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Alternative mechanisms of increased eggshell hardness of avian brood parasites relative to host species

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Obligate brood parasitic birds lay their eggs in nests of other species and parasite eggs typically have evolved greater structural strength relative to host eggs. Increased mechanical strength of the parasite eggshell is an adaptation that can interfere with puncture ejection behaviours of discriminating hosts. We investigated whether hardness of eggshells is related to differences between physical and chemical traits from three different races of the parasitic common cuckoo *Cuculus canorus*, and their respective hosts. Using tools developed for materials science, we discovered a novel correlate of increased strength of parasite eggs: the common cuckoo's egg exhibits a greater microhardness, especially in the inner region of the shell matrix, relative to its host and sympatric non-host species. We then tested predictions of four potential mechanisms of shell strength: (i) increased relative thickness overall, (ii) greater proportion of the structurally harder shell layers, (iii) higher concentration of inorganic components in the shell matrix, and (iv) elevated deposition of a high density compound, $MgCO_3$, in the shell matrix. We confirmed support only for hypothesis (i). Eggshell characteristics did not differ between parasite eggs sampled from different host nests in distant geographical sites, suggesting an evolutionarily shared microstructural mechanism of stronger parasite eggshells across diverse host-races of brood parasitic cuckoos.

Keywords: host–parasite coevolution; microhardness; puncture ejection; recognition systems

1. INTRODUCTION

Obligate parasitic birds, including the common cuckoo (*Cuculus canorus*; hereafter: cuckoo), lay their eggs into nests of other species and subsequently forgo many of the behavioural and physiological costs and evolutionary trade-offs of parental care [1]. In turn, cuckoo parasitism severely reduces the host's fitness [2,3], typically because the parasitic chick evicts host eggs and nest-mates [4,5]. This results in coevolutionary arms-races [6] of morphological, physiological and cognitive adaptations and counter-adaptations between parasites and hosts; hosts recognize and avoid parasitism and parasites reduce or circumvent rejection by hosts [1,7–13].

The rejection of parasite eggs is an effective anti-parasite defence [14,15]. Rejection requires the hosts to be able to identify parasitism, for example, by discriminating between own and foreign eggs [16,17]. The host must then also be able to respond to parasitism either by grasping or puncturing to eject parasite eggs [18,19], or by deserting parasitized nests [20,21]. Cuckoos have evolved several morphological traits to decrease the probability of egg discrimination by

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hosts; female cuckoos seek out and specialize to lay eggs in the nests of one major host species, and parasitic eggs typically resemble in avian-perceivable colour and maculation a particular host's eggs [22–26]. This has resulted in the evolution of specialized host-races (also referred to as *gentes* plural, *gens* singular) in the cuckoo, identifiable by genetic signatures, behavioural traits and differential eggshell mimicry [27–31].

In combination with, or in addition to, visual mimicry [9], the physical structure of common cuckoo eggshells may improve parasitism success through the greater eggshell strength of brood parasite eggs relative to parental, host eggs [32,33]. Comparative patterns suggest, and experimental data confirm, that increased structural strengths of parasite eggs, including those of *Molothrus* cowbirds and *Cuculus* cuckoos, function to reduce rejection rates as a result of hosts' puncture ejection of parasite eggs by increasing the hosts' cost of these rejection attempts through an increased probability of damaging own eggs [8,9,34,35]. Accordingly, cuckoo eggshell thickness across host-races varies positively with the rejection rate of its particular hosts [36]. Increased parasite egg strengths have also been hypothesized to decrease the probability of damage during rapid laying by the female parasite herself [37] or by other, competitor parasites, laying in the same host nest [38] during multiple parasitism [39]; but experimental support is lacking for the latter two functions of greater parasite eggshell strength [18].

Mechanically, eggshell strength can be directly measured as the breaking strength of whole eggs or fragments [40]. Several physical and chemical traits are known to affect, and may be used to predict, the strength of avian eggs, including the shell's thickness [33,38,41], the egg's roundness [41,42], the ratio of organic to inorganic components in the shell [41], pigment distribution [42] and the structural density of the shell matrix [37]. As predicted by evolutionary hypotheses (see above), even though parasitic cuckoo eggs resemble host eggs in size, structure and appearance [43,44], parasitic cuckoo eggs are also generally stronger than parental passerine birds' eggs, both in absolute measures and in relative metrics, compared with predictions based on shell volume [36], although not based on shell mass [45]. For example, the cuckoo lays eggs that are slightly larger or match the hosts' eggs in volume [46,47]; yet, each cuckoo host-race's eggshell is consistently thicker than its host species' eggshell [8,38,48], resulting in the consistently greater whole egg breaking strengths of specialist cuckoo host-races over their respective hosts [36]. In our analyses, therefore, we set out to examine the physical and chemical microstructural basis of greater parasite versus host eggshell strengths.

Specific mechanisms generating increased eggshell strength have been investigated in detail for parasitic cowbirds and cuckoos [37,41], including different cuckoo species and host-races [36]. Here, we studied multiple host-race systems of the common cuckoo and applied advanced materials science technology to provide a comprehensive test of the alternatives proposed by previous works. This study also integrates morphological, chemical and mechanical estimates of eggshell hardness to differentiate between alternative hypotheses of the structural correlates of greater eggshell strength in cuckoos versus hosts. We analysed a novel potential dimension of host–parasite eggshell structure evolution by applying a technique used typically in materials science, the Vickers microhardness test [49], for investigating cuckoo and host eggshell strength at the micro-scale within three different eggshell regions. We then used a variety of analytical techniques to test predictions of multiple hypotheses regarding increased shell strength in the cuckoo compared with its hosts. Accordingly, greater strength may be the result of

(i) an increase in eggshell thickness in cuckoos [36,38],
(ii) greater relative proportion(s) of structurally stronger eggshell layers in cuckoos (this study),
(iii) more inorganic relative to organic components in the shell matrix of cuckoos [41,50], and
(iv) proportionally more Magnesium carbonate ($MgCO_3$), a denser than calcite mineral ($CaCO_3$), in the eggshell matrix of cuckoos [49], or
(v) any combination of these mechanisms.

Previous work only provided strong evidence in favour of hypothesis (i) and did not test these alternatives using the same set of samples and study species. For our samples, we sourced eggshells from three host species parasitized by cuckoos from three different sites across Europe [51]. For one of these sites, we also complemented our samples with eggshells of local, non-host passerine species to provide geographically anchored comparisons between parasites, hosts and non-hosts. Our comparisons were aimed to investigate if variation in the structural basis of eggshell strength consistently differed between various specialist cuckoo host-races and their respective hosts across different geographical localities.

