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Author for correspondence:
Stephanie C. McClelland
e-mail: stephanie.mcclelland.2018@live.rhul.ac.uk

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Convergent evolution of reduced eggshell conductance in avian brood parasites

Stephanie C. McClelland[1], Gabriel A. Jamie[2,3], Katy Waters[1], Lara Caldas[1], Claire N. Spottiswoode[2,3] and Steven J. Portugal[1]

[1]School of Biological Sciences, Royal Holloway University of London, Egham, Surrey TW20 0EX, UK
[2]Department of Zoology, University of Cambridge, Downing Street, Cambridge CB2 3EJ, UK
[3]FitzPatrick Institute of African Ornithology, DST-NRF Centre of Excellence, University of Cape Town, Rondebosch 7701, Cape Town, South Africa

SCM, 0000-0002-8763-2291; GAJ, 0000-0002-2766-7687; CNS, 0000-0003-3232-9559; SJP, 0000-0002-2438-2352

Brood parasitism has evolved independently in several bird lineages, giving rise to strikingly similar behavioural adaptations that suggest convergent evolution. By comparison, convergence of physiological traits that optimize this breeding strategy has received much less attention, yet these species share many similar physiological traits that optimize this breeding strategy. Eggshell structure is important for embryonic development as it controls the flux of metabolic gases, such as O_2, CO_2 and H_2O, into and out of the egg; in particular, water vapour conductance (G_{H_2O}) is an essential process for optimal development of the embryo. Previous work has shown that common cuckoos (*Cuculus canorus*) have a lower than expected eggshell G_{H_2O} compared with their hosts. Here, we sought to test whether this is a trait found in other independently evolved avian brood parasites, and therefore reflects a general adaptation to a parasitic lifestyle. We analysed G_{H_2O} for seven species of brood parasites from four unique lineages as well as for their hosts, and combined this with species from the literature. We found lower than expected G_{H_2O} among all our observed brood parasites both compared with hosts (except for brown-headed cowbirds (*Molothrus ater*)) and compared with the expected rates given their phylogenetic positions. These findings suggest that a lowered G_{H_2O} may be a general adaptation for brood parasitism, perhaps helping the parasite nestling to develop greater aerobic fitness.

This article is part of the theme issue 'The coevolutionary biology of brood parasitism: from mechanism to pattern'.

1. Introduction

Avian brood parasites forego the costs of raising their own offspring, and instead rely on hosts to incubate their eggs and provision their young [1–3]. Interspecific obligate brood parasitism is found in approximately 1% of bird species, and has evolved independently seven times: three times in Cuculidae (cuckoos), and once each in the Indicatoridae (honeyguides), Icteridae (*Molothrus* spp., cowbirds), Viduidae (*Vidua* spp. and cuckoo finches, *Anomalospiza imberbis*) and Anatidae (black-headed ducks, *Heteronetta atricapilla*) [1]. Despite the large phylogenetic diversity of parasitic species, there are commonalities in the approaches that they adopt to ensure the hosts will incubate their eggs, and successfully rear their offspring. Since brood parasitism has arisen independently in each of these linages, this is suggestive of convergent selection pressures acting on these traits.

While the behavioural adaptations common to different lineages of brood parasites have been well studied, convergent adaptation in physiological traits has been less thoroughly investigated. However, examples of apparent convergent physiological adaptations across brood parasite species can be found at multiple stages of their development. At the egg stage, these include parasitic species having thicker egg shells than those of their hosts [4]. This is

thought to hinder the host in puncturing the parasite's egg, and hence make ejection from the nest more difficult if the host attempts to evict it [5,6]. Thicker eggshells may also function to protect the parasitic egg from fracture during the rapid laying process that is characteristic of many brood parasites [7]. In parasitic cowbirds (e.g. brown-headed cowbirds (*Molothrus ater*)) and greater honeyguides (*Indicator indicator*), thicker eggshells may function to help protect against egg puncturing by conspecifics attempting to parasitize the same nest [8,9]. Many brood parasites also have a shorter incubation period compared with their hosts [10–12], and early hatching is beneficial as it provides a competitive advantage for the parasite chick over host young [13,14]. This is achieved either through facilitating the killing or ejection of host eggs or chicks [12], or through providing a competitive edge in obtaining food from the host parents, for those species where host and parasite are reared together [15–17]. Various mechanisms have been proposed to explain the shorter developmental period seen in brood parasites, including internal incubation by the female (in some species where eggs are laid at 48 h intervals) [18] and a higher concentration of growth-promoting steroids in the yolk [19], but the precise mechanism behind early hatching is not fully understood. Finally, convergent physiological adaptations in brood parasites are also found at the chick stage. Many parasite nestlings have stronger neck or back muscles [12,20,21] that not only aid in hatching from a thicker eggshell, but also likely assist parasitic chicks to kill the hosts, chicks, in those species that are highly virulent [20,22].

These examples show that many physiological adaptations to brood parasitism occur at the egg stage. Yet, while much has been documented about the size [23], maculation [24,25] and thickness [4,6] of the eggs of avian brood parasites, comparatively less is known about their eggshell physiology. This includes traits such as the rate of exchange of respiratory gases (carbon dioxide, oxygen and water vapour) across the eggshell pores, which may play a role in the rapid development of the embryo of parasitic birds [26]. Gas exchange across the shell depends on the diffusive properties of the eggshell and, importantly, on the environmental conditions under which the egg is placed [27–30]. If the nest environment is too humid or too xeric, then either too little or too much water loss occurs, which can cause developmental abnormalities and embryonic death [28,31,32]. Gas exchange contributes to the rate of water loss, and can be measured across the eggshell as the water vapour conductance (G_{H_2O}). Therefore, G_{H_2O} must be fine-tuned such that desiccation does not endanger the embryo, while at the same time allowing sufficient water to be lost for embryo growth and air cell formation [32,33]. Because all nutrients for embryonic development are deposited by the mother into the egg prior to laying, maintaining suitable humidity levels for gas exchange and incubation temperature constitute the female's only physical control of the requirements for her offspring's embryonic development once the egg has been laid [34–37].

The physical characteristics of the nest environment are known to be important determinants of G_{H_2O}, and therefore G_{H_2O} across the eggshell of parasitic species should be expected to match that of their hosts, given that they experience identical nest environments. However, contrary to this expectation, the nanostructure of the eggs of one parasite species, common cuckoos (*Cuculus canorus*), has been demonstrated to differ greatly from that of its hosts [6]. Therefore, while the outer appearance of the parasite's eggshell might sometimes superficially match that of the hosts, eggshell physiology and gaseous exchange might be considerably different.

