 314–325.
- Balkan, M., Karakaş, R. & Biricik, M. (2006). Changes in eggshell thickness, shell conductance and pore density during incubation in the Peking Duck (*Anas platyrhynchos* f. *dom.*). *Ornis Fennica* **83**, 117–123.
- Bán, M., Barta, Z., Munoz, A., Takasu, F., Nakamura, H. & Moskát, C. (2011). The analysis of Common Cuckoo's egg shape in relation to its hosts' in two geographically distant areas. *J. Zool.* **284**, 77–83.
- Berridge, M.J. (1995). Calcium signalling and cell proliferation. *BioEssays* **17**, 491–500.
- Birkhead, T., Hemmings, N., Spottiswoode, C., Mikulica, O., Moskát, C., Bán, M. & Schulze-Hagen, K. (2011). Internal incubation and early hatching in brood parasitic birds. *Proc. Biol. Sci.* **278**, 1019–1024.
- Blom, J. & Lilja, C. (2004). A comparative study of growth, skeletal development and eggshell composition in some species of birds. *J. Zool.* **262**, 361–369.
- Booth, D.T. (1989). Regional changes in shell thickness, shell conductance, and pore structure during Incubation in eggs of the mute swan. *Physiol. Zool.* **62**, 607–620.
- Booth, D.T. & Seymour, R.S. (1987). Effect of eggshell thinning on water vapor conductance of malleefowl eggs. *Condor* **89**, 453–459.
- Brooker, M.G. & Brooker, L. (1991). Eggshell strength in cuckoos and cowbirds. *Ibis* **133**, 406–413.
- Brooks, J. & Hale, H.P. (1955). Strength of the shell of the hen's egg. *Nature* **175**, 848–849.
- Bunck, C.M., Spann, J.W., Pattee, O.H. & Fleming, W.J. (1985). Changes in eggshell thickness during incubation: implications for evaluating the impact of organochlorine contaminants on productivity. *Bull. Environ. Contam. Toxicol.* **35**, 173–182.
- Capen, D.E. (1977). Eggshell thickness variability in the white-faced ibis. *Wilson Bull.* **89**, 99–106.
- Castilla, A.M., Herrel, A., Díaz, G. & Francesch, A. (2007). Developmental stage affects eggshell-breaking strength in two ground-nesting birds: the partridge (*Alectoris rufa*) and the quail (*Coturnix japonica*). *J. Exp. Zool. A Ecol. Genet. Physiol.* **307**, 471–477.
- Castilla, A.M., Herrel, A., Robles, H., Malone, J. & Negro, J.J. (2010). The effect of developmental stage on eggshell thickness variation in endangered falcons. *Zoology* **113**, 184–188.
- Chien, Y.C., Hincke, M.T. & McKee, M.D. (2009). Ultrastructure of avian eggshell during resorption following egg fertilization. *J. Struct. Biol.* **168**, 527–538.
- Dahlgren, R.B. & Linder, R.L. (1971). Effects of polychlorinated biphenyls on pheasant reproduction, behavior, and survival. *J. Wildl. Manag.* **35**, 315–319.
- Drobniak, S.M., Dyrz, A., Sudyka, J. & Cichoń, M. (2014). Continuous variation rather than specialization in the egg phenotypes of cuckoos (*Cuculus canorus*) parasitizing two sympatric reed warbler species. *PLoS One* **9**, e106650.
- Engqvist, L. (2005). The mistreatment of covariate interaction terms in linear model analyses of behavioural and evolutionary ecology studies. *Anim. Behav.* **70**, 967–971.
- Entwistle, K., Silyn-Roberts, H. & Abuodha, S.O. (1995). The relative fracture strengths of the inner and outer surfaces of the eggshell of the domestic fowl. *Proc. Biol. Sci.* **262**, 169–174.
- Feeney, W.E., Welbergen, J.A. & Langmore, N.E. (2012). The frontline of avian brood parasite–host coevolution. *Anim. Behav.* **84**, 3–12.
- Feeney, W.E., Welbergen, J.A. & Langmore, N.E. (2014). Advances in the study of coevolution between avian brood parasites and their hosts. *Annu. Rev. Ecol. Evol. Syst.* **45**, 227–246.
- Finnlund, M., Hissa, R., Koivusaari, J., Merilä, E. & Nuuja, I. (1985). Eggshells of arctic terns from Finland: effects of incubation and geography. *Condor* **87**, 79–86.
- Forstmeier, W. & Schielzeth, H. (2011). Cryptic multiple hypotheses testing in linear models: overestimated effect sizes and the winner's curse. *Behav. Ecol. Sociobiol.* **65**, 47–55.