2. MATERIAL AND METHODS

2.1. The avian eggshell

The eggshell is composed of both organic matrix proteins and inorganic crystalline components [52]. The major constituent of the inorganic component of the shell is calcite ($CaCO_3$), but 'impurities', including phosphorus (P) and sodium (Na) in the form of their respective salts are also found in detectable quantities [49,53]. The eggshell is divided into six zones [52] and two easily distinguishable shell layers: an inner mamillary layer and an outer palisade layer. Crystal growth is initiated at organic cores positioned on the innermost side of the mamillary layer [54]. The mamillary layer is also the site of the eggshell's calcite reserve involved in calcium absorption into the developing embryo [55]. Deposition of an organic matrix begins in the palisade layer, where it reinforces the growing calcite, alters crystal orientation and increases eggshell strength [54]. In addition to calcium (Ca), magnesium (Mg) in the form of $MgCO_3$ [56] is commonly found in the mamillary layer of the eggs in a range of avian taxa and may increase the structural strength of this

Table 1. Species and cuckoo host-race IDs and associated sample sizes available to use for the parallel structural analyses. Species codes (cuckoo: each common cuckoo host-race and its local host in brackets; GRWA: great reed warbler, PHPH: common redstart, GRTI: great tit, PIFL: pied flycatcher, and Reed: reed warbler) and species types (cuckoo: parasite, host or sympatric non-host) locality, Latin names, sample sizes (number of eggshells) used for each analysis, and references for extensive details for the study site and species information from the published literature.

species/host-race	type	species	thickness	structure	hardness	elemental composition	location
cuckoo (GRWA)	cuckoo 1	*Cuculus canorus*	12	10	5	5	Hungary [17]
GRWA	host 1	*Acrocephalus arundinaceus*	14	10	5	4	Hungary [17]
cuckoo (PHPH)	cuckoo 2	*Cuculus canorus*	12	9	5	3	Finland [5]
PHPH	host 2	*Phoenicurus phoenicurus*	13	11	5	3	Finland [5]
GRTI	non-host 2	*Parus major*	5	4	0	0	Finland [5]
PIFL	non-host 2	*Ficedula hypoleuca*	5	4	5	3	Finland [5]
cuckoo (reed)	cuckoo 3	*Cuculus canorus*	5	5	5	5	Czech Republic [10]
reed	host 3	*Acrocephalus scirpaceus*	9	5	5	5	Czech Republic [10]

weaker layer, although to date, there is no conclusive evidence in support of this hypothesis [49,57].

2.2. Eggshell samples

We collected samples during the northern summers of 2006–2007, across three sites in Europe where three different host-races of the cuckoo parasitize predominantly three different host species (table 1). We focused on species- and host-race-specific differences in our statistical analyses because our sample sizes did not allow us to apply a full-scale phylogenetic analysis with just three host–parasite systems, and also because recent molecular work suggests independent evolutionary origins of each of these host–parasite systems [27]. To extend the interspecific scope of our analyses, in Finland, we also sourced eggshells from two sympatric nest-box breeding passerine species, the great tit *Parus major* (a non-host of the cuckoo) and the pied flycatcher *Ficedula hypoleuca* (a very rare host of the cuckoo), in addition to the nest-box breeding passerine host, the redstart *Phoenicurus phoenicurus*) [3]. All eggs were collected unincubated (i.e. prior to the onset of incubation of the whole clutch as assessed by daily nest monitoring), cleaned with 70 per cent ethanol and stored in a cool dark place until measurements.

All analyses were conducted on the equatorial section of eggshells because it represents the weakest and most uniform part of the eggshell, thus providing the best estimate of the lower limits of eggshell strength [58]. Also, puncture ejection by hosts is most probably focused on this part of the egg, laying on its side in the nest; thus, the equatorial section is the most exposed part of the egg to hosts' ejection attempts to break through the shell [34].

2.3. Thickness measurements

Three small fragments of the eggshells were removed, and thickness was measured once per fragment using a point micrometer (0–25 mm range, 0.01 mm graduation) following the methods described in Igic *et al.* [51]. Averages of the three micrometer measurements were calculated to produce a single thickness value per eggshell specimen. Eggshell thickness was then compared between eggs from all taxonomic groups (each host/non-host songbird species and each cuckoo host-race), using a general linear mixed model (GLMM) (JMP v. 9, SAS Institute, Cary, NC, USA; table 1), controlling for host nest ID (for the cuckoo and host eggs) and year (known to covary with eggshell morphology: [59]) as random effects; Tukey's honest significant difference (HSD) tests were used to test for post hoc comparisons.

2.4. Structural measurements

We used a Philips XL30S FEG scanning electron microscope (SEM) to visualize and measure cross sections of eggshell samples. Sample preparation procedure is described in detail in Igic *et al.* [51]. To quantify structural differences throughout the eggshell, we characterized three visually distinct layers in the eggshell cross section (figure 1 and electronic supplementary material, figures S1 and S2). Based on visible textural differences across different parts of the eggshell, and guided by the pioneering work of our research team member, Heather Silyn-Roberts, on eggshell structure [54,60], we separated the eggshell into three layers, the mamillary layer and an outer and inner palisade layer. The mamillary layer was characterized by the presence of cone structures, mamillae. The outer and inner palisade layers were differentiated by increased visual porosity and roughness of inner layer texture compared with that of the outer layer. The delineation between the layers was not always a clear line and in the case of the outer and inner palisade layers, it was usually observed as a texture gradient (see electronic supplementary material, figure S2). To check the quantitative consistency and reliability of our eggshell region delineations, we therefore compared measurement data from independent observers (see below).

Digital SEM photographs of eggshell cross sections were taken at a magnification of 1000× once per sample (figure 1). The proportions of total eggshell thickness represented by each of the three eggshell regions for each sample were measured on the photographs using the ruler functionality on IMAGEJ 1.40 g

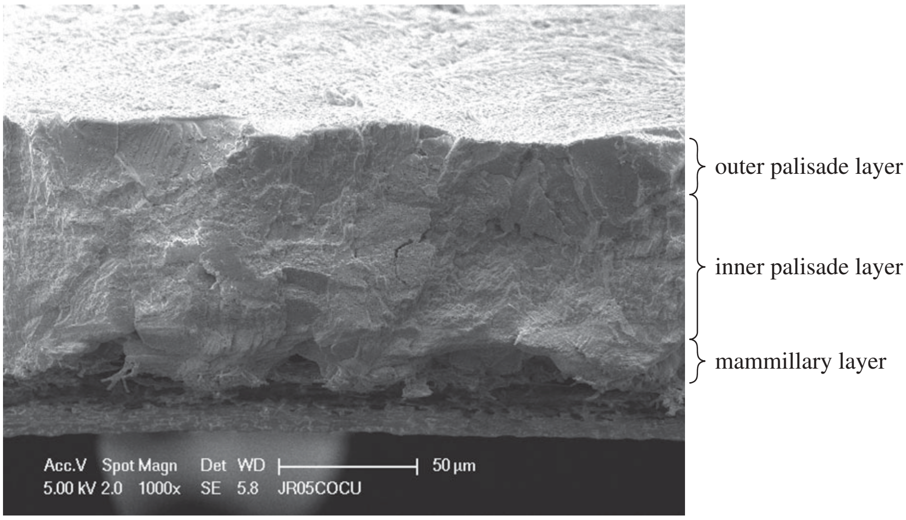

Figure 1. Scanning electron micrograph of the cross section of a common cuckoo eggshell showing the three layers used in structural analyses. Photo by B.I.

(National Institute of Health, USA; downloadable free from http://rsb.info.nih.gov/ij/). The locations of three measurements on the images were chosen randomly along the shell image, choosing locations within the zone of the most uniform thickness. Measurement sets were taken for each photograph by two different observers, and average proportions were calculated for each experimenter's measurements per eggshell. The proportion of total eggshell width (a unitless measure) assigned for each of the three regions was compared using separate GLMMs for each eggshell layer, with host/non-host species/cuckoo host-race and experimenter as fixed effects and eggshell ID as a random effect (Jmp 9).