Previous work by Portugal *et al*. [33] tested the hypothesis that brood parasites should have an elevated gas exchange across the eggshell to promote the rapid development of the embryo, as has been suggested in cowbirds based on the number of external pore openings [38]. Contrary to this expectation, they found that the G_{H_2O} of common cuckoos eggs was lower (i) than eggs of their hosts, (ii) than expected for their egg size, and (iii) than expected given the common cuckoo's phylogenetic position [33]. The lower G_{H_2O} in common cuckoo eggs was suggested to permit slower depletion of the yolk, thus providing more reserves at the end of the incubation period to assist the embryo with the energetically demanding events of hatching from an egg of great structural strength, and of evicting host eggs and nest-mates [39].

Here, we test the hypothesis that a reduced eggshell G_{H_2O} is thus an adaptation to a parasitic lifestyle, and therefore a commonality among all avian brood parasites, regardless of host identity, parasitic egg size or parasitic phylogenetic position. The unusual coevolutionary biology of brood parasites provides a unique opportunity to understand the extent to which developmental physiology is simultaneously fine-tuned to different environments while potentially dictated and/or constrained by phylogeny. Our multi-species systems comparison also proposes a framework for future studies to focus on the physiological adaptations of parasites, across multiple independently evolved taxa, that may have contributed to the dynamics of host–parasite relationships. To investigate any commonalities between avian brood parasites in eggshell G_{H_2O}, we use new data from six obligate brood-parasitic species from three independently evolved lineages of brood parasites (cuculine cuckoos, honeyguides and parasitic finches), and 10 species of hosts common to them and to related brood parasites. We combine these with data from 51 species from the literature, including a representative of a fourth lineage of brood parasites, the parasitic brown-headed cowbirds, *Molothrus ater*.

2. Material and methods

(a) Species and eggshell samples

Eggs of the following brood-parasitic species were collected from the wild in the Choma District of Zambia under permit (see tables 1 and 2 for sample sizes): lesser honeyguides (*Indicator minor*), greater honeyguides, cuckoo finches (*Anomalospiza imberbis*), pin-tailed whydahs (*Vidua macroura*) and purple indigobirds (*Vidua purpurascens*). Additionally, data on common cuckoos and brown-headed cowbirds were added from the literature (see below and electronic supplementary material, tables S1 and S2). Eggs were collected and blown within a few days of laying and stored in the dark, inside airtight plastic containers, until analysis. The time between collection and analysis varied, with some eggs stored since 2008. This is unlikely to have affected the analysis as it has been shown that G_{H_2O} of fresh eggs is not significantly different from museum specimens that have been stored over 50 years [40].

The following host species were collected from the same location (corresponding parasites in brackets following species names; see tables 1 and 2): little bee-eaters (*Merops pusillus*) (parasitized by greater honeyguides), crested barbets

Table 1. Sample sizes, collection locations and parasite strategy for the seven species of brood parasites for which eggshell conductance (G_{H_2O}) was measured. *N* refers to the number of (i) whole eggs where G_{H_2O} was measured for each species and (ii) the number of eggs that shell fragments were taken from. For example, for lesser honeyguides there were four eggs used for eggshell fragment analyses, and 14 shell fragments were used from these four eggs. Strategy refers to the approach of the parasite to dealing with the offspring of their respective hosts (see Material and methods). 'Virulent' is where the parasite kills the host's offspring, while 'outcompete' refers to a strategy whereby the parasite does not directly kill the host's offspring, but outcompetes them for resources (usually fatally, in cuckoo finches).

species	*N*	location	strategy
lesser honeyguide (*Indicator minor*)	3 whole eggs, 14 shell fragments from 4 eggs	Zambia	virulent
greater honeyguide (*Indicator indicator*)	3 whole eggs, 24 shell fragments from 4 eggs	Zambia	virulent
cuckoo finch (*Anomalospiza imberbis*)	6 whole eggs	Zambia	outcompete
pin-tailed whydah (*Vidua macroura*)	6 shell fragments from 3 eggs	Zambia	outcompete
purple indigobird (*Vidua purpurascens*)	5 shell fragments from 1 egg	Zambia	outcompete
common cuckoo (*Cuculus canorus*)	9 whole eggs 4 shell fragments from 1 egg	UK (NHM)	virulent

(*Trachyphonus vaillantii*) (parasitized by lesser honeyguides), common waxbills (*Estrilda astrild*) (parasitized by pin-tailed whydahs), Jameson's firefinches (*Lagonosticta rhodopareia*) (parasitized by purple indigobirds) and tawny-flanked prinias (*Prinia subflava*) (parasitized by cuckoo finches). Additionally, we also analysed eggs from several co-occurring estrildid species that are commonly parasitized by closely related *Vidua* spp. at the same study site or in other parts of their range: zebra waxbills (*Amandava subflava*) (elsewhere parasitized by Jambandu indigobirds, *Vidua raricola*), African quailfinches (*Ortygospiza fuscocrissa*) (elsewhere parasitized by quailfinch indigobirds, *Vidua nigeriae*), red-billed firefinches (*Lagonosticta senegala*) (locally parasitized by village indigobirds, *Vidua chalybeata*) and orange-winged pytilias (*Pytilia afra*) (locally parasitized by broad-tailed paradise whydahs, *Vidua obtusa*). For phylogenetic comparison, eggs of two related estrildid finch species (not believed to be hosts) were also collected from the same field site in Zambia: locust finches (*Paludipasser locustella*) and blue waxbills (*Uraeginthus angolensis*).

