Evolution of embryonic development and egg physiology in avian brood parasites

Thesis submitted for the degree of

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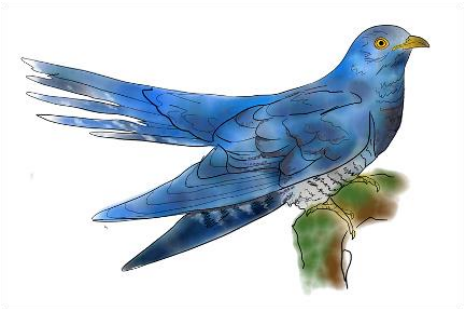

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Obligatory inspirational quote:

"All my life I have lived and behaved very much like the sandpiper - just running down the edges of different countries and continents, looking for something."- Elizabeth Bishop

Table of content

List of tables and figures

with an asterisk were collected in Zambia, but were not hosts of parasites included in this study.

Figure 2.1 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. 2006.

Table 2.2 Model support table (AICc) for the top-ranked PGLS models (model weight >0.05) of G_{H2O} which contribute to the average models. Estimates for parameters are provided to indicate inclusion in respective models. Model weight are estimate across entire set of 32 models and sum to 1. (a) displays top models for whole egg analysis, and (b) top models for shell fragment analysis.

Figure 2.2 Non-mass corrected mean G_{H2O} (mg day^{-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 in G_{H2O} both cases (*t*-test; A: $t_{13.9} = 2.39$, $p = 0.03$, B: $t_{7.8} = 3.98$, $p = 0.004$).

Figure 2.3. Top: G_{H20} (mg day^{-1} torr^{-1}) corrected by egg weight (g) for whole eggs. Brood parasites and respective host species connected by coloured lines. Note: Average G_{H20} of several hosts (n=5) of the common cuckoo is presented in the host category. Bottom: G_{H20} (mg day^{-1} torr^{-1}) corrected by egg weight (g) for shell fragments. Brood parasites and respective hosts are linked by coloured lines. Note: No G_{H2O} 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 cuckoos in the top panel.

Figure 2.4 (A) Mean G_{H20} (mg day^{-1} torr^{-1}) corrected by egg weight (g) for eggshell fragments. Three hosts species compared to with low virulence brood parasites and three high virulence brood parasites. (B) Mean G_{H20} (mg day^{-1} torr^{-1}) corrected by egg weight(g) for whole eggs. Five host species compared with two low virulence brood parasites and three high virulence brood parasites. There was no significant difference was between high virulence and low virulent species in either case (Whole eggs: $t_{2.38} = 2.70$; $p = 0.09$, Eggshell fragment: $t_{1.56} = 1.82$; $p = 0.31$).

Figure 3.1 Egg candling of a common waxbill (*Estrilda astrild*) egg. The size and shape and level of development of the embryo can be seen by shining light through the egg.

Table 3.1 Mean rate of embryo movement (EMR) per minute and standard errors at each incubation stage (1–5), for parasitic species and their hosts. Parasites are in red. Designation of high virulence or low virulence of parasite species based on Kilner 2005.

Figure 3.2 Phylogenetic tree showing the species in the phylogenetically informed mixed model. Species in red are brood parasites. Symbol shapes match brood parasites to species they parasitise. Constructed from the "Tree of life database" using the R package 'rotl' (Michonneau et al. 2016). Branch-lengths set at 1.

Figure 3.3 Rate of embryo movement over the course of incubation for all parasitic species (red) and all non-parasitic (black) species combined. Shading indicates standard error.

Figure 3.4 Rate of embryo movement over the course of incubation of common cuckoos and their hosts (great reed warbler) and relatives, white-browed coucals and African black coucals. Shading indicates standard error.

Figure 3.5 Non-significant difference between high virulence brood parasite species and low virulence species. High virulence species (that actively kill host young) comprised common cuckoos, and greater and lesser honeyguides. Low virulence species comprised brown-headed cowbirds and pin-tailed whydahs. Shading indicates standard error.

Table 4.1 Hypotheses and predictions with supporting rationale, of how eggshell calcium carbonate content in birds relates to life history strategies and eggshell characteristics. Hypothesis are divided based on Tinbergen's four question structure.

Figure 4.1 Phylogenetic tree of mean eggshell calcium carbonate content (ash % of dry eggshell mass) of species eggs**.** Phylogenetic tree of all included species (n=222) generated from the open tree of life (Michonneau et al., 2016). Branch colour represents ancestral reconstruction of eggshell calcium content (log Arcsine of eggshell calcium %) with green representing higher calcium carbonate content and orange representing a lower content. Purple

bars display log eggshell thickness (mm) of each species. Inset graph: calcium carbonate content (ash % of dry eggshell mass) predicted by (log) eggshell thickness.

Figure 4.2 Mean carbonate calcium content (ash % of dry eggshell mass) of species eggs in relation to lifespan and clutch size (eggs/nest). Mean eggshell calcium carbonate content of 222 species (Log – Arcsine transformed) calculated from 817 eggs, showing ash % decreases with increasing lifespan in species with large sized clutches, but not species with small clutches (t = 3.13, p = 0.002). The regression lines are representative of linear regression, not corrected for phylogenetic relatedness.

Figure 5.1 Profilometry 2D image of surface roughness of a background region of a cuckoo-finch (*Anomalospiza imberbis)* egg, showing higher surface points as light yellow and deeper points as darker brown. Image generated by SPIP software (Image Metrology, Denmark). Mean surface roughness (S_a) is shown along the top of the image.

Figure 5.2 Sessile water droplet on the surface of a chestnut-winged cuckoo (*Clamator coromandus*) eggshell showing left and right water droplet contact angles. The green line illustrated the extrapolated curve of the surface and the blue lines illustrate the curve of the droplet. Image generated by Advance software (Krüss, Germany).

Figure 5.3 Phylogenetic tree generated from the online tree of life (Michonneau et al., 2016), representing all species sampled for eggshell roughness. All other measurements and analysis were performed on a subset of these species. Species labels in red text represent obligate brood parasites.