2.5. Elemental composition

The carbon (C), Ca and Mg composition of the different eggshell layers were quantified using the energy-dispersive X-ray spectroscopy (EDAX) function on the SEM (following the thickness measurements, see above). Only the composition of these three elements were quantified because relative proportions of C and Ca composition can be used to estimate the relative major organic (C-rich and Ca-free protein matrix) and inorganic (predominantly $CaCO_3$) constituents of the eggshell matrix, respectively. In turn, Mg was quantified to estimate relative $MgCO_3$ concentration of the mamillary layer only, which is hypothesized to affect eggshell strength [49]. A single location per eggshell layer per egg sample specimen was analysed using the EDAX function of the SEM. The relative positions of the analysis locations were chosen haphazardly, constrained by the requirement that relatively flat eggshell portions were required to give an accurate EDAX measurement. All analyses were conducted at a magnification of 12 000× using a 10×10 µm area section, a 20 kV beam and a spot size of four and were run for 150 s. Elemental composition was quantified as a percentage per 100 atoms (C, Ca and Mg only) using the native EDAX Genesis software.

GLMMs were constructed with relative elemental composition (either Ca : C or % Mg) as a response and species/host-race as a fixed effect and nest ID and year as random effects across each eggshell layer (outer/inner palisade, mamillary) separately (Jmp 9).

2.6. Microhardness measurements

To measure microhardness across different parts of an eggshell cross section, we sourced new equatorial fragments of the shells from our cuckoo and host egg collection (table 1). Fragments were mounted using epoxy resin to allow measurements on eggshell cross sections. A plastic hook kept the eggshell stable and to prevent it from breaking during sample preparation. Diamond pads with grit sizes varying from 500 to 1200 µm were used to grind down the samples that were then polished using 9 µm struers DP Plan and 3 µm DP Mol cloths on a semiautomatic polishing machine (Struers Abramin). Microhardness was measured using a Vickers hardness tester (LECO M-400 Hardness Tester). Trials were conducted using a 30 g load for 10 s. This load weight and time were chosen to most accurately measure hardness without indents going over the eggshell region boundaries (see below). Because of the instrumentation, we faced a lack of visible details to distinguish exactly the three eggshell layers (described above as outer and inner palisade layers, mamillary layer) based on textural differences under the apparatus used for the Vickers hardness testing and the filer micrometer (in contrast to the SEM where the layers were well distinguishable). Thus, we were constrained to approximate the three eggshell layers described above by dividing the shell transects into three evenly spaced 'regions' (figure 2). As a result of this technical limitation, our microhardness measurements of eggshell regions are only approximations to true microhardness measurements of the three shell layers defined in our structural analysis above. Direct comparisons between the results of these two analyses should be done with caution.

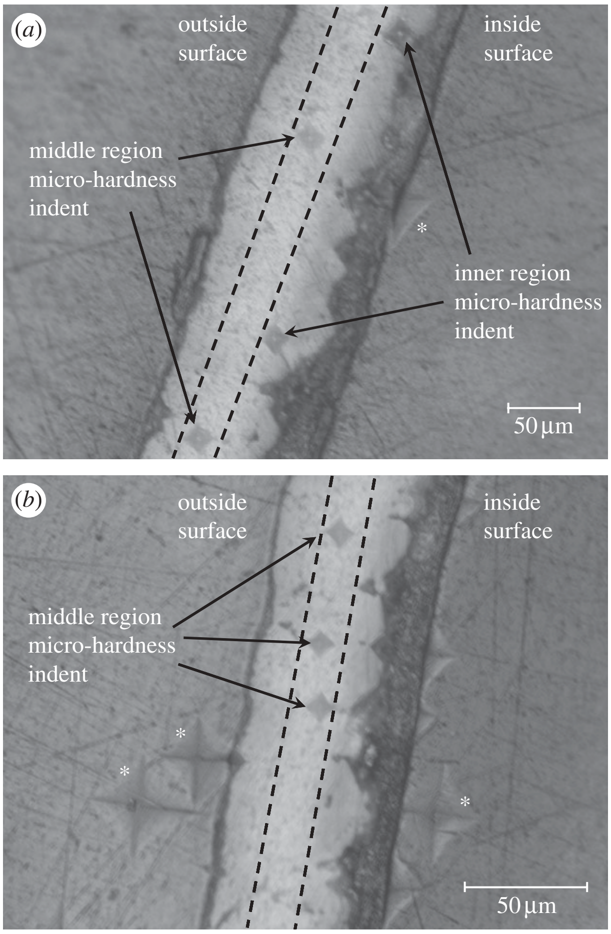

Figure 2. (*a*) Common cuckoo host-race specializing on common redstarts and (*b*) common redstart host eggshell cross sections under a filer micrometer examination following microhardness testing. Dashed lines represent theoretical division of the three eggshell regions. Asterisks denoting the outlier appearing in the figure is a location where the indenter has erroneously missed the eggshell, data from such measurements were not analysed. Photos by K.B.

Hardness was measured across four sites per eggshell region per egg sample specimen. A Vickers hardness number (VHN) was calculated by measuring the two diagonal impressions using a filer micrometer (figure 2). Microhardness indents per region were then made consecutively in a straight line across the length of each eggshell region but only on uniform and undamaged locations to avoid measurement biases. Indents always started with the outer region and ended with the inner region because the inner region was irregular and difficult to measure in a straight line.

First, global patterns of microhardness of eggshells between the different species and host-races, irrespective of shell regions, were compared using GLMMs with (i) species and host-races nested within egg-source geographical locality as fixed effects, and sample ID as a random effect. We then examined potential variation of microhardness across the eggshell's cross section with an analysis of (ii) species/host-race and eggshell region as fixed effects, and sample ID as a random effect (JMP 9). Pearson product–moment correlation coefficients between hardness of the three layers were calculated and tested for significance using R 2.9.0 (statistical package).

3. RESULTS

3.1. Eggshell thickness

Eggshell thickness overall differed significantly between cuckoo host-races relative to host and non-host species ($F_{7,63} = 44.64$, $p < 0.001$; figure 3). The thickest eggs were sourced consistently from the different cuckoo host-races whereas the host and non-host species were found to have the thinnest eggs. As predicted from the published literature [8,36,45,46], cuckoo eggs from each of the three host-races in our collection of eggshell samples were found to be significantly thicker than both their local host and local rare/non-host eggs; in turn, host and non-host species' eggs were not statistically different from each other in thickness (figure 3).

3.2. Structural measurements

We detected an observer effect for proportional measurement of outer ($F_{1,56} = 17.54$, $p < 0.0001$), and inner palisade layers ($F_{1,56} = 7.62$, $p = 0.008$), but not the mamillary layer ($F_{1,56} = 1.71$, $p = 0.197$), measured as relative thickness in relation to the entire eggshell's thickness. This was because one observer consistently underestimated the size of the outer palisade layer and overestimated the size of the inner palisade layer. When accounting for these observer effects statistically, we again detected statistical differences between host/non-host species and cuckoo host-races with respect to the proportions of total thickness of outer palisade layers ($F_{7,49} = 8.18$, $p < 0.0001$) and inner palisade layers ($F_{7,49} = 3.62$, $p = 0.003$), but not mamillary layers ($F_{7,49} = 0.50$, $p = 0.83$). However, the post hoc tests revealed that these effects were only associated with the great tit eggshells having a smaller proportion of outer palisade layer and a greater proportion of inner palisade layer, relative to all other species and host-races showing similar proportion of the three eggshell layers (figure 4).