(b) Whole-egg conductance measurements

For eggs that were collected shortly after laying and blown in the field, the whole egg was used to measure G_{H_2O}. The G_{H_2O} of the eggs was measured following the same standard protocol [33,41–44] that was used by studies that were the source of comparative literature data (see electronic supplementary material, table S1), and researchers were blind to which species each egg came from during the initial process of measuring G_{H_2O}. Eggshells were filled with water to capacity via a syringe and fine-gauge needle (using water instead of fresh egg contents has been shown to have no effect on G_{H_2O}; [45]). The eggs had been blown following collection, so to cover the blow hole, we glued on a small section of impermeable plastic cut to size to cover the hole, using Loctite™ superglue (Consumer Products Henkel Corporation, Ohio, USA). The plastic covering the blow hole made up, on average, less than 2.5% of the total egg surface area, and previously we demonstrated that this was an effective way of covering the blow hole [44]. The glue was left to dry for 4 h, before the eggs were weighed to the nearest 0.0001 g (Sartorius 1265 MS, Göttingen, Germany) and placed in a desiccator (Camlab, Cambridge, UK) filled with self-indicating silica gel. The desiccator was housed in a constant temperature cabinet (Porkka, Hertfordshire, UK) at $25 \pm 1^{\circ}$C. After 24 h, the eggs were weighed to the nearest 0.1 mg before being returned to the desiccators. The eggs were then weighed at the same time of day on three successive days to provide two values of 24 h G_{H_2O}, and a mean was taken. Any mass loss was assumed to be the result of water loss [33,40,44,46]. Calculation of G_{H_2O} was as previously described [40,44,46,47]. Briefly, the G_{H_2O} of a shell can be calculated as:

$$G_{H_2O} = \frac{M_{H_2O}}{\Delta P_{H_2O}}, \tag{2.1}$$

where G_{H_2O} = water vapour conductance (mg d^{-1} torr^{-1}), M_{H_2O} = the rate of mass loss (mg d^{-1}) and ΔP_{H_2O} = water vapour pressure difference across the shell (torr). Internal P_{H_2O} was assumed to be 23.8 torr (water vapour pressure of saturation at the egg temperature, 23.8 torr at 25°C), and external P_{H_2O} to be 0 torr, owing to the desiccator atmosphere being close to zero humidity.

(c) Fragment eggshell conductance measurements

Previously we demonstrated that eggshell fragments can be used to measure G_{H_2O} across eggshells [40,44,46]. For eggs that had been broken in the field, or had cracks present, fragments were used to determine G_{H_2O}. Eggshell fragments were glued to the top of Eppendorf tubes (surface area of 24.4 mm^2) that had been previously filled with 200 µl of water. Loctite glue was applied via a syringe and needle to the circumference of the Eppendorf, before placing the eggshell fragment on top, inside surface down, ensuring that the top of the tube was entirely covered with eggshell. The eggshell fragment was then gently pushed down to ensure contact with the glue and left for 4 h to dry (following the manufacturer's recommendations). Most eggshell fragments were taken from the equatorial portion of the egg in order to get a relatively flat shell section with a large enough diameter to cover the opening of the Eppendorf tube. The Eppendorf tubes were housed in PCR preparation racks (Cole-Parmer, St Neots, UK) to aid transport and weighing, and to ensure the Eppendorf was upright at all times. Once the glue had dried, each eggshell fragment was checked to ensure that it was adhered securely, before superglue (RS Components, Corby, UK) was applied to its underside, around the join of the Eppendorf circumference and the eggshell. The superglue was allowed to dry for 2 h, then the tops of the eggshells were brushed gently with a dry artist's paintbrush to remove any particulate dust. The efficiency of the seal between the eggshell and the Eppendorf tubes can be established through examining the repeatability of the mass loss between weighing sessions. Samples that showed an abnormally high rate of mass loss were checked for cracks in the shell fragment or an incomplete glue seal, and discarded if a defect was found [40]. All other aspects of G_{H_2O} measurement and analyses were identical to the whole-egg protocol.

All G_{H_2O} species values extracted from the literature were measured using the same methods to our study and hence are

Table 2. Sample sizes, and primary parasite for the 11 species of hosts for which eggshell conductance (G_{H_2O}) was measured. *N* refers to (i) the number of whole eggs where G_{H_2O} was measured for each species and (ii) the number of shell fragments and numbers of eggs from which these were taken. For example, for little bee-eaters there was one egg used for eggshell fragment analyses, and six shell fragments were used from this egg. Host eggs were collected from the same location as their respective parasites (table 1).

species	*N*	parasite
little bee-eater (*Merops pusillus*)	2 whole eggs, 6 fragments from 1 egg	greater honeyguide
crested barbet (*Trachyphonus vaillantii*)	1 whole egg	lesser honeyguide
zebra waxbill[a] (*Amandava subflava*)	6 whole eggs, 9 fragments from 3 eggs	Jambandu indigobird (West Africa only)
blue waxbill[a] (*Uraeginthus angolensis*)	2 whole eggs, 8 fragments from 2 eggs	none
common waxbill (*Estrilda astrild*)	4 whole eggs, 34 fragments from 14 eggs	pin-tailed whydah
African quailfinch[a] (*Ortygospiza fuscocrissa*)	1 fragment from 1 egg	quailfinch indigobird (West Africa only)
locust finch[a] (*Paludipasser locustella*)	2 fragments from 1 egg	none
Jameson's firefinch (*Lagonosticta rhodopareia*)	1 whole egg, 18 fragments from 9 eggs	purple indigobird
red-billed firefinch[a] (*Lagonosticta senegala*)	2 fragments from 2 eggs	village indigobird
orange-winged pytilia[a] (*Pytilia afra*)	14 fragments from 5 eggs	broad-tailed paradise whydah
tawny-flanked prinia (*Prinia subflava*)	35 whole eggs, 2 fragments from 1 egg	cuckoo finch

[a]Collected in Zambia, but were not hosts of parasites included in this study.

comparable. See the electronic supplementary material, tables S1 and S2 for a full list of the literature sources.

(d) Life-history and ecological data

A total of 43 species was used for whole-egg analyses, and 36 species for fragment analyses (see electronic supplementary material, tables S1 and S2 for full species list and the literature sources). Species were restricted to passerines or near-passerine families for similarity in egg size. Life-history and ecological data were gathered primarily from *Handbook of the birds of the world*, volumes 1–13 [48], and cross referenced with *Birds of the western Palearctic* [49]. In addition, electronic supplementary material was obtained from family and species-specific monographs, and field guides to nests (sources available on request).

We restricted the number of life-history traits included to those that have been found to have a significant effect on G_{H_2O} [44]. These were as follows (all scored from the literature): adult body mass (g), mean fresh egg weight (g), median breeding range (degrees latitude), nest type (cup/non-cup), ground nesting (no/yes), diet (calcium-rich/herbivore) and whether the parental foraging style meant that adults returned habitually to the nest with wet plumage (no/yes wet incubating parent; see Results; see [50] for full description). Body mass of adult birds was taken as a mean for both sexes, primarily from [51]. Breeding latitude was compiled from data tabulated by Orme *et al*. [52].