Figure 5.4 a) Surface roughness (S_a nm) of the eggshells of avian brood parasites (left, blue) and of non-parasite species (right, red). b) Mean surface contact angle of water droplet (CA) on the eggshell of avian brood parasites (left, blue) and of non-parasite species (right, red). Red dotted line at 90° demonstrates threshold between hydrophobic values above and hydrophilic values below. No significant difference was determined between parasite and non-parasite eggs for either trait (surface roughness: t = 0.09, p = 0.26; hydrophobicity: t = 0.59, p = 0.79). Boxplots show interquartile range and median value as a line, with whiskers encompassing values within to 1.5 times the interquartile value. *Distribution of data is shown in scatterplots (overlaid) and frequency plots (alongside each pairing).*

Figure 5.5 Association between surface roughness and mean contact angle in either parasites (orange) or non-parasite (green). R^2 for parasite eggs was -0.07, and R^2 for non-parasite eggs was -0.003.

Figure 5.6 Differences in surface roughness (S_a nm) between maculated and non-maculated eggshell regions of the eggs of cuckoo finches and their hosts, tawny-flanked prinias. "**" signifies a significant difference with a p value < 0.005. "n.s." signifies no significant differences between groups. Boxplots show interquartile range and median value as a line, with whiskers encompassing values within to 1.5 times the interquartile value. Distribution of data is shown in scatterplots (overlaid) and frequency plots (alongside each pairing).

Figure 5.7 Differences in hydrophobicity (mean contact angle $CA°_m$) between brood parasites and their hosts (right, blue) were not different from differences between brood parasites and a randomly allocated non-host egg (left, red) (t_{372} = 0.219, p = 0.827). Boxplots show interquartile range and median value as a line, with whiskers encompassing values within to 1.5 times the interquartile value.

Figure 5.8 A) Calcium carbonate content of eggshell of brood parasite species compared to that of non-parasite species. Boxplots illustrate the group mean and interquartile range, and whiskers cover 1.5 times the interquartile range. Species of each measurement are differentiated by colour on the scatterplot. B) Calcium carbonate range for each species sampled, differentiated between parasites and non-parasites.

Table 6.1 List of species and numbers of eggs recorded for this study. Virulence definition based on descriptions in (Kilner, 2005). Mean incubation lengths per species extracted from the Handbook of birds of the world (Del Hoyo, J.; Elliot, S.A. & Sargatal, 1992).

Figure 6.1 Phylogenetic tree of parasite and non-parasitic species included in this study. High virulence parasitic species are in red, low virulence in light blue and non-parasites in dark blue. Tree generated from the online tree of life using the R package 'rotl' (Michonneau et al., 2016). Maximum branch length set to 1.

Figure 6.2 Metabolic rate corrected for egg mass (CO_2 per gram of egg, ml/min) of parasitic and non-parasitic species. Parasitic species are split between high virulence and low virulence based on Kilner (2005), see Table 6.1. High virulence brood parasites differ significantly from both other groups.

Figure 6.3 Metabolic rate at discrete incubation stages (see Methods) of parasitic and non-parasitic species. Metabolic rate is recorded as CO_2 production and corrected for mass (CO_2 per gram, ml/min). Parasitic species are split between high virulence and low virulence based on Kilner (2005). High virulence brood parasites differ significantly from both other groups at stage 4, and additionally, differ from non-parasitic species at stage 3.

*Supplementary and appendices figures and tables are not listed.

Contribution of authors

I, Stephanie C McClelland, declare all work in this thesis to be my own, with the exception of collaborator contributions detailed below.

Chapter 2

Convergent evolution of reduced eggshell conductance in avian brood parasites.

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Conceptualisation by S.J.P. and **S.C.M**; methodology conceived by **S.C.M,** C.N.S, S.J.P; resources provided by C.N.S. and S.J.P, samples provided by G.A.J. and C.N.S. and S.J.P; data was collected by **S.C.M,** K.W, L.C. and S.J.P.; formal analysis by **S.C.M**; The original draft of the paper was written by **S.C.M**, with contribution and edits by all authors. All authors provided intellectual input, read, and approved the final manuscript.

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Chapter 3

More frequent embryonic movement during development in avian brood parasites: Preparation for the demands of early parasitic life?

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Chapter 4

How much calcium to shell out? Life history strategy influences eggshell calcium content across avian families.

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Conceptualisation by S.J.P. and P.C.; data were collected by S.J.P. and G.M; Collection of life history traits by **S.C.M**; data analysis by **S.C.M**; the original draft of the paper was written by **S.C.M**, with contribution and edits by all authors. All authors provided intellectual input, read, and approved the final manuscript.

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Chapter 5

Eggshell composition and surface properties in avian brood parasites compared to non-parasitic species

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Chapter 6

Patterns of embryo metabolic rate in avian brood parasites: Highly virulent brood parasites exhibit a defined 'plateau' stage similar to precocial birds.

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CHAPTER 1. **General Introduction**

For centuries biologists have been fascinated by the co-evolutionary dynamics of brood parasites and their hosts. Brood parasites are species that lay their eggs in the nest of another (host) species, thereby forgoing the cost of raising their own young (Davies and Quinn, 2000; Soler, 2017). Research has focused on the coevolutionary arms race between parasites and their hosts, and the impressive range of tricks and mimicry employed by brood parasites to convince their unwitting host to adopt their offspring (Langmore et al., 2003; Feeney et al., 2014; Soler, 2014). Yet despite decades of study, new aspects of these interspecies relationships are still coming to light and many features of their evolution are still unknown.

While much of the focus of avian brood parasitic research has focused on egg mimicry, much less research has delved into the topic of the physiological adaptions of embryonic and hatchling brood parasites – essentially, what is going on inside the shell. This life stage is somewhat over-looked in comparison to the adaptions and behaviour of parent brood parasites, yet is equally, if not more, important for the success of the parasitism (Grim, 2007; Honza et al., 2015; Igic et al., 2015b). Even though they are far from being a monophyletic group (brood parasitism has evolved at least seven times (Krüger and Pauli, 2017), many obligate brood parasites have converged on a number of physiological mechanisms in egg development that support the success of their offspring in the nest of the host (Croston and Hauber, 2010; Hamilton and Orians, 1965; Spottiswoode, 2010). Among these are early hatching (Birkhead et al., 2011; Portugal et al., 2014), thicker eggshells (Krüger, 2007; Antonov et al., 2012), and stronger and more developed muscles for hatching stage (Honza et al., 2015). Each of these bestows the offspring with an advantage that allows it to better exploit their foster parents. During this project I explore the mechanisms behind these adaptions.