3.3. Elemental composition

The composition of the outer palisade layer, measured by the ratio of Ca : C in this layer of the eggshell, was similar between host/non-host species and cuckoo host-races (overall mean Ca : C ratio ± s.d.: 0.99 ± 0.46; $F_{1,20} = 1.36$, $p = 0.257$). Similarly, the composition of the inner palisade layer measured by the ratio of Ca : C was statistically similar across all samples (overall mean Ca : C ratio ± s.d.: 1.00 ± 0.55; $F_{1,20} = 2.67$, $p = 0.118$). Finally, the composition of the mamillary layer was not statistically different between the samples when measured by either Mg concentration (overall mean Mg% ± s.d.: $0.68\% \pm 0.40\%$; $F_{1,19} = 1.72$, $p = 0.205$) or by the Ca : C ratio (overall mean Ca : C ratio ± s.d.: 0.89 ± 0.42; $F_{1,19} = 1.30$, $p = 0.269$).

3.4. Microhardness measurements

Overall, cuckoo host-races' eggshells combined were found to have a significantly greater overall microhardness compared with either host or non-host eggshells ($F_{2,29} = 11.20$, $p = 0.0002$; figure 5). Similarly, the overall GLMM analyses revealed statistical differences

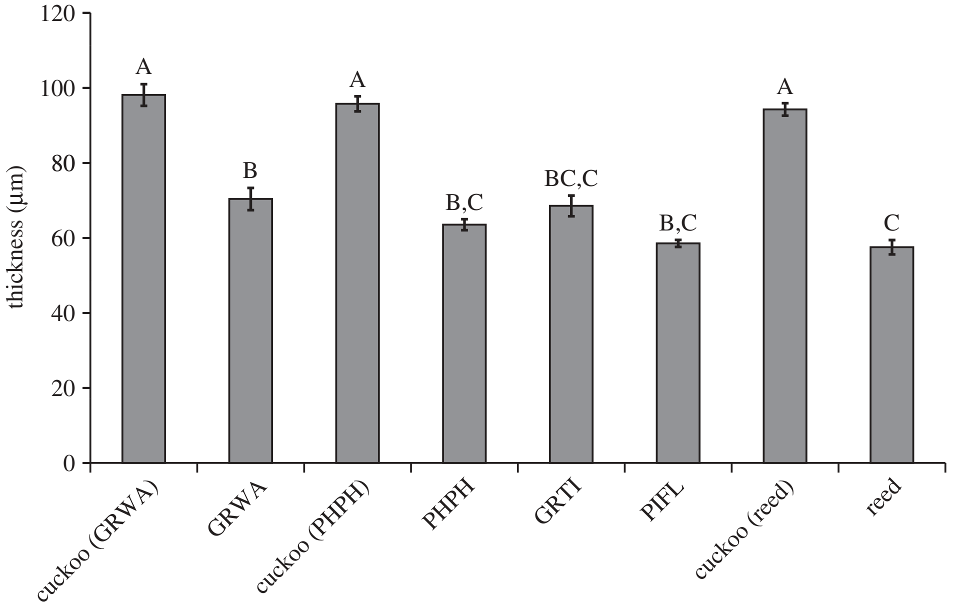

Figure 3. Eggshell thickness (mean ± s.e.) for brood parasitic common cuckoo host-races, host and non-host species. Groups not connected by the same letter were significantly different in a general linear mixed model (GLMM) analysis, using post hoc Tukey's HSD test ($\alpha = 0.05$). See table 1 for species codes.

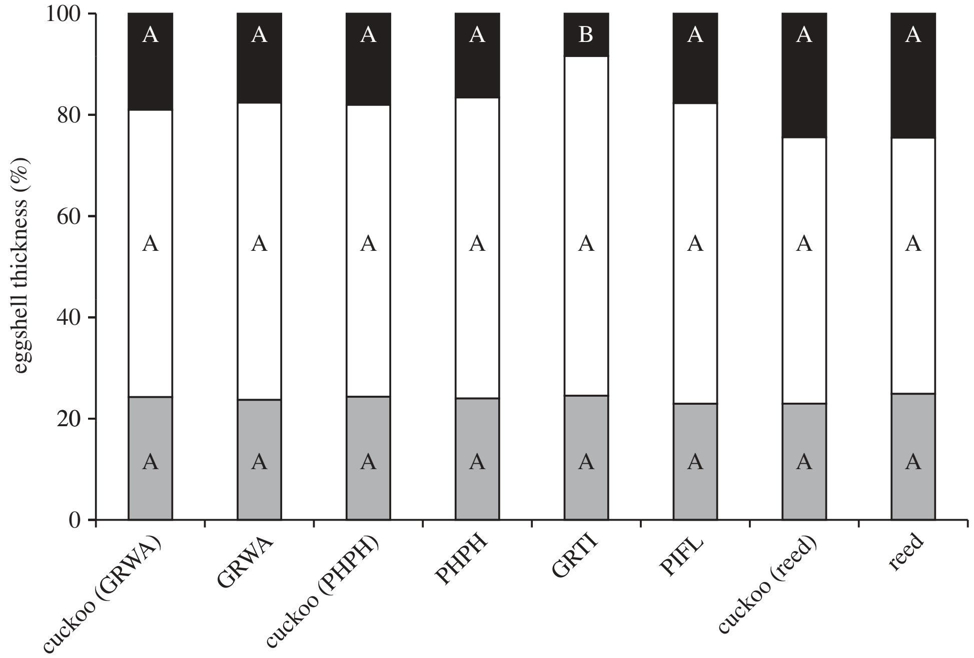

Figure 4. Average relative thickness of the three eggshell layers from total eggshell thickness for all host and non-host species and common cuckoo host-races measured in the structural analysis. For species codes, please refer to table 1. Bar coloration indicates black, outer palisade layer; white, inner palisade layer; and grey, mamillary layer. Groups not connected by the same letter were significantly different in a GLMM analysis, using post hoc Tukey's HSD test ($\alpha = 0.05$).

between the various cuckoo host-races and the host/non-host species ($F_{6,29} = 4.03$, $p = 0.005$; see electronic supplementary material, figure S3).

In a second analysis incorporating eggshell regions, we again confirmed statistically significant differences across the three eggshell regions between the various species and host-races sampled ($F_{2,391} = 91.45$, $p < 0.0001$; figure 6), and also detected consistent positive correlations between the hardness measures of all three layers within samples (table 2). Specifically, the middle region (mean VHN: 121.54 ± 3.93 s.e.) was found to be the hardest, followed by the outer region (mean VHN: 95.07 ± 3.93 s.e.), and the inner region (mean VHN: 83.70 ± 3.93 s.e.) being the weakest (figure 6).

In our post hoc pairwise comparisons of parasite host-races and their sympatric hosts/non-hosts, the pairwise comparisons of increased microhardness in cuckoo host-race compared with host were not found to be statistically significant when combining data across all eggshell regions, except for the statistically

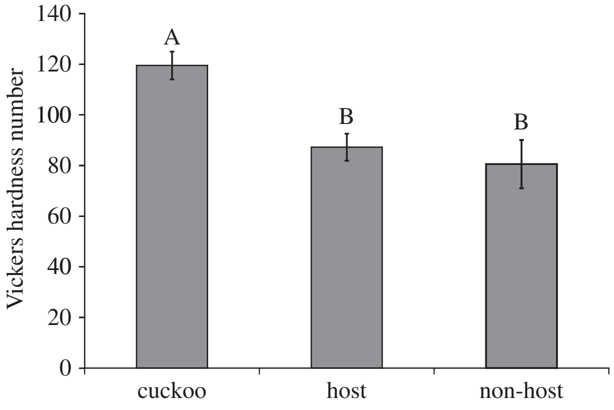

Figure 5. Global eggshell Vickers hardness numbers (VHNs; least square means ± s.e., across all geographical sites, host/non-host species and cuckoo host-race, combining data from all eggshell regions) extracted from a GLMM. Groups not connected by the same letter were significantly different in a GLMM analysis, using post hoc Tukey's HSD test ($\alpha = 0.05$).