- Freeman, B.M. & Vince, M.A. (1974). *Development of the avian embryo. A behavioural and physiological study*. London: Chapman and Hall.
- Garrison, J.C. & Terepka, A.R. (1972). Calcium-stimulated respiration and active calcium transport in the isolated chick chorioallantoic membrane. *J. Membr. Biol.* **7**, 128–145.
- Gaston, A.J. (1976). Brood Parasitism by the pied crested cuckoo *Clamator jacobinus*. *J. Anim. Ecol.* **45**, 331–348.
- Geltsch, N., Bán, M., Hauber, M.E. & Moskát, C. (2016). When should common cuckoos *Cuculus canorus* lay their eggs in host nests? *Bird Study* **63**, 45–51.
- Gloag, R., Keller, L.-A. & Langmore, N.E. (2014). Cryptic cuckoo eggs hide from competing cuckoos. *Proc. Biol. Sci.* **281**, 20141014.
- Gosler, A.G., Higham, J.P. & James Reynolds, S. (2005). Why are birds' eggs speckled? *Ecol. Lett.* **8**, 1105–1113.
- Grim, T., Samaš, P., Moskát, C., Kleven, O., Honza, M., Moksnes, A., Røskft, E. & Stokke, B.G. (2011). Constraints on host choice: why do parasitic birds rarely exploit some common potential hosts? *J. Anim. Ecol.* **80**, 508–518.
- Handrich, Y. (1989). Incubation water loss in king penguin egg. I. Change in egg and brood pouch parameters. *Physiol. Zool.* **62**, 96–118.
- Hargitai, R., Moskát, C., Bán, M., Gil, D., López-Rull, I. & Solymos, E. (2010). Eggshell characteristics and yolk composition in the common cuckoo *Cuculus canorus*: are they adapted to brood parasitism? *J. Avian Biol.* **41**, 177–185.
- Hargitai, R., Costantini, D., Moskát, C., Bán, M., Muriel, J. & Hauber, M.E. (2012). Variation in plasma oxidative status and testosterone level in relation to egg-ejection effort and age of brood-parasitic common cuckoo nestlings. *Condor* **114**, 782–791.
- Hanley, D., Šulc, M., Brennan, P.L.R., Hauber, M. E., Grim, T. & Honza, M. (2016). Dynamic egg color mimicry. *Ecol. Evol.* **2016**; **62**: 4192–4202.
- Honza, M., Picman, J., Grim, T., Novák, V., Čapek, M. Jr & Mrlík, V. (2001). How to hatch from an egg of great structural strength. A study of the common cuckoo. *J. Avian Biol.* **32**, 249–255.
- Honza, M., Feikusová, K., Procházka, P. & Picman, J. (2015). How to hatch from the common cuckoo (*Cuculus canorus*) egg: implications of strong eggshells for the hatching muscle (*musculus complexus*). *J. Ornithol.* **156**, 679–685.
- Igic, B., Hauber, M.E., Galbraith, J.A., Grim, T., Dearborn, D.C., Brennan, P.L., Moskát, C., Choudhary, P.K. & Cassey, P. (2010). Comparison of micrometer-and scanning electron microscope-based measurements of avian eggshell thickness. *J. Field Ornithol.* **81**, 402–410.
- Igic, B., Braganza, K., Hyland, M.M., Silyn-Roberts, H., Cassey, P., Grim, T., Rutila, J., Moskát, C. & Hauber, M.E. (2011). Alternative mechanisms of increased eggshell hardness of avian brood parasites relative to host species. *J. R. Soc. Interface* **8**, 1654–1664.
- Igic, B., Cassey, P., Grim, T., Greenwood, D.R., Moskát, C., Rutila, J. & Hauber, M.E. (2012). A shared chemical basis of avian host–parasite egg colour mimicry. *Proc. Biol. Sci.* **279**, 1068–1076.