(e) Phylogenetic methods and analyses

R statistical software was used to conduct all statistical analysis and production of figures (R Studio Team 2015) [53]. Values of G_{H_2O} produced from whole-egg analysis ($n = 43$ species) and fragmented eggs ($n = 36$ species) were analysed separately. However, a number of species ($n = 12$) were included in both analyses for which both whole- and fragmented-egg values were available. The number of eggs and eggshell fragments measured per species can be found in tables 1 and 2 and tables 3 and 4, respectively.

Since species are not statistically independent, we modelled G_{H_2O} while taking into account their shared phylogenetic history [54–56]. We downloaded 1000 phylogenetic trees for each of our species subsets (43 species for whole eggs and 36 for fragmented eggs) from www.birdtree.org, which used a backbone tree based on Ericson *et al*. [57]. For each set of trees, a maximum clade credibility tree was produced using the function maxCladeCred from the R package phangorn [58,59], and these trees were used for subsequent phylogenetically informed analysis (figure 1).

We estimated Pagel's λ for G_{H_2O} on each tree, using the function phylosig (from the R package phytools [60,61] on 999 phylogenetic simulations. Pagel's λ ranges between 0 and 1 and is an indication of the strength of the phylogenetic signal of a trait across a phylogeny. A Pagel's λ value of 1 or close to 1 indicates that the evolution of a trait is best described by a Brownian motion model of trait evolution (and thus corresponds

Table 3. Top models for whole-egg analysis. Model support table (AICc) for the top-ranked pgls models (model weight $>$ 0.05) of G_{H_20} that contribute to the average models. Estimates for parameters are provided to indicate inclusion of these parameters in respective models. Model weights are estimates across the entire set of 32 models and sum to 1.

model no.	intercept	parasite status	shell thickness	nest type	wet parent	mean breeding latitude	parasite status/shell thickness interaction	AICc	δAICc	model weight	logLik
1	0.06	−0.71		1.34		0.01		83.50	0.00	0.20	−35.928
2	0.40	−0.65				0.01		84.20	0.76	0.14	−38.812
3	0.65	−0.73						84.90	1.47	0.10	−40.323
4	0.38	−0.81		0.96				85.80	2.32	0.06	−38.375
5	0.05	−0.73		1.42	−0.11	0.01		86.00	2.57	0.06	−35.856
6	0.31	−0.67	0.00			0.01		86.10	2.58	0.05	−38.502
7	0.03	−0.72	0.00	1.29		0.01		86.10	2.63	0.05	−35.886

Table 4. Top models for shell fragment analysis. Model support table (AICc) for the top-ranked pgls models (model weight $>$ 0.05) of G_{H_20} that contribute to the average models. Estimates for parameters are provided to indicate inclusion of these parameters in respective models. Model weights are estimates across the entire set of 32 models and sum to 1.

model no.	intercept	parasite status	shell thickness	nest type	wet parent	mean breeding latitude	parasite status/shell thickness interaction	AICc	δAICc	model weight	logLik
1	−0.58		−4.30		−0.57			36.5	0.00	0.14	−14.857
2	−0.61	−1.57	−4.33		−0.56		16.54	36.5	0.04	0.14	−12.253
3	−0.69		−3.91					37.6	1.09	0.08	−16.595
4	−0.51		−4.13		−0.53	0.00		37.8	1.35	0.07	−14.264
5	−0.53	−1.53	−4.20		−0.52	0.00	15.42	38.3	1.87	0.06	−11.721

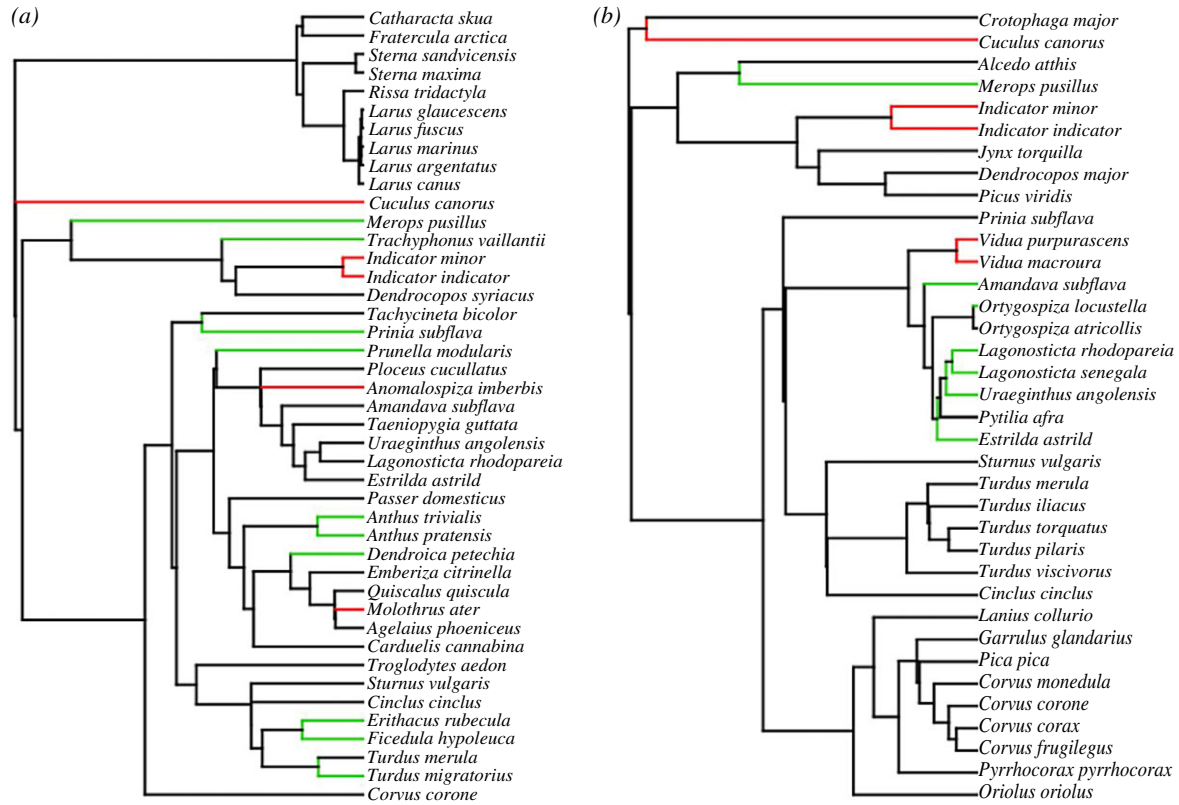

Figure 1. Phylogenetic trees with representatives for (*a*) whole-egg analysis and (*b*) shell fragment analysis. Red lines indicate brood-parasitic species, and green lines indicate frequent hosts of parasites in each subset. Trees generated using a backbone tree from Ericson *et al*. [57] (see Material and methods).

closely to expectations given through shared patterns of relatedness), whereas a value close to 0 indicates little or no phylogenetic signal in the distribution of the trait among tip species [54].