Background to Brood parasitism

Brood parasitism can be either facultative, where the female may also raise a clutch of her own young, or obligate, where the female completely foregoes nest building and parental care (Stevens, 2013). Brood parasites can also be split between those who parasitize others of their own species (intraspecific parasitism) or birds of another species (interspecific parasitism) (Croston and Hauber, 2010; Davies, 2011). This thesis deals primarily with interspecific, obligate brood parasites, where adaptions for a parasitic lifestyle are more pronounced.

Although brood parasitism is not exclusive to birds (it is found in insects and fish), they are certainly the most prevalent and well known (Sato, 1986; Zink, 2000; Soler, 2017). It is seen is many separate clades of birds across almost all continents, including the Old World cuckoos (*Cuculinae),* New World cuckoos (*Neomorphinae),* the American cowbirds (*Icteridae)* and Black-headed ducks *(Anatidae*), the African honeyguides (*Indicatoridae*) and the indigobirds and their allies (*Ploceidae*) (Croston, 2010). Approximately 1% of all birds are known to be obligate brood parasites (Krüger and Pauli, 2017), and facultative parasitism is significantly more common (Petrie and Møller, 1991; Zink, 2000). Of these, the cuckoos and the cowbirds have received the most attention in both the popular and scientific literature.

The benefits of brood parasitism are quite clear. By avoiding the cost of raising their own young, parasites can invest more of their energy reserves in laying a greater number of eggs. Brood parasites are extremely prolific layers; common cuckoo (*Cuculus canorus*) females lay, on average, eight eggs per season, but will often exceed 15 (Davies and McCallum, 2015). Even more extreme are brown-headed cowbirds (*Molothrus ater*) that can lay over to 40 eggs per season (Davies and Quinn, 2000; Reetz, 2008) and the record from a single captive female is 77 eggs (Holford and Roby, 1993). In comparison, a similar sized species with parental care might be expected to raise at most two clutches of four eggs (Böhning-Gaese et al., 2000). Additionally, avoiding parental care provides other advantages for brood parasites such as avoiding the cost of nest building and allowing them to migrate earlier (Davies and Quinn, 2000; Payne, 2005). Of course, the benefits to the brood parasite entail costs to their host, either a total loss of their breeding success for the season (in species that evict or kill host offspring), or a reduction in the fitness of their offspring (when they fail to compete with the brood parasite chick) (Hauber, 2003; Scharf et al., 2021). In addition, energetic costs of raising a demanding parasite chick may influence the chance of survival of the adult hosts to the next breeding season (Payne and Payne, 1998), however a recent studies have suggested no survival costs to

raising a parasite compared to raising the hosts own young (Samaš et al., 2018, 2019). Regardless, there is strong selection both on host species to recognize and reject brood parasite eggs, and for brood parasites to produce eggs that are less recognizable, resulting in an on-going arms race between species.

This arms race has resulted in amazing adaptions in egg appearance by both hosts and parasites. Many brood parasites have evolved to produce eggs that mimic the shape and colour of their host to such a degree that they are indistinguishable from host eggs to the human eye (Langmore and Spottiswoode, 2013; Attard et al., 2017; Stoddard and Hauber, 2017) (Figure 1.1). For example, females common cuckoos are separated into different gentes (subgroups that parasitise a specific host species), each which produces eggs of an appearance to match that of their host (Davies and McCallum, 2015). Interestingly, male common cuckoos are believed to mate with females of multiple gentes, and hence father offspring raised by different hosts. Many generalist parasites there is no geographical isolation to prevent gene flow between the host-gentes of common cuckoos (Fossøy et al., 2011). This presents the question of how gentes maintain distinct egg appearance despite genetic exchange. One likely solution is that as the heterogametic sex, females could carry the genes for egg patterning on the female-specific W chromosome which it passes on to its daughter, creating a maternal lineage that share a particular host mimetic egg pattern (Gibbs et al., 2000). However, Fossøy et al. 2011 identified biparental inherited markers suggesting paternity is also important for maintaining genetic differentiation between cuckoo gentes.

Figure 1.1 Mimetic cuckoo finch eggs (outside ring) and eggs of their host, tawny-flank prinias (inner ring). Photo adapted from Spottiswoode and Stevens 2012.

In response to egg mimicry by brood parasites, many hosts have evolved a greater degree of inter-clutch variation and/or a smaller degree of intra-clutch variation making accurate mimicry more difficult (Stokke et al., 2002; Krüger, 2007; Caves et al., 2021). Some host species have higher rates of rejection of parasites, such as the Australian superb fairy-wrens (*Malurus cyaneus*) that reject 40% of Horsfield-bronze cuckoo (*Chrysococcyx basalis*) chicks (Langmore et al., 2003), whereas others such as dunnocks (*Prunella modularis*), a host to the European common cuckoo, will rarely recognize and reject foreign eggs (Davies and McCallum, 2015). Rejection can also be costly to the host, both in the energy expended in recognising and evicting parasitic eggs but also the risk of damaging or mistakenly removing the hosts' own eggs (Davies and McCallum, 2015; Ruiz-Raya et al., 2015). Reed warblers have been shown to evict the wrong egg in up to 30% of cases due to the effective mimicry of common cuckoo eggs (Davies and Quinn, 2000). In some cases acceptance of parasitic eggs is a better strategy for hosts than rejection. Brown-headed cowbirds have been shown to use retaliatory "mafia behaviour" against hosts that reject their eggs by destroying the nest and remaining host eggs (Hoover and Robinson, 2007), which stimulated the host to renest and so provides another opportunity for the cowbird to parasitise them (Soler et al., 2017). As cowbird nestlings do not evict the host offspring, in such cases hosts may have greater success raising the cowbird alongside its own young than risk losing the whole clutch.

Egg size is another trait that appears to be under selection by the brood parasitic lifestyle. Brood parasites frequently parasitize species which are much smaller than themselves in body and egg size, to which they need to match their eggs (Krüger and Davies, 2004; Spottiswoode, 2013), as size can also be used as a cue for recognition by hosts. Some brood parasites achieve this through a reduction in adult body size (body size and egg size being strongly allometrically linked) such as the cuckoos of the *Chrysococcyx* genus, while others produce a much smaller egg that would be expected for their body size (Krüger and Davies, 2004). Small egg size also introduces a constraint on the energy contents of the egg and the hatching mass of nestlings, which is a significant price to pay considering the energetically demanding activity of hatchling brood parasites (Hargitai et al., 2010; Igic et al., 2015b).