Table 2. Correlation matrices of Vickers microhardness numbers for the three eggshell regions (OUT, outer; MID, middle; and INR, inner); significant correlations (*$0.01 > p > 0.01$; **$p < 0.001$) are indicated with an asterisk; (*a*) all samples, (*b*) cuckoo host-races only and (*c*) host/non-host songbird species only.

	(*a*)		(*b*)		(*c*)	
	OUT	MID	OUT	MID	OUT	MID
OUT						
MID	0.62**		0.64*		0.44	
INR	0.60**	0.60**	0.58*	0.59*	0.42	0.42

significant difference between the Finnish cuckoo host-race and the local rare/non-host, the pied flycatcher's eggshells (see electronic supplementary material, figure S3). By contrast, post hoc tests within layers revealed that hardness measures were consistently greater for cuckoo host-races than for host/non-host species for the inner region (table 3 and figure 6). Although total eggshell thickness was also positively correlated with inner region hardness across all samples combined ($r = 0.52$, $t_{26} = 3.13$, $p = 0.004$), this pattern did not hold when restricting analysis within cuckoo host-races only ($r = 0.10$, $t_{11} = 0.32$, $p = 0.75$) or within host/non-host species only ($r = -0.28$, $t_{13} = -1.06$, $p = 0.31$).

4. DISCUSSION

Our results confirm that the main structural correlate of increased eggshell strength of the brood parasitic common cuckoo involves greater absolute eggshell thickness relative to hosts (hypothesis (i)), as had been reported previously [32,33,36]. Increased absolute thickness also typically involves greater relative-to-size thickness of the cuckoo compared with its hosts (e.g. [46], also shown here for Hungarian cuckoo and great reed warbler eggshells that are similar to each other in volume: [61]). In turn, we did not find support for the alternative hypotheses of greater eggshell hardness of cuckoos related to relatively thicker regions of the microstructural stronger components (hypothesis (ii)) or other, chemical mechanisms (hypotheses (iii) and (iv)).

However, based on our application of microhardness measurements from material science to comparisons of avian eggshells across species and eggshell regions, a main contribution of our study is that we detected consistently stronger inner regions, or mamillary layers, of cuckoo eggshells relative to hosts and sympatric non-hosts, which resulted in a global statistical pattern of greater microhardness of cuckoo versus host eggshells. Since we detected no proportional difference of the width of the three visually distinct eggshell layers (outer/inner palisade, mamillary) between host and parasite lineages (our findings possibly imply a greater investment into the hardness of this otherwise weaker eggshell layer (mamillary layer) by cuckoos. Taken together, these lines of evidence provide new evidence in support of the hypothesis that both the macro- (eggshell thickness) and microstructure (microhardness) of brood parasites' eggs have evolved as an adaptation to generate structurally stronger shells, compared with host eggs [8,36,62]. To address this hypothesis explicitly, future work should, therefore, use comparative analyses to focus on the eggshell traits [47], including the shell matrix strength and the chemical composition between parasitic and non-parasitic cuckoos [45]. However, proximately, because of the general size similarity of parasitic cuckoos' and their respective hosts' eggs [47], we predict that the increased thickness in cuckoo eggshells is most probably owing to a shorter phase of crystal nucleation and a longer phase of eggshell deposition during crystallization, as was reported for the formation of the thicker eggshells of the helmeted guineafowl *Numida meleagris* relative to domestic hens *Gallus g. domesticus* [63].

One of the specific structural mechanisms evaluated here was the prediction that cuckoos had greater relative proportions of their structurally stronger eggshell layers, identified here as the outer and inner palisade layers and smaller proportion of the structurally weaker inner mamillary layer (this study) than host/non-host species. Contrary to this prediction, the relative proportions of total shell thickness assigned to the three regions were similar between host species and cuckoo host-races (figure 4). Instead, our microhardness analysis revealed that the inner region of eggshells, and possibly the hardness of the mamillary layer, was greater in the eggs of cuckoo host-races, relative to their respective hosts and non-host songbirds, sampled in this study. Assuming that microhardness of the different eggshell layers contributes positively to the overall breaking strength of the whole eggshell, the predicted outcome of this novel structural mechanism is greater eggshell strength of the cuckoo eggs compared with hosts (figure 5), as had been reported or estimated in empirical studies of whole eggshells [8,34,36,46,64].

From the cuckoo embryo's perspective, the increased hardness of the inner region of cuckoo

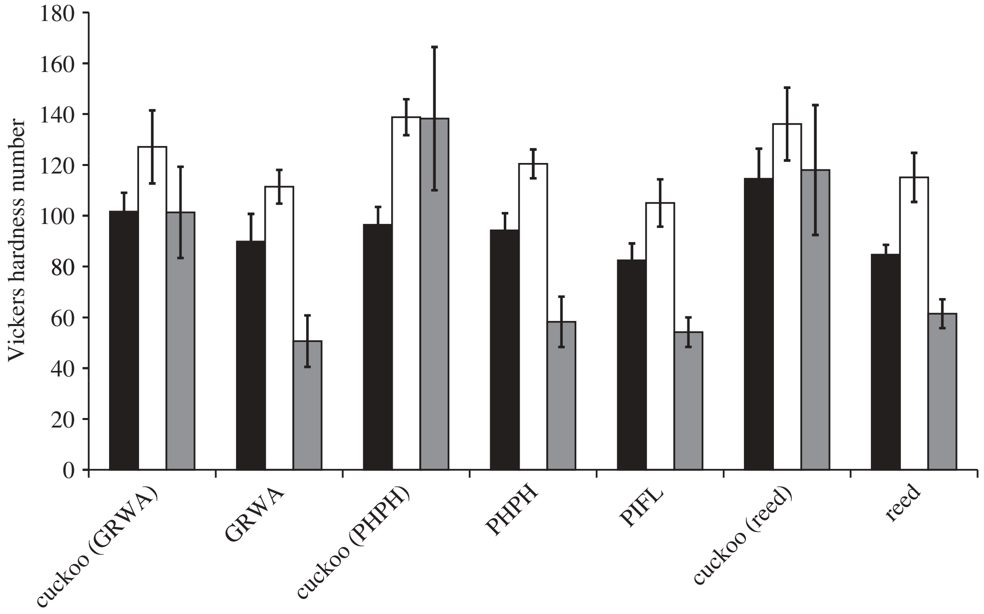

Figure 6. Average relative strength (mean ± s.e.) as measured by VHNs of the three eggshell regions for all host and non-host species and common cuckoo host-races. Bar coloration indicates black, outer region; white, middle region; and grey, inner region. For species codes see table 1. For statistical results see table 3.