We constructed phylogenetic generalized least-squares (pgls) models using the pgls command in the package caper [62]. These models incorporate the expected similarity between sister taxa by producing a covariance matrix of how they are expected to covary based on their position on the phylogeny and the strength of the phylogenetic signal (Pagel's λ). For each of our pgls models, Pagel's λ was assigned to the value produced by the phylosig function for that dataset. Using these pgls models, we tested the effect of being a brood parasite on observed G_{H_2O}, while controlling for other life-history traits expected to influence this value. Full models included eggshell thickness, nest type (see above for categories), whether or not parents are wet when returning to the nest, and mean breeding latitude. Interactions between shell thickness and parasitic status were also included in the full models, because brood-parasitic species tend to have thicker eggshells than expected for their size [5,6]. Despite this pattern in brood parasites, adult body mass and fresh egg weight were highly correlated with eggshell thickness (explaining greater than 75% of variance in all cases), and, therefore, only eggshell thickness was included in the models.

Model selection was performed by creating models including all possible variables listed above, and assessing model performance based on AICc (Akaike Information Criterion corrected for small sample sizes). Model estimates with relative weights of all models with an AICc of less than 2 are presented in tables 3 and 4. Subsequently, a model averaging technique was applied, constructing an average model including all models that could not be rejected with 95% certainty based on model weighting. Model selection and averaging was done using the R package MuMIn [63]. Plots and phylogenetic trees produced using R packages ggplot2 [64] and phytools, respectively. A Welch two sample *t*-test was used to initially compare G_{H_2O} between parasites and common hosts (host species listed above in §2a, with the addition of eight host species from the literature (electronic supplementary material, table S2); however, owing to the small number of species, this analysis did not take phylogeny into account. For one species we unfortunately had only a single egg sample. Therefore, we repeated our analysis of whole eggs without this species; excluding these data did not change the significance of the models (electronic supplementary material, Supplementary information S1 Additional analyses.).

3. Results

(a) Comparison of G_{H_2O} in parasites and their respective hosts

Brood parasites had a significantly lower G_{H_2O} when compared in a pairwise manner with common host species. For whole eggs, G_{H_2O} (mean $\pm$ s.e.m.) was 0.76 ± 0.12 mg d^{-1} torr^{-1} for brood parasite species ($n = 5$) and 1.34 ± 0.20 mg d^{-1} torr^{-1} for host species ($n = 16$; $t_{13.9} = 2.39$, $p = 0.031$). For eggshell fragments, G_{H_2O} was 0.39 ± 0.05 mg d^{-1} torr^{-1} for brood parasite species ($n = 5$) and 0.74 ± 0.06 mg d^{-1} torr^{-1} for host species ($n = 10$; $t_{7.8} = 3.98$, $p = 0.004$; figures 2 and 3).

Owing to potentially confounding effects of egg size on G_{H_2O}, comparisons were also undertaken correcting for species mean egg weight. The results were similar to non-mass-corrected values, with hosts having significantly higher G_{H_2O} per gram than brood parasites (for whole eggs: $n = 16$, $t_{12.2} = 3.51$, $p = 0.004$, and for eggshell fragments: $n = 10$, $t_{5.2} = 3.51$, $p = 0.015$; figure 3). No statistically significant difference was found in G_{H_2O} or mass-corrected G_{H_2O} between virulent and non-virulent brood parasites ($p > 0.05$ for whole eggs and eggshell fragments), although there was

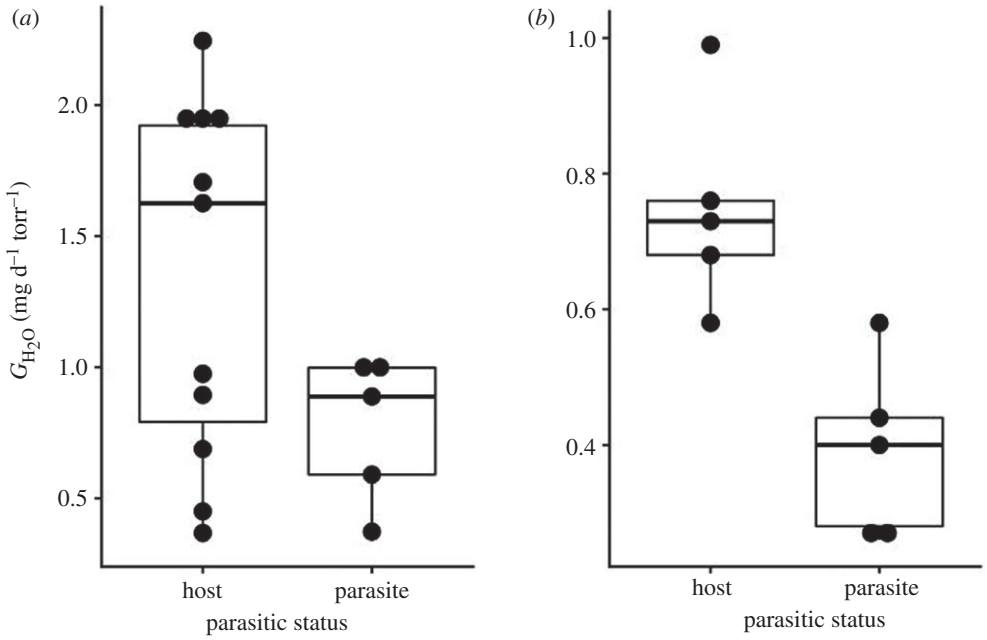

Figure 2. Non-mass-corrected mean G_{H_2O} (mg d^{-1} torr^{-1}) of avian brood parasites and common hosts. For whole eggs (*a*), five parasitic and 11 host species were compared, and for shell fragments (*b*), five parasitic and five hosts species were compared. Host species had significantly higher G_{H_2O} in both cases (*t*-test: (*a*) $t_{13.9} = 2.39$, $p = 0.03$; (*b*) $t_{7.8} = 3.98$, $p = 0.004$).