Most brood parasites hatch earlier than the eggs of their host species (Birkhead et al., 2011). Common cuckoo eggs need approximately 11 days of incubation, compared to 12-13 for their hosts (Davies and McCallum, 2015). One theory for how this is achieved is internal incubation. Birkhead et al. (2011) showed that several species of brood parasites retain the egg in the

oviduct for an additional 24-hours after it is fully formed, giving the embryo a head-start on development. By dissecting eggs shortly after laying and before incubation by the host, the authors found that the developmental state of the brood parasites embryo was approximately 31 hours more advanced than would be expected without internal incubation. The reason this estimate is greater than the 24-hours the egg is retained is that body temperature (internal incubation) is higher than external incubation. Another factor that may facilitate early hatching could be the reduced egg size of brood parasites. Smaller eggs may contain less resources which are quickly depleted by the growing embryo forcing it to hatch early (Kattan, 1995; Igic et al., 2015b). A recent suggestion has been that the thicker eggshells of brood parasites means that they allow the egg to retain heat longer when the host is away from the nest, and hence development proceeds at a faster rate during incubation disturbance (Yang et al., 2018).

Hatching earlier that host eggs is advantageous for the parasitic nestling, both those that evict their hosts eggs, and those that must compete with the host brood. Many species of brood parasites, upon hatching, will proceed to remove any potential competitors from the nest. The most well studied is the behaviour of cuckoo nestlings, who within a day of hatching, will locate any other eggs and chicks in the nest and use their back and shoulders to lift them over the edge and out of the nest (Krüger and Davies, 2002; Anderson et al., 2009; Honza et al., 2007) (Figure 1.2). The cuckoo chick will often have emptied the nest within 24-hours of hatching (Davies and Quinn, 2000). This requires an extraordinary strength for a newly hatched young, as the egg and chicks may weigh close to the nestlings own body mass (Honza et al., 2007). This is energetically expensive for the nestling, but the costs have been found to be recoverable in terms of nestling growth (Anderson et al., 2009; Hargitai et al., 2012), even when the number of host eggs to be evicted in experimentally increased (Medina et al., 2019). However, despite compensatory growth, evicting cuckoo chicks pay a cost in later fledging (Grim et al., 2009a) and higher oxidative stress (Hargitai et al., 2012).

Other parasites have a more vicious method of removing competition. Nestling honeyguides, as well as striped cuckoos (*Tapera naevia*), are born with sharp hook-like protrusions on their upper and lower jaws (Morton and Farabaugh, 1979; Spottiswoode and Koorevaar, 2012). The freshly hatched honeyguide chicks use these protrusions to stab and puncture any other young sharing their nest, often grasping shaking the young for several minutes (Spottiswoode and Koorevaar, 2012). Like with cuckoos, this is energetically expensive behaviour for a new

hatchling, but provides the substantial benefit of them monopolising the parental resources (Spottiswoode and Colebrook-Robjent, 2007).

For both chick-killing and nest-mate tolerant brood parasites an additional challenge of their reproductive strategy may be hatching from an unusually strong eggshell. Many brood parasite species lay eggs with shells that are thicker than expected for the size of the egg. However, this increased the difficulty for the chick to hatch from the egg if the internal breaking strength of the shell is likewise increase. Evidence of this is seen in common cuckoos, which have an enlarged hatching muscle (Honza et al., 2015) and require a greater number of pecks to pip from the egg (Honza et al., 2001). Likewise in brown-headed cowbirds, the duration of hatching activity was longer compared to a non-parasitic icterid host (Yoon, 2013). Whether, or how, the embryo development of brood parasites differs to enable this hatching behaviour has not been investigated.

This raises the question of how brood parasites acquire the strength and energy required to perform both this strenuous hatching and the difficult physical tasks of early parasite life. Hatchling birds are often described in terms of the precocial-altricial spectrum (Starck and Ricklefs, 1998). This scale describes the level of development and independence an animal has at birth. Brood parasites (barring the black-headed duck (*Heteronetta atricapilla*), fall on the altricial end of the scale (Yom-Tov and Geffen, 2006; Krüger, 2007). Altricial nestlings hatch in a relatively early state of development, whereby they are blind, unable to thermoregulate, have little coordination of movement, and are entirely reliant on parental care for nutrition and protection (Starck, 1993). Conversely, precocial offspring are born already quite advanced in their development, sighted, mobile, often already with a layer of down and generally able to leave the nest and forage shortly after hatching (Starck, 1993; Starck and Ricklefs, 1998). However, while brood parasites young appear altricial in most aspects, the eviction behaviour suggests a level of muscular strength and coordination that is more akin to precocial species. This suggests that the in-ovo development of brood parasites differ from other species in key aspects that are yet to be discovered.

This project explores the mechanisms and traits of eggs structure and embryonic development in brood parasites that may facilitate their life history and compares these to that of their non-parasitic relatives and hosts.

Figure 1.2 Common cuckoo chicks evicting eggs and nestlings of hosts (great reed warblers *Acrocephalus arundinaceus*). Image credit: Anderson et al. 2009.

Brood parasite trait evolution

The phylogenetic range over which brood parasitism occurs is quite extensive. The approximately 1% of bird species that exhibit obligate brood parasitism includes 57 cuckoos, 20 species of finch, 17 honeyguides and a single species of duck (Davies and Quinn, 2000). Studies of comparative physiology need to take into account the phylogenetic relationship of the species being compared, as they cannot be considered independently from each other as ancestry will shape the range of traits that can and have evolve (Garland et al., 2005). As such we need to consider the evolutionary history of different clades in order to recognise any physiological adaptions of brood parasitism.