Table 3. t-Statistics calculated from Bonferroni-corrected two-sample t-tests for Vickers hardness number (VHN) comparisons for each eggshell region (OUT, outer; MID, middle; and INR, inner); asterisks correspond to the level of significance (*0.1 $> p >$ 0.05; **0.05 $> p$). Italicized comparisons indicate specialist cuckoo race and its respective host/non-host species; for species codes, please refer to table 1. These analyses did not include great tit (GRTI) samples because of logistical constraints of time and funding.

comparison	OUT	MID	INR
cuckoo(GRWA) versus cuckoo(PHPH)	0.51	−0.73	−1.1
cuckoo(GRWA) versus cuckoo(Reed)	−0.92	−0.44	−0.53
cuckoo(GRWA) versus GRWA	0.89	0.99	2.46**
cuckoo(PHPH) versus cuckoo(Reed)	−1.31	0.17	0.53
cuckoo(PHPH) versus PIFL	1.45	2.89	2.92**
cuckoo(PHPH) versus PHPH	0.23	2.02	2.68**
cuckoo(Reed) versus Reed	2.39**	1.22	2.16*
PIFL versus PHPH	−1.24	−1.41	−0.35

eggshells could compromise the hatching ability of the young cuckoo, as this layer is structured in a way so as to facilitate pipping [52]. This microstructural difference between host and parasite eggs may provide an additional explanation for behavioural adaptations reported in cuckoo hatchlings, involving greater overall numbers of pecks on the inside of the eggshell, to facilitate a successful hatching process from the structurally stronger parasite eggshells [48]. Alternatively, cuckoo eggshell regions may undergo thus far untested compositional and, hence, structural changes between laying and hatching. This is suggested by ontogenetic differences in the compositional and structural changes between species that differ in embryonic development rates [65]. For example, greater calcium absorption by a developing cuckoo embryo may facilitate hatching from a thicker and a stronger eggshell, but this again requires further investigation.

Even though the mamillary layer may have an overall greater organic content [66], one of the main differences between the mamillary and palisade layers is the presence of an organic matrix that reinforces the growing calcite in the palisade layer, producing a structurally stronger material [52,54], possibly through mechanisms involved reducing calcite crystal size [67]. Another difference is the presence of a calcium reserve body in the mamillary layer [68] that may be associated with eggshell hardness in the inner region. Therefore, there may be specific processes involving calcite crystal growth in the mamillary layer during eggshell development, awaiting further research. These processes may then parallel those acting on formation of the palisade layer to increase structural strength of mamillary layer of parasitic cuckoo eggs, and perhaps also of other brood parasites eggs, in addition to the general pattern of thicker and stronger eggshells in parasites. Alternatively, the eggshell strength is found to increase as the preferential orientation of calcite crystals decreases as this may reduce simple propagation of cracks through the shell [69], and this may be a mechanism used by the cuckoo to increase microhardness. Here, we found no evidence for the presence of different relative amounts of organic versus inorganic components, and reported a lack of differences in the proportional amounts of Mg, relative to Ca, in the cuckoos' eggshell matrix. Therefore, we again predict that mechanisms involving differences in the calcite crystalline structure underlie the greater structural hardness of mamillary layers in cuckoo eggshells relative to host eggs; however, these alternatives will require further investigation using X-ray diffraction analyses [60].

Although no pairwise statistically significant differences were observed in our samples for hardness of the two outer regions (palisade layer), a pattern of cuckoo eggs being overall structurally harder was observed (figure 5; see electronic supplementary material, figure S3). Even so, we found no statistical pattern that this small observed difference is related to the ratio of organic to inorganic components (i.e. C : Ca) in these two regions. We therefore suggest that if cuckoo eggs across cuckoo-host systems are generally structurally harder in these two regions, increased strength is not related to increases in inorganic content [41], but possibly to changes in specific protein composition throughout the eggshell [70–72]. The organic matrix is responsible for controlling crystal orientation [54] and possibly crystal size [67] in the palisade layer, and it is therefore likely that protein matrices of cuckoo and host eggs differ in relation to their interactions with the growing calcite crystals [71,72] or the composition of specific shell matrix proteins [67]. We suggest further qualitative and quantitative analyses of protein composition between cuckoo and host eggshells as most previous proteomic analyses have involved either fowl or ratite eggshells ([72] but see [73]).

In light of the differences found between the cuckoo and its hosts, but also between the cuckoo and its non-hosts, our results are consistent with the possibility that the eggshell patterns reported here are taxon-specific. Because the extent of the microstructural differences adaptations associated with increased cuckoo eggshell strength did not differ between cuckoo host-races, these eggshell traits may not be the outcome of host-race specific, coevolving traits; a similar pattern of consistent host-parasite differences but not host-race-specific variation in eggshell shapes was also recently reported for the cuckoo [74]. This suggestion is feasible because we studied host–parasite pairs from distant geographical regions, where conditions may also vary in environmental and biological factors that impact egg coloration and eggshell strength [42,53,59]. Alternatively, our sample sizes and the specific choice of species and host-race, may have been insufficient to detect an intraspecific effect within our small sample of cuckoo host-races, contrary to both predictions [31] and recent empirical support for the positive covariation of parasite eggshell thickness, host eggshell thickness and host egg rejection rates across different cuckoo species and host-races [36].

Despite its comparative limitations, our study employed novel integration of multiple techniques to test alternative proximate hypotheses regarding a well-known aspect of the natural history of the common cuckoo and its egg morphology, namely that parasite eggs have structurally stronger eggshells compared with their hosts' eggs to withstand puncture rejection behaviours [32,41]. Our study is also novel in that much of the previous literature has focused on investigating eggshell characteristics of galliform and ratite bird species. We therefore highlight the need for a broader comparative coverage of avian eggshell studies, as many predictions made in this study in regards to mechanistic processes involved in eggshell strength between cuckoo and host are reliant on the assumption of evolutionarily shared characteristics [75].

We suggest a previously undescribed mechanism of increased eggshell strength in the common cuckoo through its structurally stronger inner region, possibly the mamillary layer, relative to host and non-host songbird species. An added value of the novel application of established microhardness measurement technique [49] to biological questions [50,64] is that it explores structural effects independent of eggshell thickness *per se*, and enables the hardness testing of different eggshell regions separately. Our study therefore guides future investigations of the mechanistic basis of coevolution and the resulting morphological, perceptual and behavioural arms-races between brood parasites and hosts [6,76].

B.I. and M.E.H. conceived and designed this study. J.R., M.E.H., P.C., T.G. and C.M. collected samples in the field. B.I. collected thickness measurements, conducted SEM/EDAX and structural analyses, with the guidance of M.M.H. and H.S.-R. K.B. conducted microhardness analysis with the guidance of M.M.H. and H.S.-R. B.I. analysed all data. B.I. and M.E.H. wrote the first draft, and the final version of manuscript, and all other authors contributed to writing and editing. Our research was conducted under local and institutional research permits. For assistance in the laboratory, we are grateful to J. Galbraith and C. Hobbis. We thank two anonymous reviewers for helpful comments. For major funding, we thank the Human Frontier Science Program (to T.G., P.C. and M.E.H.).

