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

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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.

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

Introduction

Obligate brood parasitic birds lay their eggs into nest of other bird species and use these hosts to raise parasitic offspring at the expense of the hosts' own fitness (Davies 2000; 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 (Moksnes, Røskaft & Braa 1991; 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 rejection when detected (Brooker & Brooker 1991; Antonov *et al.* 2012).

The eggshells of brood parasitic birds are unusually strong for their egg's 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 erroneously damage their own eggs in the process (Spaw & Rohwer 1987; Rohwer, Spaw & Røskaft 1989; Røskaft, Rohwer & Spaw 1993; 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 weaker (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, eggs 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. For instance, the eggshell thickness of different common cuckoo (*Cuculus canorus*) and diederik cuckoo (*Chrysococcyx caprius*) gentes (host races) are positively correlated with the discrimination abilities or eggshell thickness of their respective host species (Spottiswoode 2010; but see Igic et al, 2011 and Drobniak *et al.* 2014). Coevolution with brood parasites may also select for thicker-shelled eggs in hosts (Spottiswoode & Colebrook-Robjent 2007). In addition to eggshell thickness, other characteristics may also contribute to the greater breaking strength of parasitic eggs, including a rounder egg shape, a greater density of inorganic components in the eggshell, and the size or orientation of the eggshell's crystalline components (Picman 1989; Picman & Pribil 1997; Bán *et al.* 2011). Independently of overall eggshell thickness, the inner-most (mammillary) layers of common cuckoo 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). Similarly, brown-headed cowbird (*Molothrus ater*) hatchlings take longer to hatch and produce more clicks (suggesting higher pulmonary respiration) during hatching relative to hatchlings of its red-winged blackbird host (*Agelaius phoeniceus*; Yoon 2013). 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). By contrast, the egg teeth of brown-headed cowbird hatchlings are smaller than those of red-wing blackbird hatchlings (Yoon 2013). Physiological mechanisms may also help common cuckoo hatchlings hatch from thicker-shelled eggs, including heavier egg yolks that contain greater concentrations of anti-oxidants (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 remains unknown.

Although common cuckoo eggshells are thicker than those of their hosts soon after being laid, it is unclear whether they remain thicker than hosts' eggshells at the hatching stage. Avian embryos derive most of the calcium required for growth by decalcifying the calcium carbonate from the inner-most (mammillary) 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). Therefore, it is possible that cuckoo embryos decalcify a greater portion of their eggshells during development relative to their hosts, which would 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 regarding the structural changes to eggshells of brood parasites associated with embryogenesis (although see Karlsson & Lilja 2008).

We examined and 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 warblers for comparison because they lay eggs of a comparable size to those of cuckoos but with significantly thinner eggshells (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; Török *et al.* 2004; Hargitai *et al.* 2010; Honza *et al.* 2015; 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 (Table S1) and from 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). We then either cleaned, and stored in a dark dry place immediately after collection (early-stage eggs) or placed 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 450x and 1600x. 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: R = 0.96; 95% C.I: [0.95, 0.98]) and taken on images of the same eggshell at different locations (12 randomly chosen eggshells imaged and measured twice: R = 0.87; 95% C.I: [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 (8 total combinations; 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 & 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 trans-site 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 (Table S2), and therefore was not used in our final models. We used re-sampling analyses to confirm that our unbalanced dataset did not influence our results (Supplementary Materials; 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 (Figure 1). Hatched warbler eggs were on average 4.82 µm (± 1.96 s.e.) thinner than early-stage warbler eggs ($P = 0.049$; Table 1; Figure 1), whereas hatched cuckoo eggs were on average 5.69 µm (± 2.27 s.e.) thinner than early-stage cuckoo eggs ($P = 0.04$; Table 1; Figure 1). This difference between eggshell 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 µm ± 2.72 s.e; 95% C.I: [−6.29, 4.54]; $t_{88} = -0.32$; $P = 0.75$). Early-stage cuckoo eggshells were 16.21 µm (± 1.78 s.e.) thicker than early-stage warbler eggshells, whereas hatched cuckoo eggshells were 17.09 µm (± 2.12 s.e.) 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; Figure 2) and not because of differences in the thicknesses of their palisade layers ($P = 0.55$; Table 2; Figure 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 µm ± 1.29 s.e; 95% C.I: [−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 all stages of development. As eggshell thickness is the strongest contributor to eggshell breaking strength (Brooks & Hale 1955; Ar, Rahn & Paganelli 1979), our findings imply that cuckoo eggshells maintain a greater breaking strength than warbler eggshells throughout embryonic 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 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 warbles, 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 (Figures 1 & 2).

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 hypercalcemia 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 punctured (Antonov *et al.* 2008). 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 undergo 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 relatives (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 and 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 & Křivánek 2014).

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

	Total eggshell thickness				
Predictor	**Estimate (s.e.)**	**95% C.I.**	**Wald *t***	**df**	***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 (s.e.)**	**95% C.I.**	**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.

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.