A number of studies have constructed evolutionary models in order to determine the behavioural, ecological and physiological factors that have encouraged or resulted from the evolution of brood parasitism. For example, Krüger *et al.* (2002) modelled at the evolution of brood parasitism across 136 species of cuckoos in relation to ecological parameters and egg size. They determined that the evolution of brood parasitism was preceded by changes in migration behaviour, a switch to more open habitat types, and an expansion in the size of breeding range. However, their model indicated that the reduction in egg size came about after the switch from parental care to parasitism. They suggest that parasitizing small-bodied hosts has selected for smaller egg size both to increase likelihood of acceptance and improve the effectiveness of host incubation. Similarly Mermoz and Ornelas 2004 compared traits of parasitic cowbirds using phylogenetically independent contrasts across the Icteridae family. They considered changes in egg size, energy reserves of the eggs, shell thickness and nestling growth rate over evolutionary time, and they determined only eggshell thickness to be significantly related to brood

parasitism. They suggest, however, that increased eggshell thickness arose in a non-parasitic ancestor originally and preadapted this lineage to parasitism, rather than coming about as a result of the reproductive strategy. However, Spottiswoode's (2010) findings that eggshells are thicker in cuckoo gentes which parasitize more discriminating hosts might suggests that in these species eggshell strength has evolved as a result of host rejection as a selective pressure after the advent of brood parasitism.

In many cases it can be difficult to disentangle the effect of shared ancestry from convergence between parasites and hosts selected for through coevolution. In the case of Viduidae finches, the close phylogenetic relationship between vidua finches and their estrildids hosts makes it difficult to determine whether mimetic gape pattern is inherited from a shared ancestor, or has evolved and been selected for after the establishment of brood parasitism (Sorenson and Payne, 2001). While commonality of ancestry likely aided in the success of early parasitizing of related hosts, examples from other brood parasites that target distantly related hosts provide evidence that effective mimetic traits, such as egg patterning, can also arise as a result of co-evolutionary selection with hosts (Sorenson and Payne, 2001; Soler, 2014).

Study species

Part of what will lend this project a unique perspective is the phylogenetic distribution of study species. This research covers species within four avian lineages which are believed to have evolved brood parasitism independently of each other (Yom-Tov and Geffen, 2006). The main parasite-host species (those appearing in all chapters) included in this study are discussed below. For further details of field sites and breeding biology of these species see **Appendix item 1**. The eggs of other parasite and host species were included in **Chapter 2,** and **5**, and are detailed in the relevant sections.

1. Cuckoos (Family *Cuculidae*)

As well as being the most well know family for brood parasitism, *Cuculidae* probably has the greatest variety of parental strategies of any bird family; they are believed to have evolved brood parasitism three times independently (Krüger and Davies, 2002) (Figure 1.3). This makes them a pivotal group to study the physiology of brood parasitism.

- Common cuckoo (*Cuculus canorus*)

Common cuckoo are an evictor species of obligate brood parasite, divided into different gentes (host-specialist races, singular:"gens"), parasitizing dunnock (*Prunella modularis*), reed warblers (*Acrocephalus scirpaceus*) and meadow pipits (*Anthus pratensis*) among others (Davies and McCallum, 2015). Fieldwork for this thesis focused on a population of common cuckoos (containing both the Eurasian reed warbler gens and great reed warbler gens) near Hodonın in the south-eastern part of the Czech Republic, at a field site maintained by our collaborator Dr Marcel Honza (Academy of Sciences of the Czech Republic).

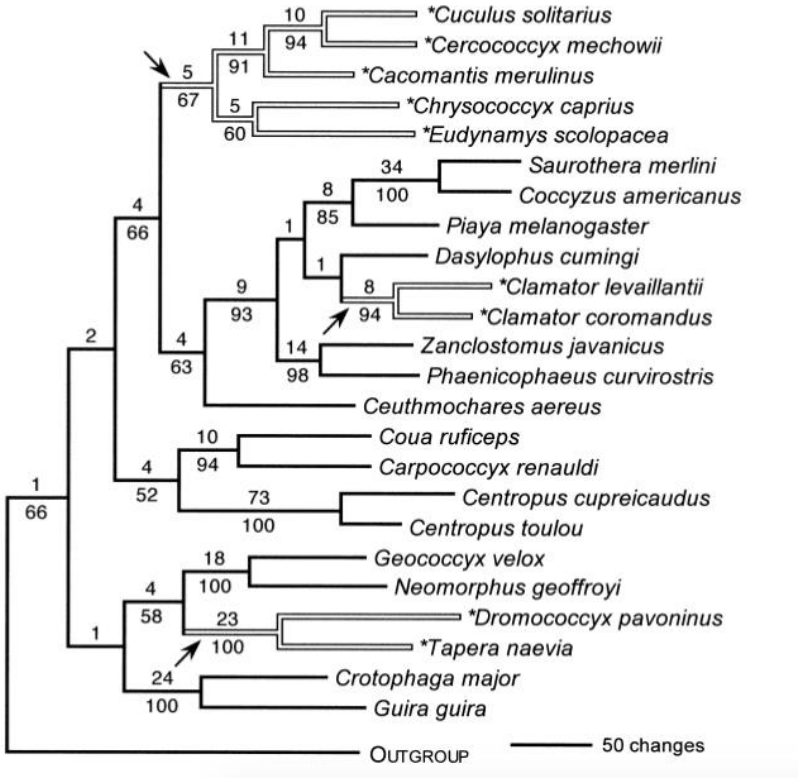

Figure 1.3. A phylogeny of a subset of cuckoo species based on mitochondrial DNA. Arrows indicate the three unique origins of brood parasitism. Asterisk and outlining of branches indicates species which are obligate brood parasites. Figure adapted from Sorenson and Payne 2002.

2. Cowbirds (Family *Icteridae*)

- Brown-headed cowbird (*Molothrus ater*)

Brown-headed cowbirds are non-evicting obligate brood parasites. They are extreme generalist in their host choices and have been known to lay eggs in the nest of over 200 species, including

occasional inappropriate hosts such as of hummingbirds (*Trochilidae*), ducks (*Anatidae*) and raptors (*Falconidae*) (Sealy, 2015), however, they most commonly target small passerines. This thesis studied a population in Southern Illinois in collaboration with Dr Mark Hauber (University of Illinois Urbana-Champaign, USA). The primary host in this population is the prothonotary warbler (*Protonotaria citrea*).

3. Honeyguides (Family *Indicatoridae*)

Honeyguides are a family of near-passerine species found in Africa. All 17 species are believed to be obligate brood parasites (Spottiswoode and Koorevaar, 2012). All honeyguides hatch with hooked beaks with which they puncture the eggs or nestlings of the host (Short and Horne, 2001; Spottiswoode and Koorevaar, 2012) (Figure 1.4).