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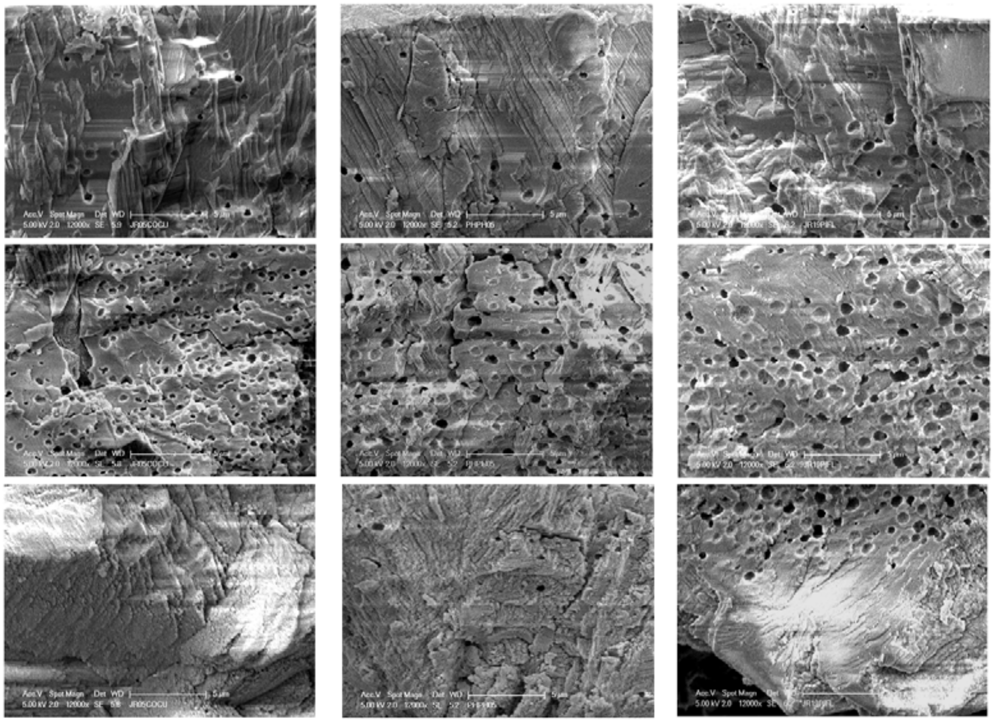
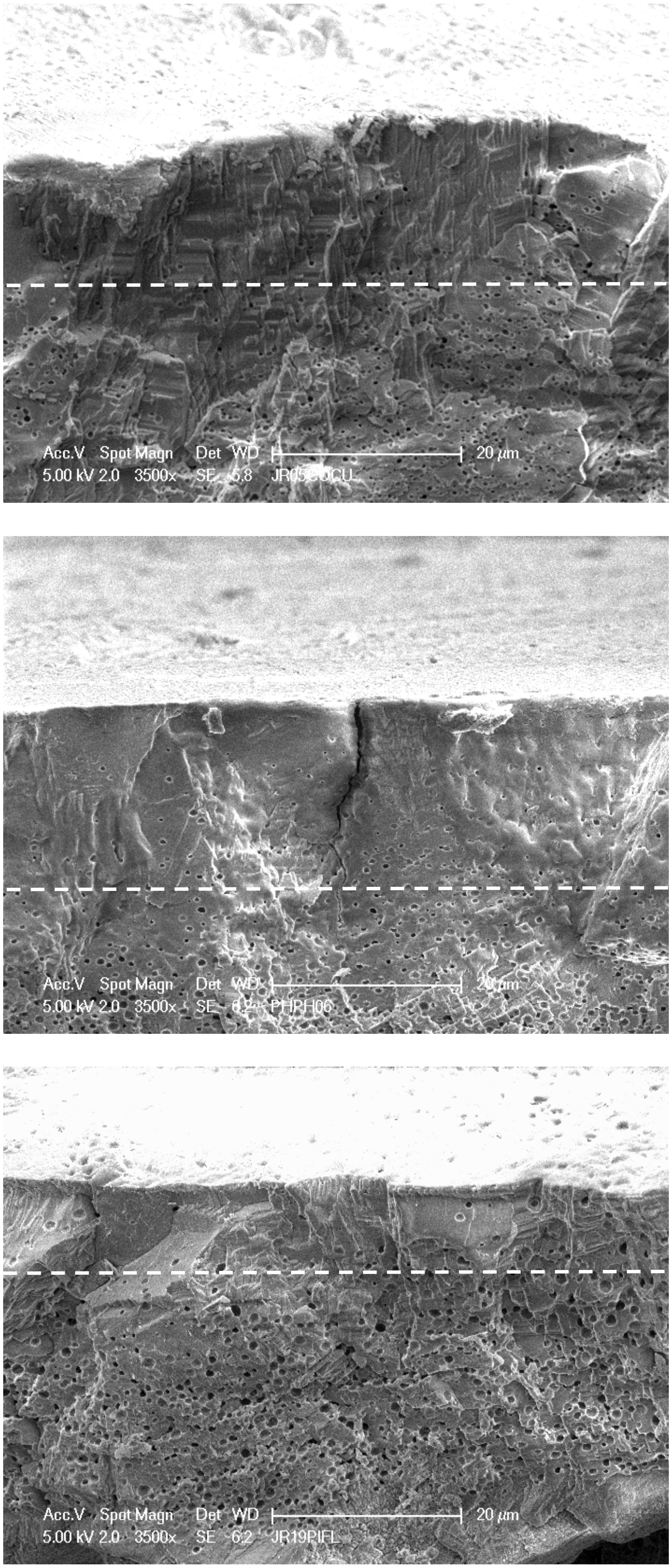
Supplementary Figures

Supplementary Figure 1: Scanning electron micrographs of the eggshell layer cross-sections. From left to right is redstart-specific host-race of the common cuckoo [Cuckoo (PHPH)], host species common redstart (PHPH), and a sympatric non-host species, the pied flycatcher (PIFL). From top to bottom is the outside palisade layer, inner palisade layer, mammillary layer. Photos by BI.



Supplementary Figure 2: Scanning electron micrographs showing boundaries between the outer and inner palisade layers of eggshell layer cross-sections. From top to bottom is redstart-specific host-race of the common cuckoo [Cuckoo (PHPH)], host species common redstart (PHPH), and a sympatric non-host species, the pied flycatcher (PIFL). Dashed line represent theoretical boundaries between the outer and inner palisade layers used in the structural analysis. Photos by BI.