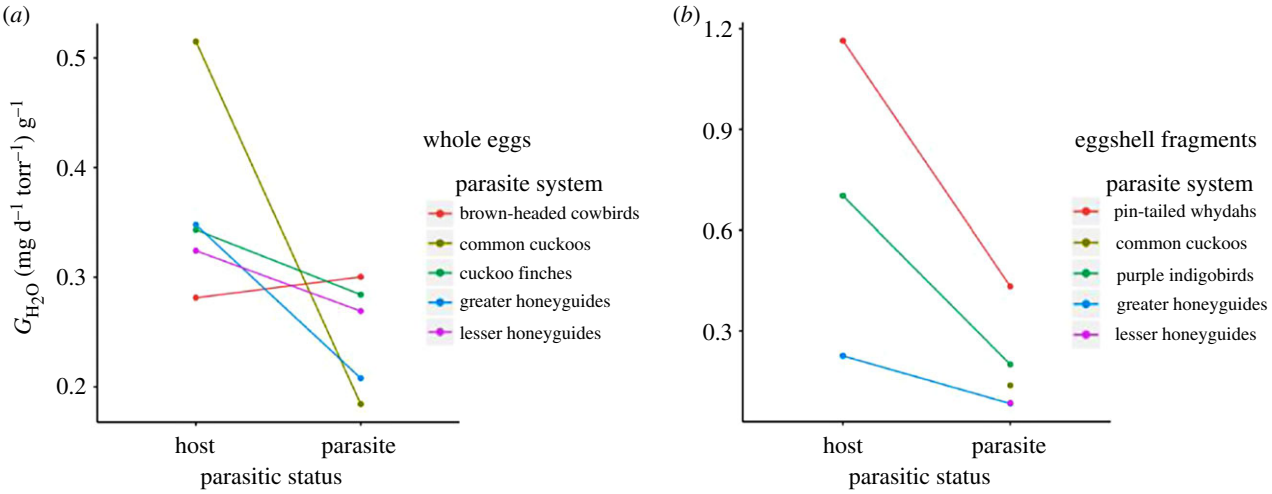

Figure 3. (*a*) G_{H_2O} (mg d^{-1} torr^{-1}) corrected by egg weight (g) for whole eggs. Brood parasites and respective host species connected by coloured lines. Note: Average G_{H_2O} of several hosts ($n = 5$) of the common cuckoo is presented in the host category. (*b*) G_{H_2O} (mg d^{-1} torr^{-1}) corrected by egg weight (g) for shell fragments. Brood parasites and respective hosts are linked by coloured lines. Note: No G_{H_2O} values are available for shell fragments for hosts of common cuckoos or lesser honeyguides. An average value was calculated for five species of hosts of the common cuckoo in (*a*).

a strong trend for greater G_{H_2O} in the eggs of non-virulent parasites (tables 1 and 2; figure 4). However, owing to the small sample size (virulent brood parasites: $n = 3$, non-virulent brood parasites: $n = 2$), a non-significant *p*-value may be the result of insufficient power for this analysis.

(b) Phylogenetic signal

Pagel's λ for G_{H_2O} for both whole eggs and eggshell fragments was 0.71. In both cases, this value was significantly different from 0 and 1 ($p > 0.001$), implying that while there is an effect of phylogeny on G_{H_2O}, it is primarily driven by an evolutionary process that is weaker than would be seen with a Brownian motion model of trait evolution, meaning that phylogeny alone is not determining the patterns seen in this trait.

(c) Whole-egg conductance

For whole eggs, the average model (table 3) of the pgls analysis for mean G_{H_2O} retained the predictors parasitic status (binary), eggshell thickness, nest type ('scrape', 'cup', 'shallow'), wet parent (binary) and mean breeding latitude (degrees). The interaction term of parasite status and eggshell thickness was not retained in any of the viable models, indicating that this interaction was neither significant in affecting G_{H_2O} nor improved the fit of the models to the data. Parasitic status explained a significant amount of variance in G_{H_2O} ($z = 2.24$, $p = 0.025$), with parasitic species displaying lower G_{H_2O} than would be expected given their phylogenetic position ($n = 5$ of 43). Nest type also significantly predicted mean G_{H_2O} ($z = 1.96$, $p = 0.050$), with shallow and scrape style nests having a lower G_{H_2O} than cup nests. However despite being retained in the average model based on AICc,

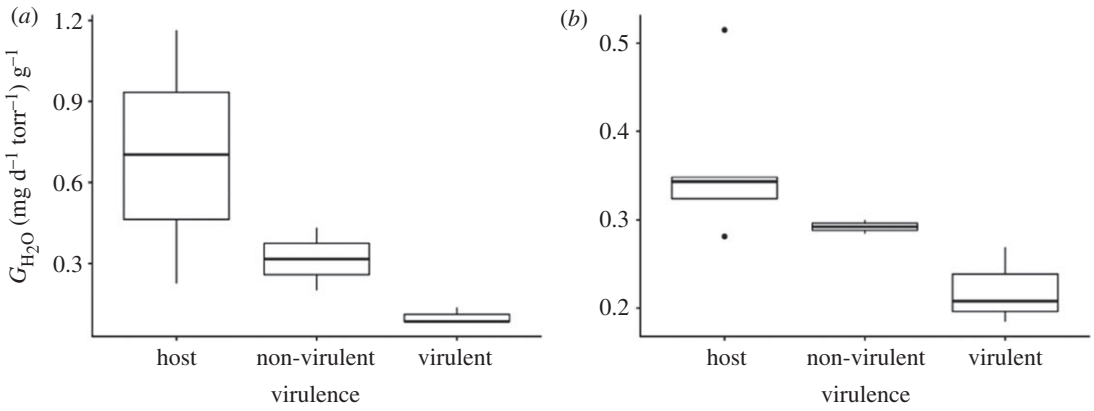

Figure 4. (*a*) Mean G_{H_2O} (mg d^{-1} torr^{-1}) corrected by egg weight (g) for whole eggs. Five host species compared with two non-virulent brood parasites and three virulent brood parasites. (*b*) Mean G_{H_2O} (mg d^{-1} torr^{-1}) corrected by egg weight (g) for eggshell fragments. Three host species compared with two non-virulent brood parasites and three virulent brood parasites. There was no significant difference between virulent and non-virulent species in either case (whole eggs: $t_{2.38} = 2.70$; $p = 0.09$, eggshell fragment: $t_{1.56} = 1.82$; $p = 0.31$).

neither eggshell thickness, wet parent, nor mean breeding latitude had a significant effect ($p > 0.05$) in the average model. Parasitic status was found to have the largest relative variable importance, because it was retained in all seven contributing models. The next most important variable in model fit was mean breeding latitude, which occurred in five of the models, despite being non-significant.