- Greater honeyguide (*Indicator indicator*) and lesser honeyguide (*Indicator minor*)

The greater honeyguides parasitize burrow-nesting bee-eaters (*Meropidae*), whereas lesser honeyguides parasitise black-collared barbets (*Lybius torquatus*). Lesser and greater honeyguides differ somewhat in their parasitism strategy. Greater honeyguides females will puncture the eggs of the host when laying which limited the number of host offspring their chick needs to kill (Spottiswoode and Koorevaar, 2012; Spottiswoode, 2013). Whereas Lesser honeyguides do not puncture eggs, as the lay very rapidly to avoid host detection and aggression. This increases the effort the lesser honeyguide chick must expend in chick killing. Data on these species were collected in Choma region, Zambia, during the dry season (September to November) at the fieldsite of our collaborator Prof. Claire Spottiswoode, with the assistance of local field assistants.

Figure 1.4 Greater honeyguide nestling with beak hook used to kill host young. Photo by Prof. Claire Spottiswoode.

4. Parasitic finches (Family *Viduidae)*

- Pin-tailed whydah (*Vidua macroura*)
- Cuckoo finch (*Anomalospiza imberbis*)

The indigobirds and whydahs (*Vidua*), and the sister group the cuckoo finches (*Anomalospiza*), are passerine obligate brood parasites, which do not evict or kill the host offspring, however in the case of cuckoo finches will almost always starve out the host chicks. Based on mitochondrial DNA, it is believed these species shared a common ancestor approximated 20 million years ago. While the *Vidua* species all specialise on parasitising a specific species of estrildid finches (family *Estrildidae*), the cuckoo finches parasitise a number of species of old-world warblers (family *Sylviidae*). At the field site relevant to this thesis, the primary hosts of cuckoo finches are tawny-flanked prinias (*Prinia subflava*) and zitting cisticola (*Cisticola juncidis*). These species were also studied at Prof. Claire Spottiswoode's fieldsite in Zambia, however these birds breed during the wet season (November to April).

Avian embryo respiration and development

Unlike developing mammals which are supplied nutrients regularly from the mother, all the components necessary to make a new organism are contained within a bird's egg at the point of laying. The only external elements to the process after laying are heat, which is generally

acquired from an incubating parent, and oxygen, which diffuses through the shell of the egg (Mortola, 2009; Proctor and Lynch, 1993). The content of the egg is composed of the albumen, which provides water and protein to the developing embryo and acts as a shock absorber against physical damage, and the yolk, which is the main source of energy in the form of lipids, as well as other substances such as hormones and anti-oxidants (Mueller et al., 2015). The energy available to the developing embryo for both growth and maintenance is, therefore, constrained by what the mother has deposited in the egg (Igic et al., 2015). The unusually small eggs relative to adult body size of brood parasites suggest less, rather than more, reserves are potentially available (Krüger and Davies, 2004). However, the relative size of the yolk has been found by Török et al. (2004) to be larger in common cuckoos relative to their hosts, which the authors suggested provides a greater energy source for the developing embryo. Contrary to this, however, Igic et al. (2015) examined the composition of common cuckoo yolks and determined that the proportion of triacylglycerols, the main energy reserve lipid, was actually lower in cuckoos than their hosts. Whether this is a pattern across multiple species of brood parasites has not yet been explored.

The egg content at laying is only one element of the developmental process. Another component is how effectively the embryo metabolizes resources to optimize development during incubation. Metabolic processes inside the egg are driven by the exchange of metabolic gases with the environment. Oxygen (O_2) diffuses into the egg due to the partial pressure gradient created by the developing embryo's consumption, while water vapour and carbon dioxide (CO_2) diffuse out of the egg (Mueller et al., 2015; Rahn and Paganelli, 1990). A double lining of membrane develops on the inner side of the egg which connects to the growing embryo to expedite the exchange of gases when diffusion becomes insufficient for growth (Mortola, 2009). As water is lost, a pocket of air develops between these two membranes, and this air cell is eventually penetrated by the embryo (internal pipping) so that it can begin pulmonary respiration in addition to diffusion, during hatching (Menna and Mortola, 2002).

Oxygen consumption and/or carbon dioxide production can be used as a proxy for estimating the metabolic rate of the embryo (Lighton, 2008; Mortola, 2009), and given the rate of development is constrained by this exchange of metabolic gases, we can predict that rapidly developing brood parasites may have evolved mechanisms to optimize respiration. As gas exchange is a passive process, the structure of the eggshell is the most important factor determining this rate (Portugal et al., 2010; Jaeckle et al., 2012). Eggshells are perforated by a

system of pores to enable this exchange, and the porosity of eggshells is known to be negatively related to incubation length in some species (Zimmermann et al., 2007). Both common cuckoos and brown-headed cowbirds have a pore structure that is either more dense (in the former) or larger in individual pore size (in the latter) (Hargitai et al., 2010; Jaeckle et al., 2012). Jaeckle et al. (2012), suggesting that brown-headed cowbirds should have a greater rate of gas exchange than other Icteridae based on the size and number of pores observed, though this was not expressly tested. However, both of these species and other brood parasites have eggshells that are thicker than average, compared to their hosts and egg size, so a modified pore structure might be necessary to compensate for the greater diffusion distance (Hargitai et al., 2010; Antonov et al., 2012). The benefits of thicker eggshells for brood parasitism is discussed in more detail below, however, it would be expected to impede gas exchange by increasing the distance for diffusion (Jaeckle et al., 2012).

An increased number of pores does not automatically result in an increased rate of gas exchange. In particular, the shell of common cuckoos has a branching pore structure, meaning that multiple external pore opening may converge to only a single opening on the inner shell surface, and thereby not increase the overall number diffusion pathways available (Portugal et al. 2014; Board 1982). Portugal et al. (2014) measured the diffusion properties of common cuckoo eggshells by measuring water vapour conductance (G_{H20}) of museum and freshly blown eggs. They found that cuckoo eggshells had a lower G_{H20} than (1) the eggs of their host species, (2) expected for their egg size, and (3) predicted for their phylogenetic position. While this suggests cuckoos do not have an increased rate of gas exchange and may not explain early hatching in this species, it has not yet been determined in living eggs. This is important as eggs have the potential to change in their porosity with the thinning of the eggshell that occurs during development, as the embryo sequesters calcium from the inner eggshell for bone formation (Orłowski and Hałupka, 2015; Igic et al., 2017;).