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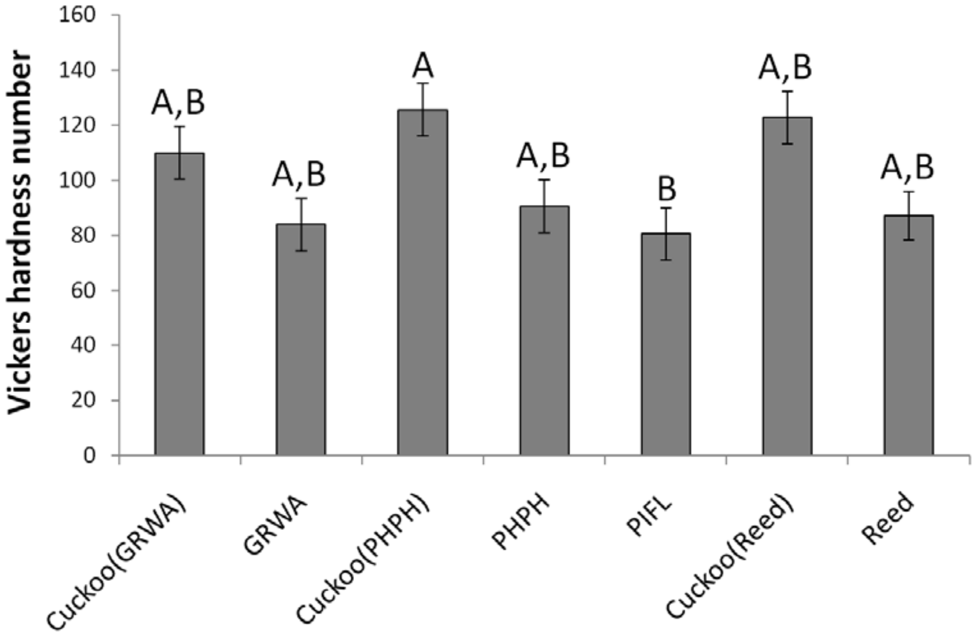
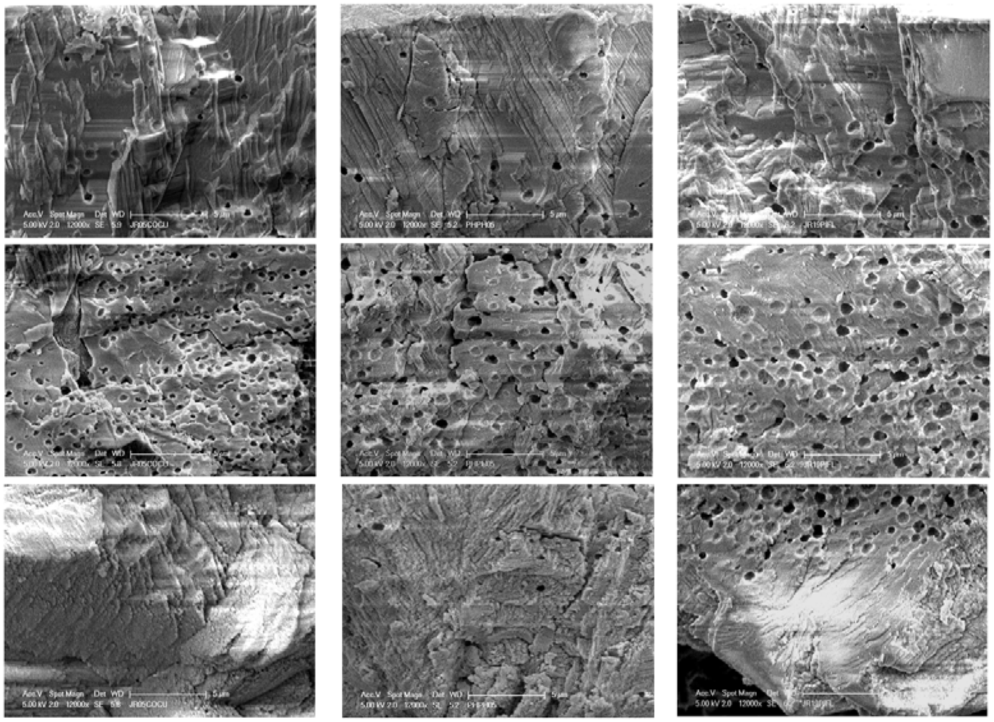
Supplementary Figure 3: Global eggshell Vickers hardness numbers (least square means ± S.E, combined across all eggshell regions) of host/non-host species and cuckoo host-races extracted from a generalised linear mixed model (GLMM). Groups not connected by the same letter were significantly different in a GLMM analysis, using post-hoc Tukey's HSD test ($\alpha = 0.05$). For species codes, please refer to table 1.

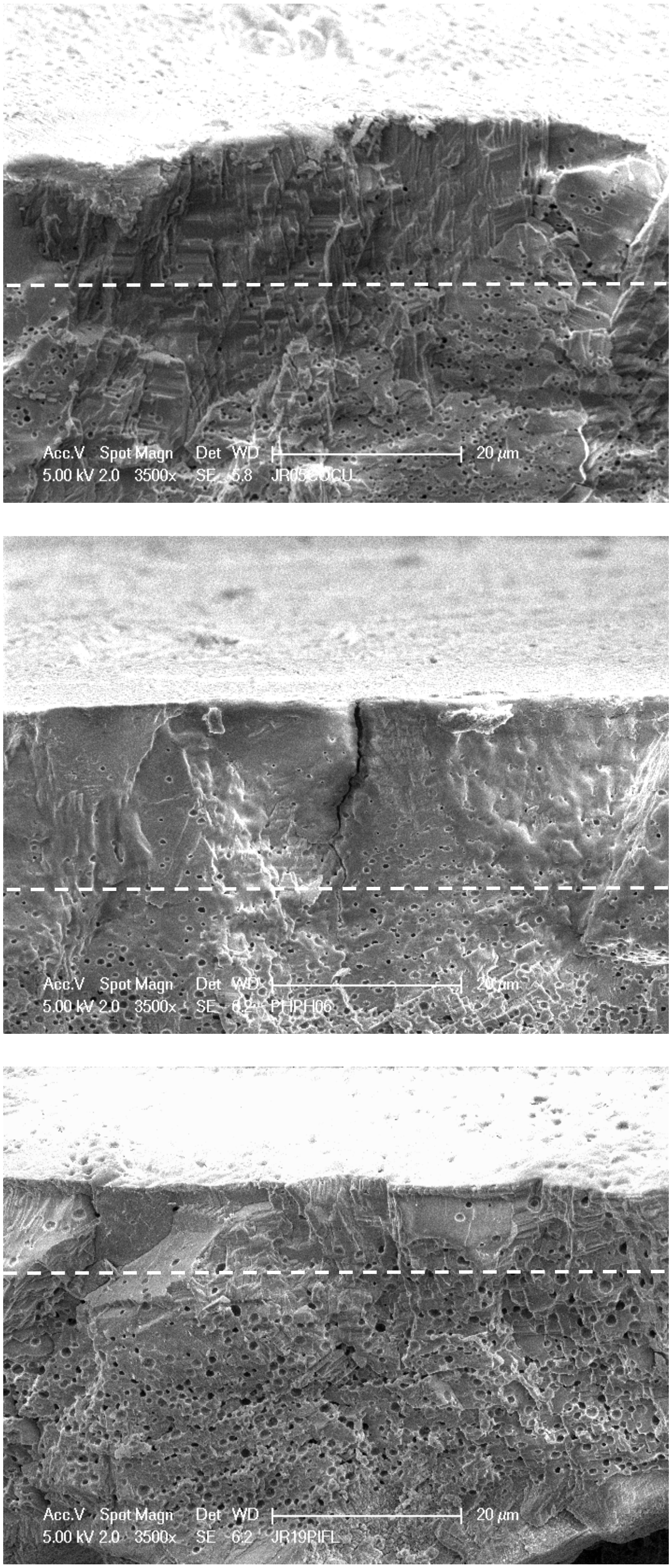


Supplementary Figures

Supplementary Figure 1: Scanning electron micrographs of the eggshell layer cross-sections. From left to right is redstart-specific host-race of the common cuckoo [Cuckoo (PHPH)], host species common redstart (PHPH), and a sympatric non-host species, the pied flycatcher (PIFL). From top to bottom is the outside palisade layer, inner palisade layer, mammillary layer. Photos by BI.



Supplementary Figure 2: Scanning electron micrographs showing boundaries between the outer and inner palisade layers of eggshell layer cross-sections. From top to bottom is redstart-specific host-race of the common cuckoo [Cuckoo (PHPH)], host species common redstart (PHPH), and a sympatric non-host species, the pied flycatcher (PIFL). Dashed line represent theoretical boundaries between the outer and inner palisade layers used in the structural analysis. Photos by BI.



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Supplementary Figure 3: Global eggshell Vickers hardness numbers (least square means ± S.E, combined across all eggshell regions) of host/non-host species and cuckoo host-races extracted from a generalised linear mixed model (GLMM). Groups not connected by the same letter were significantly different in a GLMM analysis, using post-hoc Tukey's HSD test (α = 0.05). For species codes, please refer to table 1.