- Igic, B., Zarate, E., Sewell, M.A., Moskát, C., Cassey, P., Rutila, J., Grim, T., Shawkey, M.D. & Hauber, M.E. (2015). A comparison of egg yolk lipid constituents between parasitic common Cuckoos and their hosts. *Auk* **132**, 817–825.
- Johnson, D.H. (2002). The importance of replication in wildlife research. *J. Wildl. Manag.* **66**, 919–932.
- Karlsson, O. & Lilja, C. (2008). Eggshell structure, mode of development and growth rate in birds. *Zoology* **111**, 494–502.
- Kern, M.D., Cowie, R.J. & Yeager, M. (1992). Water loss, conductance, and structure of eggs of pied flycatchers during egg laying and incubation. *Physiol. Zool.* **65**, 1162–1187.
- Kreitzer, J.F. (1972). The effect of embryonic development on the thickness of the egg shells of coturnix quail. *Poult. Sci.* **51**, 1764–1765.
- Krüger, O. & Davies, N. (2002). The evolution of cuckoo parasitism: a comparative analysis. *Proc. Biol. Sci.* **269**, 375–381.
- Krüger, O. & Davies, N.B. (2004). The evolution of egg size in the brood parasitic cuckoos. *Behav. Ecol.* **15**, 210–218.
- Mermoz, M.E. & Ornelas, J.F. (2004). Phylogenetic analysis of life-history adaptations in parasitic cowbirds. *Behav. Ecol.* **15**, 109–119.
- Mikhailov, K. E. (1997) *Avian eggshells: an atlas of scanning electron micrographs*. Newbury: British Ornithologists' Club Occasional Publications, No. 3. The Nature Conservancy Bureau Limited.
- Nedomová, Š., Buchar, J. & Křivánek, I. (2014). The effect of the egg's shape on the stress distribution in the eggshell at internal pressure loading. *Acta Univ. Agric. Silv. Mendel. Brun.* **55**, 129–142.
- Orłowski, G. & Hałupka, L. (2015). Embryonic eggshell thickness erosion: a literature survey re-assessing embryo-induced eggshell thinning in birds. *Environ. Pollut.* **205**, 218–224.
- Packard, M.J. & Packard, G.C. (1993). Water loss from eggs of domestic fowl and calcium status of hatchlings. *J. Comp. Physiol. B.* **163**, 327–331.
- Payne, R.B. (1974). The evolution of clutch size and reproductive rates in parasitic cuckoos. *Evolution* **28**, 169–181.
- Picman, J. (1989). Mechanism of increased puncture resistance of eggs of brown-headed cowbirds. *Auk* **106**, 577–583.
- Picman, J. & Pribil, S. (1997). Is greater eggshell density an alternative mechanism by which parasitic cuckoos increase the strength of their eggs? *J. Ornithol.* **138**, 531–541.
- Portugal, S.J., Hauber, M.E., Maurer, G., Stokke, B.G., Grim, T. & Cassey, P. (2014). Rapid development of brood-parasitic cuckoo embryos cannot be explained by increased gas exchange through the eggshell. *J. Zool.* **293**, 219–226.
- Rasmussen, J.L., Sealy, S.G. & Underwood, T.J. (2009). Video recording reveals the method of ejection of brown-headed cowbird eggs and no cost in American robins and gray catbirds. *Condor* **111**, 570–574.
- Rohwer, S., Spaw, C.D. & Røskft, E. (1989). Costs to northern orioles of puncture-ejecting parasitic cowbird eggs from their nests. *Auk* **106**, 734–738.