One of the aims of this thesis is to explore whether there is a pattern in the metabolic rates of brood parasite embryos, by measuring gas exchange rates across a multitude of host and parasite species, over the course of incubation. The expectation being that brood parasite eggs will have increased gas exchange during at least one component of their incubation period, most likely the latter part. To determine whether this is an adaption that has arisen as a result of brood parasitism, we will be comparing brood parasites with diverse evolutionary histories from phylogenetically distant lineages, as well as contrasting them with closely related non-

parasitic relatives. In this way, we hope to be able to determine whether there has been convergent evolution of embryo respiration rates among brood parasites.

Eggshell structure

As discussed above, the eggshell is a critical component in controlling the development of the embryo. The eggshell provides mechanical protection, regulation of gas exchange and is an important source of calcium for the developing chick (Blom and Lilja, 2004; Maurer et al., 2015). The eggshell structure may also play a role in retaining heat (Yang et al., 2018b). The importance of eggshell structure for the speed and success of developing embryos means that it is under selective pressure to adapt towards an optimum structure. The eggshells of brood parasites exhibit adaptions which may improve the competitive ability of the hatchling. Most reported of these is the increased thickness of brood parasite eggs. Common cuckoos lay eggs with a shell that is approximately 2.2 times thicker than similarly sized host eggs (Honza et al., 2015). This thicker shell is not a trait exclusive to the cuckoo family; cowbirds lay eggs that are 30% thicker than expected for their egg size, and honeyguides eggs are likewise structurally stronger than their hosts (Spaw and Rohwer, 1987; Spottiswoode, 2010). The benefit of structurally stronger eggshells is still uncertain, but there are a number hypotheses in this regard. The "*laying damage hypothesis*" (Antonov et al., 2012), for example, suggest a stronger eggshell both protects the egg from damage during rapid laying (the laying time for cuckoos can be less than 10 seconds (Davies and McCallum 2015)), and potentially to damage the host eggs through collision. Great spotted cuckoo's (*Clamator glandarius*) laying behaviour is suggestive of this as it is observed to lay it's eggs at a distance from the nest, "shooting" them against the host eggs, resulting in visible damage to the host but not the cuckoos eggs (Soler and Martínez, 2000). Similarly, the "*puncture resistance hypothesis*" suggests that stronger shell make it harder for host to eject parasitic eggs (Sealy, 1996; Antonov et al., 2006). Since many hosts have beaks that are too small to grasp an egg it needs to remove, they rely on puncturing the egg. However, stronger shells make this more difficult and costly, and also increase the risk of the host damaging their own eggs (Antonov et al., 2006).

As for disadvantages, stronger eggshell requires greater effort for the chick to hatch from. It has been shown, for cowbirds at least, that their structurally stronger eggshells are equally difficult to puncture from the interior (Picman, 1997). As mentioned above, this requires a stronger hatching muscle in the chick, and a greater overall number of pecks from the chick in

order to hatch (Honza et al., 2001, 2015). While overall thickness appears to be the primary factor for the increase in strength of brood parasite eggs, Igic et al. (2011) suggests changes in the micro-structure of the eggshell as an alternative mechanism. Eggshells are made up of organic and inorganic components, the inorganic component is constructed primarily of calcium carbonate ($CaCO_3$) in the form of columnar crystals and the organic component determines the size and orientation of the developing crystals (Mortola, 2009; Igic et al., 2011). Igic et al. (2011) propose that the inner mammillary layer of cuckoo eggs has a different structural composition (such as crystal size) that strengthens this generally weaker layer of the eggshell.

As mentioned above, thicker eggshell would be predicted to slow gas exchange through the eggshell, which seems counter intuitive for species which exhibit potentially accelerated development. However, this could be countered by a modified pore structure, which appears to be the case in cowbirds, and possibly, common cuckoos (Hargitai et al., 2010; Jaeckle et al., 2012). Although Portugal et al. (2014) demonstrated reduced water conductance in un-incubated common cuckoo eggs, it has not been explored how the pore structures change over development. During incubation, developing embryos leach calcium from the mammillary layer, thereby changing the structure of the shell. Igic et al. 2017 studied the process of eggshell thinning in cuckoo eggs verses their hosts, to test the hypothesis that cuckoos thin their eggshells to a greater extent than hosts. While Igic et al. (2017) found this not to be the case (cuckoo eggs were still relatively thicker at hatching), they did not explore whether the mechanism of decalcification affected the structure of the egg in a way that would influence gas exchange. If brood parasites thinned the mammillary layer in such a way as to boost gas exchange at key points in development, this could be an adaption towards enhanced hatching condition.

Embryo movement

The surprising strength of newly hatched brood parasites has frequently been commented on in literature, but surprisingly little has been done to assess how it is achieved. A novel route of this investigation is to assess the movement of the brood parasite embryo *in-ovo*. In all vertebrate species, movement and muscular contractions during embryo development occur and effect the formation of the musculoskeletal system (Pollard et al. 2017; Pollard, Mcgonnell and Pitsillides 2014). It has been suggested that embryonic movement may even cause

epigenetic changes in the skeletal structure of limbs between and within species (Botelho et al., 2015a; Felsenthal and Zelzer, 2017; Pollard et al., 2014). In fact, toe orientation in altricial birds is believed to be largely controlled by embryo movement (Botelho et al., 2014, 2015b). Botelho et al. (2014) showed that zygodactyl toe orientation (two digits facing back, two forward) in Psittaciformes is produced by muscle action on the forming foot during early development, and a lack of movement leads to the ancestral (anisodactyl) orientation. Studies that have experimentally caused paralysis of embryos *in-ovo* have found deformation in the growth of limbs, such as absence of essential joint cavities (Pitsillides, 2006), and misshape of the femur (Nowlan et al., 2010; Roddy et al., 2010). Conversely, hyperactivity of the chick embryo (pharmacologically induced) has been shown to increase body mass and the number of muscle fibres in leghorn chickens (Heywood et al., 2005).