(d) Eggshell fragment conductance

The average model for eggshell fragment analysis contained most of the same predictors as for the whole-egg analysis, with the exceptions that nest type was excluded, and that the interaction term for parasitic status and eggshell thickness was included. As with the whole-egg analysis, parasitic status was found to significantly predict G_{H_2O} (z-value = 2.17, $p = 0.03$), with lower values for parasitic species than would otherwise be predicted for their phylogenetic position ($n = 5$ of 36). Additionally, there was a significant effect of eggshell thickness ($z = 2.35$, $p = 0.01$) and its interaction with parasitic status ($z = 2.09$, $p = 0.03$), with a negative correlation between G_{H_2O} and shell thickness for non-parasites but not for parasitic species. Shell thickness, followed by wet parent, contributed to the most models in the retained subset (five and four, respectively).

4. Discussion

Consistent with findings for common cuckoos [33], the eggs of brood parasite species in the present study generally had lower G_{H_2O} than expected for their size and phylogenetic position. All species of brood parasites had lower G_{H_2O} than their hosts, with the exception of brown-headed cowbirds, whose mean G_{H_2O} was higher than the host species with which it was compared. However, brown-headed cowbirds are extreme generalists [1,2] that parasitize a large variety of host species, and as such the single host species for which we had G_{H_2O} values to compare (American yellow warblers, *Setophaga petechia*) may not have been representative of other frequent hosts. As such, we are not able to conclusively determine whether brown-headed cowbirds are an exception to the pattern seen for other brood parasites.

This commonality among brood parasites is striking given their geographical spread, distant relatedness and the diversity of nesting environments of the hosts they exploit. It raises the question of whether reduced G_{H_2O} serves an adaptive purpose for embryo development in parasitic species, regardless of their hosts and nest habitat. The magnitude of the difference between hosts and parasites in G_{H_2O} was notably greater for egg fragments than whole-egg analyses. This is likely due to the eggshell fragments coming primarily from the equator region of the shell, which may exacerbate the difference between hosts and parasites [40].

(a) A lack of fine tuning of brood parasite G_{H_2O} to their hosts' nest environment?

Brood parasites all shared lower than expected eggshell G_{H_2O} despite their eggs developing in a variety of different nest types that their hosts inhabit. This lack of adaptation to the nest environment is contradictory to numerous empirical studies demonstrating that G_{H_2O} is finely tuned to the nest environment to ensure optimal water loss (see [44] and references therein). How then are parasitic eggs able to develop successfully under the nest conditions of their respective hosts? Traditionally, species that deviate from the expected allometric relationships between egg weight and G_{H_2O} [26] are what have been considered as 'extreme nesters', that is, species that nest in extremely damp or dry conditions, or in sites with extremely abnormal O_2 and CO_2 concentrations. Examples include black-necked grebes (*Podiceps nigricollis*), whose eggs are often partially submerged in water or covered in rotting vegetation during incubation [65,66]. Similarly, eggs that are typically found in environments with very low humidity (e.g. deserts) or high barometric pressure (e.g. montane environments) have reduced G_{H_2O} to minimize water loss through the shell [67,68]. The eggs of these 'extreme nesters' typically exhibit an increase in G_{H_2O} to ensure that the optimal amount of water is lost during the incubation period, despite these extreme conditions.

Eggshell G_{H_2O} is largely considered to be species-specific [26], and proposed to be genetically controlled [69]. Among brood parasites, there is little variation within species G_{H_2O} ([33], present study), suggesting G_{H_2O} is not fine-tuned to a specific host.

This is particularly interesting as cuckoos of different gentes differ according to their host with respect to other genetically controlled traits such as eggshell colour, pattern

and thickness [4,70]. Future studies might specifically compare G_{H_2O} of different cuckoo gentes, or of other brood parasites comprising multiple lineages of host specialists, such as honeyguides [71].

(b) What is the adaptive significance of reduced water vapour conductance across the eggshell in brood parasites?

The seven species of avian brood parasites studied here belong to four phylogenetically distinct groups that last shared a common ancestor approximately 79 Ma [72], suggesting that their shared physiological trait of a low G_{H_2O} is not due to common ancestry, but rather arises from the selection pressures of a parasitic lifestyle. However, it is not clear exactly what the adaptive advantage of a reduced G_{H_2O} is to brood parasites.

One possible adaptive explanation for low G_{H_2O} in parasites is that it confers benefits to the development of the cardiovascular system of nestlings, helping them to break out from their thicker eggshells and to eject or outcompete host nest-mates. Studying domestic turkeys (*Meleagris gallopavo*), Christensen *et al*. [73] established that eggs with lower conductance of H_2O, O_2 and CO_2 experienced reduced heart rates and improved embryo survival, compared with eggs with higher conductance (both relative to a mean species level G_{H_2O}). Given the increased effort required for most species of brood parasite to hatch from an egg of greater structural strength [12], a reduction in cardiac function towards the end of incubation in brood parasites would be detrimental, and likely lead to an increase in embryo mortality. Furthermore, embryos from eggs with low conductance were able to pump more oxygenated blood to growing tissues in one heartbeat than were embryos from eggs with high conductance, whose hearts were beating at a high frequency but with a smaller stroke volume. This scenario is akin to athletes who have lower resting heart rates yet pump more blood per heartbeat (e.g. [74]). Taken together, this may suggest that the embryos of avian brood parasites could be of higher aerobic fitness than those of their hosts.