- Romanoff, A.L. (1967). *Biochemistry of the avian embryo*. New York: Wiley.
- Şahan, Ü., Altan, Ö., Ipek, A. & Yilmaz, B. (2003). Effects of some egg characteristics on the mass loss and hatchability of ostrich (*Struthio camelus*) eggs. *Br. Poult. Sci.* **44**, 380–385.
- Sato, N.J., Tokue, K., Noske, R.A., Mikami, O.K. & Ueda, K. (2010). Evicting cuckoo nestlings from the nest: a new anti-parasitism behaviour. *Biol. Lett.* **6**, 67–69.
- Sealy, S.G. & Neudorf, D.L. (1995). Male northern orioles eject cowbird eggs: implications for the evolution of rejection behavior. *Condor* **97**, 369–375.
- Soler, M., Soler, J.J. & Martinez, J.G. (1997). Great spotted cuckoos improve their reproductive success by damaging magpie host eggs. *Anim. Behav.* **54**, 1227–1233.
- Sotherland, P.R., Packard, G.C., Taigen, T.L. & Boardman, T.J. (1980). An altitudinal cline in conductance of cliff swallow (*Petrochelidon pyrrhonota*) eggs to water vapor. *Auk* **97**, 177–185.
- Spaw, C.D. & Rohwer, S. (1987). A comparative study of eggshell thickness in cowbirds and other passerines. *Condor* **89**, 307–318.
- Spottiswoode, C. (2010). The evolution of host-specific variation in cuckoo eggshell strength. *J. Evol. Biol.* **23**, 1792–1799.
- Spottiswoode, C.N. (2013). A brood parasite selects for its own egg traits. *Biol. Lett.* **9**, 20130573.
- Spottiswoode, C.N. & Colebrook-Robjent, J.F. (2007). Egg puncturing by the brood parasitic greater honeyguide and potential host counteradaptations. *Behav. Ecol.* **18**, 792–799.
- Stoddard, M.C. & Stevens, M. (2010). Pattern mimicry of host eggs by the common cuckoo, as seen through a bird's eye. *Proc. Biol. Sci.* **277**, 1387–1393.
- Stoddard, M.C. & Stevens, M. (2011). Avian vision and the evolution of egg color mimicry in the common cuckoo. *Evolution* **65**, 2004–2013.
- Terepka, A., Stewart, M.E. & Merkel, N. (1969). Transport functions of the chick chorio-allantoic membrane: II. Active calcium transport, *in vitro*. *Exp. Cell Res.* **58**, 107–117.
- Török, J., Moskát, C., Michl, G. & Péczely, P. (2004). Common cuckoos (*Cuculus canorus*) lay eggs with larger yolk but not more testosterone than their great reed warbler (*Acrocephalus arundinaceus*) hosts. *Ethol. Ecol. Evol.* **16**, 271–277.
- Wyllie, I. (1981). *The cuckoo*. London: BT Batsford.
- Yoon, J. (2013). Comparative hatching characteristics of nonparasitic and parasitic icterids: is the hatching of cowbird young constrained by an unusually thick eggshell? *J. Ethol.* **31**, 35–40.