	Mammillary layer					**Palisade layer**				
Predictor	**Estimate (s.e.)**	**95% C.I.**	**Wald *t***	**df**	***P***	**Estimate (s.e.)**	**95% C.I.**	**Wald *t***	**df**	***P***
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.

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

[a] Unincubated measurement taken with membrane, hatched measurement taken without membrane.

[b] Undeveloped eggs without chorioallantois versus developed eggs with chorioallantois.

[c] 6 day-old eggs versus 17 day-old eggs (4 days prior to hatching).

[d] Early incubation (little to no embryo development) versus late incubation (shortly before hatching).

The only brood parasitic species studied to date is underlined.

Figure Captions:

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: 10µm. (b) Mean eggshell thickness (± standard error) 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.

Figure 2. (a) Scanning electron microscope images of the inner-most mammillary eggshell layers of early-stage and hatched great reed warbler and eggshells common cuckoo. Dashed lines delineate the outer palisade (above) and inner mammillary (below) eggshell layers identified by the presence of spherical vesicles in the palisade. Scale bar: 10µm. (b) Mean mammillary layer thickness (± standard error) 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.

Fig. 1

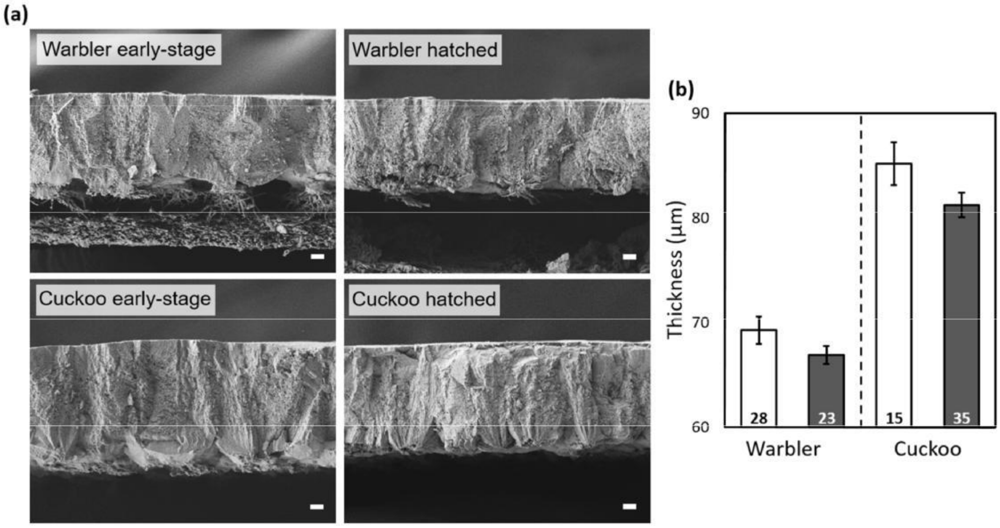
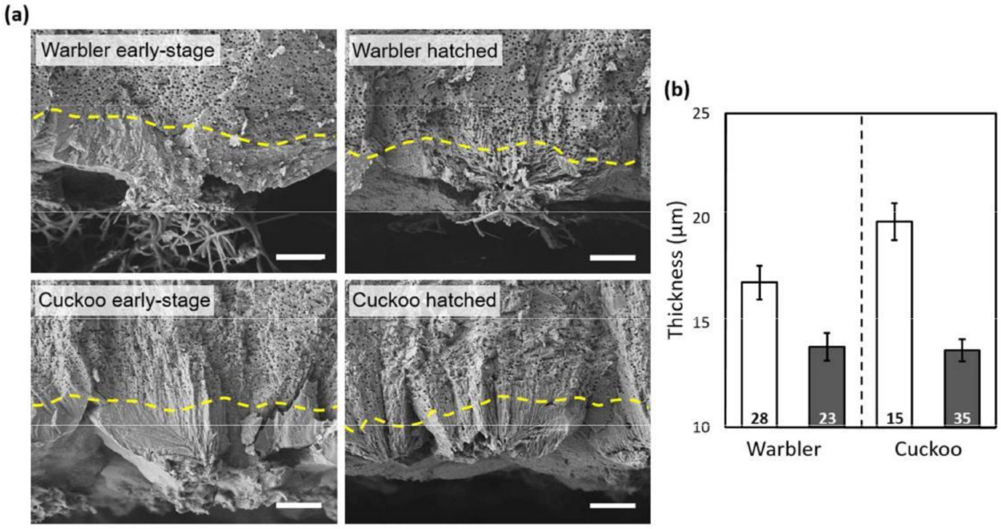

(a)
Warbler early-stage
Warbler hatched
Cuckoo early-stage
Cuckoo hatched
(b)
90
80
70
60
Thickness (µm)
28
23
15
35
Warbler
Cuckoo

Fig. 2


(a)
Warbler early-stage
Warbler hatched
Cuckoo early-stage
Cuckoo hatched
(b)
Thickness (μm)
25
20
15
10
28
23
15
35
Warbler
Cuckoo