Intense investigations into the relationships between eggshell conductance (G_{H_2O}, O_2 and CO_2), growth parameters and other physiological correlates such as heart rate have only been conducted under artificial conditions with domesticated species [73,75]. In addition, results are contradictory, with an earlier study showing no such relationship [76]. Therefore, measuring heart rate continuously through the incubation process of both parasites and hosts would provide further insight into how the two competing species differ in their physiological development, and whether a low heart rate is synonymous with a low G_{H_2O}. Such experiments could be coupled with non-destructive body composition scanning techniques to track the development of the heart and other vital organs. This would establish whether brood parasites develop a larger than predicted heart mass for their body size, which may provide more oxygenated blood per heartbeat in embryos of parasites in comparison with that of their hosts. Interestingly, we did not detect a statistically significant difference in G_{H_2O} between brood-parasitic species that could be considered highly virulent (that is, evict and/or kill the young of the host) versus less virulent (that is, outcompete host young, not necessarily fatally; see tables 1 and 2; figure 4), suggesting that reduced G_{H_2O} is not an adaptation for life after hatching. However, the small sample size means this comparison should be interpreted with caution. A stronger test of whether G_{H_2O} is specifically adapted to the energetic demands of being a highly virulent brood parasite would be enlightening.

(c) Trade-off between shell hardness and G_{H_2O}

The hypothesis above proposes an adaptive explanation for the surprisingly low G_{H_2O} of brood-parasitic eggshells. However, depending on the mechanisms underlying variation in G_{H_2O}, a brood-parasitic lifestyle may also impose constraints on G_{H_2O}, even if a low G_{H_2O} is itself not adaptive. The need for brood parasites to maintain hard eggshells might impose a strong constraint on G_{H_2O}, which could partially explain why it is surprisingly low: if high G_{H_2O} requires either a thinner shell or more numerous pore openings on the outer surface of the eggshell, and if this affects the structural integrity of the shell, then brood parasites may not be able to afford high G_{H_2O} even were it adaptive for other reasons. This hypothesis could be readily testable using a combination of biomechanical and physiological tests on eggshells.

(d) Is G_{H_2O} measured on fresh eggs representative of conductance throughout incubation?

Most eggs are collected shortly after laying, as blowing eggs becomes more difficult when substantial embryo development begins. As such, G_{H_2O} measurements are generally representative of G_{H_2O} at the onset of incubation. However, G_{H_2O} may not be consistent throughout development. Two possible mechanisms could generate changes in G_{H_2O} as incubation proceeds; we will consider each in turn.

First, G_{H_2O} may increase during incubation as eggshell thickness decreases. The eggshells of avian brood parasites are thicker than those of their hosts, and those of their closest non-parasitic relatives [4,77]. While thinning of the eggshell over incubation occurs in all bird species, it has been proposed that brood-parasitic eggshells (focused mainly on cuckoos) should undergo more dramatic thinning, and hence experience more substantial increases in G_{H_2O} during later development [78]. If so, then G_{H_2O} measured in freshly laid eggs is not necessarily representative of the incubation period as a whole [44], as differences between parasites and hosts may change further along the course of development. If more dramatic thinning of the eggshell over incubation is a general property brood parasites, then brood-parasitic embryos may have access to more calcium from the shell during the incubation period. This could allow the development of stronger bones and muscles that should assist in hatching from a thicker shell, and in ejecting/killing host chicks and eggs. If this hypothesis is correct, G_{H_2O} should increase more rapidly as shells thin during development, potentially supporting the more rapid development of the parasite. However, the precise relationship between eggshell thickness and G_{H_2O} is unclear, and recent studies suggest it is likely to be more complex than G_{H_2O} simply increasing when an eggshell thins. Moreover, it is unclear whether parasitic eggshells do thin more rapidly: Igic *et al*. [78] established

that the degree of eggshell thinning experienced by common cuckoo eggs was similar to that of their hosts.

Second, G_{H_2O} may also change over incubation if eggshell pore structure changes. For example, the erosion of calcitic crystals during incubation shortens the pathway for gas diffusion across the eggshell in malleefowl (*Leipoa ocellata*), by increasing pore diameter and reducing pore length [46]. Any such changes in pore geometry may trade-off against the continued requirements for structural hardness, as discussed above [78]. This trade-off may be exacerbated in common cuckoos, which have furcated eggshell pores that might open up into more pathways for diffusion as the inner mammillary layer erodes, potentially at the cost of weakening the shell's structural integrity [41].

5. Conclusion

We found that brood parasites have lower G_{H_2O} than their phylogenetic position and egg size would predict. Moreover, other than brown-headed cowbirds, all had lower G_{H_2O} than their host species, despite experiencing identical nesting conditions. The adaptive significance of this remains unclear. We suggest that it may allow parasite nestlings to develop stronger cardiovascular systems and make them better competitors; however, it may also be partially explained by a non-adaptive physical constraint for brood parasites to produce structurally hard eggs. These findings highlight some of the gaps in our knowledge regarding the important period of *in ovo* development for brood parasites. While the behavioural adaptations of brood parasites during the nestling and adult stages of their life have received much attention, there has been relatively little investigation into how their embryonic development may be fine-tuned to a parasitic lifestyle. Parasitic eggs may be under potentially competing selective demands to develop quickly and successfully in a wide range of nesting habitats, temperatures and humidity, while also retaining structural strength, and producing highly competitive chicks able to kill or outcompete their nest-mates. This highlights the potential for conflicting selection on embryo physiology driven by environmental conditions, such as nesting habitat, and the requirements of a brood-parasitic lifestyle. This is potentially an example of how the selective demands of the coevolutionary arms-race between hosts and parasites may drive a trait in a direction counter to what would otherwise be optimal under certain environmental conditions.

Ethics. All eggs were collected under permission from the Zambia Wildlife Authority.

Data accessibility. The authors declare that the data supporting the findings of this study are available from the corresponding author on request, and as part of the electronic supplementary material.

Authors' contributions. Conceptualization: S.J.P.; methodology: S.C.M., C.N.S. and S.J.P.; resources: C.N.S. and S.J.P.; sample collection: G.A.J. and C.N.S.; data collection: S.C.M., K.W., L.C. and S.J.P.; formal analysis: S.C.M.; writing—original draft: S.C.M. and S.J.P.; writing, reviewing and editing: S.C.M., G.A.J., K.W., L.C., C.N.S. and S.J.P.

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