Supplementary Materials for:

Igic B., Hauber M. E., Moskát C., Grim T., Shawkey M. D., Procházka P. and Honza M. Brood parasite and host eggshells undergo similar levels of decalcification during embryonic development.

Additional details on sample collection procedures

We found the majority of the nests during the building stage and checked them daily until clutch completion. Egg of common cuckoos and great reed warbler were previously found to be comparable in size (cuckoo vs. warbler egg volume: $3.11 \text{ cm}^3 (\pm 0.26 \text{ SD})$ vs. $3.08 \text{ cm}^3 (\pm 0.25 \text{ SD})$, $P = 0.5$, Török *et al.* 2004; $3.07 \text{ cm}^3 (\pm 0.20 \text{ SD})$ vs. $2.94 \text{ cm}^3 (\pm 0.31 \text{ SD})$, $P = 0.24$, Hargitai *et al.* 2010) but to differ slightly in shape (cuckoo vs. warbler egg length: $22.26 \text{ mm} (\pm 0.09 \text{ SE})$ vs. $23.00 \text{ mm} (\pm 0.1 \text{ SE})$, $P < 0.01$; cuckoo vs. warbler egg breadth: $16.57 \text{ mm} (\pm 0.06 \text{ SE})$ vs. $16.25 \text{ mm} (\pm 0.03 \text{ SE})$, $P < 0.01$; Moskát & Honza 2002) and largely in eggshell thickness (Hargitai *et al.* 2010; Igic *et al.* 2011). To confirm that the cuckoo and great reed eggs used in our study were similar in size, we measured the length and breadth for a subsample of our collected eggs using calipers (to the nearest 0.1mm) and estimated their egg volumes using Hoyt's formula (volume = length x breadth² x 0.51 ; Hoyt 1979). Any mechanical strength differences should be primarily due to differences in eggshell thickness (however see Igic *et al.* 2011). For ethical reasons, we only collected eggs from nests that were abandoned during the egg laying period, usually in response to cuckoo parasitism. All eggs were collected during the egg laying period and before the onset of incubation, and therefore before embryonic development and the onset of eggshell decalcification which occurs at later stages (Bond, Board & Scott 1988; Orłowski *et al.* 2015). Moreover, for ethical reasons (e.g. greater risk of mortality during embryonic development) and methodological difficulty, we did not collect eggshell fragments from the same eggs at the two stages of development. Instead, we used separate samples of eggs for our early-staged and hatched stage groups. To minimize the risk of including multiple eggs from the same female cuckoo, we only included cuckoo eggs that varied in their visual appearance. Great reed warblers were colour-banded in the Czech Republic and we only included eggs from different individuals. In total, we

collected 57 great reed warbler and 49 cuckoo eggs (Table S1). When multiple warbler eggs were collected from a single clutch, we calculated and used average values per clutch for our analyses (see main text). Ten of these cuckoo eggs were collected from reed warbler nests in the Czech Republic as the same cuckoo genus parasitizes both warbler species (Igic *et al.* 2012; Drobniak *et al.* 2014). Eggs were obtained and transported with the permission of governmental and institutional research committees.

Collected eggs were broken, cleaned, and stored in a dark dry place immediately after collection (early-stage eggs) or placed into incubators (Octagon 20, Brinsea, UK; or FAVORITA, Germany) to allow embryos to complete development and hatch (Czech samples only; Honza *et al.* 2001; 2015). These hatched eggshells were then immediately cleaned and stored with the early-stage eggshells for later analysis, whereas the hatchlings were placed back into suitable host nests.

All manipulations adhered to the Animal Care Protocol of the Academy of Sciences of the Czech Republic and were in compliance with the current Czech Law on the Protection of Animals against Mistreatment. The permission to perform these experiments was issued by the Ministry of Environment of the Czech Republic (Permit No. OOP/847/99-V418), by the Regional Authority of the South Moravian Region (JMK 115874/2013), by the Nature Conservation Agency of the Czech Republic (00638/PA/2007) and by the Municipal Office in Hodonín (Permits No. MUHOCJ 67363/2006, MUHOCJ 63962/2007, MUHOCJ 41433/2012 and MUHOCJ 34437/2014). The Department for Nature Conservation of the Government Office for Pest County provided permission for field work in Hungary (Permit No. PE/KTF/17190-3/2015).

Re-sampling analyses

We used re-sampling analyses to test whether our statistical outcomes were influenced by our unequal sample sizes across the two species and development stages. For these procedures, we randomly sampled 15 observations per species \times stage from our full dataset without replacement and constructed linear mixed models as specified above. We repeated this over 1000 iterations and calculated average estimates and standard errors for each predictor using the 1000 model fits. These re-sampling analyses produced qualitatively the same statistical outcomes as our full dataset models, confirming that the unbalanced dataset did not influence our results (Table S3).

General statistical analysis details

All statistical analyses were conducted in R v3.0.1 (R Development Core Team 2013). We constructed linear mixed models using the `lme()` function of the `nlme` package (Pinheiro *et al.* 2012) and used the `glht()` function of the `multcomp` package to conduct post-hoc tests with *P*-value adjustment (Hothorn, Bretz & Westfall 2008). We used q-q and residual plots to confirm normality and equal variance of residuals for all our fitted models. Repeatability was calculated using the `rpt.remLMM()` function of the `rptR` package using 1000 bootstrap replicates and 1000 permutations (Nakagawa & Schielzeth 2010).

Table S1. Collection summary and sample sizes used in analyses of common cuckoo and great reed warbler eggshells.

Species	Stage	Year	Location	Number of eggs	Mean thickness μm (s.e.)
Cuckoo	Early	2006	Czech Republic	5	78.91 (1.32)
	Early	2012	Czech Republic	1	94.15
	Early	2015	Czech Republic	3	89.62 (1.91)
	Early	2015	Hungary	6	86.71 (3.94)
	Hatched	2012	Czech Republic	5	73.77 (3.31)
	Hatched	2013	Czech Republic	7	76.93 (2.77)
	Hatched	2015	Czech Republic	22	84.32 (1.07)
Warbler	Early	2000	Czech Republic	2	67.62 (5.34)
	Early	2001	Czech Republic	3	71.85 (3.60)
	Early	2008	Czech Republic	5	59.27 (2.71)
	Early	2015	Czech Republic	11	71.60 (1.40)
	Early	2015	Hungary	7	72.20 (1.78)
	Hatched	1999	Czech Republic	7	66.81 (1.89)
	Hatched	2015	Czech Republic	22	66.71 (0.95)

Table S2: Linear mixed models comparing total eggshell thickness and thickness of the mammillary layers for cuckoo and great reed warbler eggshells collected either soon after laying (early-stage) or after eggs have hatched, and including their date of collection.

Predictor	Total thickness					Mammillary 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	80.14 (3.79)	[72.60, 87.68]	21.15	80	< 0.0001	10.90	[6.91, 14.90]	5.43	80	< 0.0001
Collection Date	-0.04 (0.07)	[-0.18, 0.10]	-0.52	80	0.60	0.04	[-0.2, 0.11]	1.33	80	0.19
Species (host – cuckoo)	-16.21 (1.79)	[-19.78, -12.65]	-9.06	80	< 0.0001	-0.73	[-2.43, 0.96]	-0.86	80	0.39
Stage (early stage – hatched)	5.23 (2.61)	[0.02, 10.43]	2.00	80	0.05	5.71	[3.16, 8.25]	4.46	80	< 0.0001
Species × Stage	-1.27 (2.78)	[-6.81, 4.28]	-0.45	80	0.65	-1.43	[-4.06, 1.20]	-1.07	80	0.28

Estimates and standard errors are expressed as differences in μm . As our predictions were focussed on testing the interactions between species and stage, we did not exclude these non-significant from the models.

Table S3: Summary of 1000 linear mixed models fitted using re-sampling analyses with 15 observations per treatment group. Models compare total eggshell thickness and thickness of the mammillary layer of cuckoo and great reed warbler eggshells collected either soon after laying (early-stage) or after eggs have hatched.

Predictor	Total thickness					Mammillary 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	78.99 (2.41)	[74.14, 83.85]	32.72	48	< 0.0001	12.49 (1.30)	[9.87, 15.11]	9.59	48	< 0.0001
Species (host – cuckoo)	-15.69 (2.35)	[-20.43, -10.96]	-6.66	48	0.03	-0.36 (1.13)	[-2.63, 1.91]	-0.32	48	0.75
Stage (early stage – hatched)	5.94 (2.65)	[0.61, 11.26]	2.24	48	< 0.0001	5.21 (1.29)	[2.62, 7.79]	2.62	48	0.0002
Species × Stage	-1.43 (3.36)	[-8.18, 5.32]	-0.43	48	0.67	-1.98 (1.61)	[-5.22, 1.26]	-1.26	48	0.22

Estimates and standard errors are expressed as differences in μm . As our predictions were focussed on testing the interactions between species and stage, we did not exclude these non-significant from the models.

Literature Cited

- Bond, G. M., Board, R. G. & Scott, V. D. (1988) A comparative study of changes in the fine structure of avian eggshells during incubation. *Zool. J. Linn. Soc.* **92**, 105-113.
- Drobniak, S. M., Dyrzcz, A., Sudyka, J. & Cichoń, M. (2014) Continuous variation rather than specialization in the egg phenotypes of cuckoos (*Cuculus canorus*) parasitizing two sympatric reed warbler species. *PLoS ONE* **9**, e106650.
- Hargitai, R., Moskát, C., Bán, M., Gil, D., López-Rull, I. & Solymos, E. (2010) Eggshell characteristics and yolk composition in the common cuckoo *Cuculus canorus*: are they adapted to brood parasitism? *J. Avian Biol.* **41**, 177-185.
- Honza, M., Picman, J., Grim, T., Novák, V., Čapek Jr M. & Mrlík, V. (2001) How to hatch from an egg of great structural strength. A study of the common cuckoo. *J. Avian Biol.* **32**, 249-255.
- Hothorn, T., Bretz, F. & Westfall, P. (2008) Simultaneous inference in general parametric models. *Biom. J.* **50**, 346-363.
- Hoyt, D. F. (1979) Practical methods of estimating volume and fresh weight of bird eggs. *Auk*, 73-77.
- Igic, B., Braganza, K., Hyland, M. M., Silyn-Roberts, H., Cassey, P., Grim, T., Rutila, J., Moskát, C. & Hauber, M. E. (2011) Alternative mechanisms of increased eggshell hardness of avian brood parasites relative to host species. *J. R. Soc. Interface* **8**, 1654-1664.
- Igic, B., Cassey, P., Grim, T., Greenwood, D. R., Moskát, C., Rutila, J. & Hauber, M. E. (2012) A shared chemical basis of avian host–parasite egg colour mimicry. *Proc. Roy. Soc. B* **279**, 1068-1076.
- Moskát, C. & Honza, M. (2002) European cuckoo *Cuculus canorus* parasitism and host's rejection behaviour in a heavily parasitized great reed warbler *Acrocephalus arundinaceus* population. *Ibis* **144**, 614-622.
- Nakagawa, S. & Schielzeth, H. (2010) Repeatability for Gaussian and non-Gaussian data: a practical guide for biologists. *Biol. Rev.* **85**, 935-956.
- Orłowski, G., Hałupka, L., Klimczuk, E. & Sztwiertnia, H. (2015) Shell thinning due to embryo development in eggs of a small passerine bird. *J. Ornithol.* **157**, 565-572.
- Pinheiro, J., Bates, D., DebRoy, S. & Sarkar, D., (2012) nlme: linear and nonlinear mixed effects models. R package version 3.1-106. <http://cran.r-project.org/web/packages/nlme/index.html>
- R Development Core Team, (2013) R: a language and environment for statistical computing.v3.0.1. Available online at <http://www.R-project.org/>
- Török, J., Moskát, C., Michl, G. & Péczely, P. (2004) Common cuckoos (*Cuculus canorus*) lay eggs with larger yolk but not more testosterone than their great reed warbler (*Acrocephalus arundinaceus*) hosts. *Ethol. Ecol. Evol.* **16**, 271-277.