This is a promising explanation for the observed hatchling strength of brood parasites. Honza et al. (2015) found that common cuckoos have a greater density of muscle fibres in the hatching muscle, *musculus complexus,* which controls flexion of the neck. This adaption is likely to be necessary in order to hatch from the structurally stronger eggs. This doesn't seek to explain the ontogeny of this musculature, but given what has been shown in other studies, mechanical stimulation of muscle growth through embryonic movement is a plausible hypothesis (Heywood et al., 2005). In particular, the relatively large intra-individual variation in the density and cross-sectional area of muscle fibres that was observed in this study could be explained by different movement patterns of individual embryos (Felsenthal and Zelzer, 2017). Whether muscles used in eviction behaviour are similarly enlarged is not known. To the best of my knowledge, it has not been tested whether the hatching muscle is employed by common cuckoos during eviction, however this is plausible considering the involvement of the neck and back.

In this thesis I explore the hypothesis that brood parasites exhibit a greater amount of movement *in-ovo* compared to the embryos of non-parasitic species that hatch in a similar altricial state, which would suggest a role of movement in development of their hatching phenotype.

Conclusion and scope

The developmental strategy of developing young greatly effects their adult form and function. Precociality is the norm for most vertebrate species, and is believed to be the ancestral state from which altricial development has evolved in some lineages (Dial and Carrier, 2012; Dyke

and Kaiser, 2010; Starck, 1993). While altricial young are less costly to produce they require a greater level of parental care, partly due to the energy costs of their accelerated post-natal growth (Starck and Ricklefs, 1998). In this way, brood parasites have shed the bulk of parental costs by not raising their own young. While nearly all obligate brood parasites are altricial at hatching (Yom-Tov and Geffen, 2006), the offspring of many brood parasites display physiological traits more akin to precocial offspring such as their coordinated movement and strength. I predict that they may achieve this through an alternation in the in-ovo developmental process and adaptations to their eggshells. By adjusting the content, structure and usage of the egg components, brood parasites may be able to achieve a partially precocial development prior to hatching. This would allow them to invest in certain muscular and structural development, while still remaining altricial overall so as to best exploit their host. This type of partitioning of ontogenetic strategies has been suggested in other avian species (Aourir et al., 2016; Dial and Carrier, 2012).

This laboratory and field techniques, making use of international and multi-institutional collaborations and resources, in order to further our collective knowledge of evolution of this fascinating group of birds. I hope that this work will contribute significantly to the fields of ecology, conservation, and evolutionary physiology among others, and provide validation for new techniques for the study of embryo development.

Thesis structure

In **Chapter 2** I investigate the water vapour conductance (G_{H2O}) of brood parasite eggshells in the laboratory, using eggs either collected in the field or provided from the destructive collection of the Natural History Museum of London. I combine these data with species values and species life-history traits extracted from published literature, and use phylogenetically informed models to compare parasitic species to their hosts and relatives.

In **Chapter 3** I explore embryo movement rates of brood parasites and their hosts developing under their normal nest conditions. Embryo movement is measured non-invasively using an infrared laser monitor ('EggBuddy") which allows movement rates to be tracked over the full course of incubation and compared across a diverse range of parasites and their hosts.

Chapter 4 looks at the eggshell composition of a wide range of birds. The proportion of the eggshell that is inorganic (calcium carbonate) compared to organic is measured by exposure to extreme heat to incinerate the organic component. I map the calcium carbonate content of birds to their life history and egg physiology to investigate how this trait varies, particularly in relation to reproductive investment.

In **Chapter 5**, I measure several physical traits in eggshells of brood parasites and hosts relating to their eggshell surface properties (roughness and wettability) and composition (calcium carbonate content as in **Chapter 4**). This chapter makes use of collaboration with engineering labs at Open University (Milton Keynes, UK) to apply material engineering techniques to assess eggshell properties using microscopy methods.

In **Chapter 6** I compare metabolic rate of developing embryos of brood parasites and hosts over the course of their development. Using a portable flow-through respirometry set-up I was able to measure metabolic rate, as CO_2 production, of embryos at the nest at several time point in their development. Thanks to collaboration with research groups internationally, these data is gathered over several lineages of brood parasites.

CHAPTER 2. Convergent evolution of reduced eggshell conductance in avian brood parasites

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Abstract

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 optimise this breeding strategy has received much less attention, yet these species share many similar physiological traits that optimise 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_{H2O}) 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_{H2O} compared to 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_{H2O} 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_{H2O} 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_{H2O} may be a general adaptation for brood parasitism, perhaps helping the parasite nestling to develop greater aerobic fitness.

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 (Davies and Quinn, 2000; Kilner and Langmore, 2011; Krüger, 2007). 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 finch, *Anomalospiza imberbis*) and Anatidae (black-headed duck, *Heteronetta atricapilla*) (Davies and Quinn, 2000). 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 lineages, 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 eggshells than those of their hosts (Spottiswoode, 2010). 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 (Antonov et al., 2006; Igic et al., 2011). Thicker eggshells may also function to protect the parasitic egg from fracture during the rapid laying process that is characteristic of many brood parasites (Sealy et al., 1995). 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 parasitise the same nest (Carter, 1986; Spottiswoode, 2013). Many brood parasites also have a shorter incubation period compared to their hosts (Briskie and Sealy, 1990; Honza et al., 2001; Kattan, 1995), and early hatching is beneficial as it provides a competitive advantage for the parasite chick over host young (Davies, 2011; Payne, 1977). This is achieved either through facilitating the killing or ejection of host eggs or chicks (Honza et al., 2001), or through providing a competitive edge in obtaining food from the host parents, for those species where host and parasite are reared together (Hauber and Kilner, n.d.; Kilner et al., 2004; Payne, 1998). 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-hour intervals) (Birkhead et al., 2011) and a higher concentration of growth-promoting steroids in the yolk (Cao et al., 2018), 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 (Honza et al., 2015, 2001; Yoon, 2013) that not only aid in hatching from a thicker eggshell, but also likely assist parasitic chicks to kill hosts chicks, in those species that are highly virulent (Honza et al., 2015; Spottiswoode and Koorevaar, 2012).

These examples show that many physiological adaptations to brood parasitism occur at the egg stage. Yet, while much has been documented about the size (Grim, 2005), maculation (Brooke and Davies, 1988; Stoddard and Stevens, 2010) and thickness (Igic et al., 2011; Spottiswoode, 2010) 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 (Ar et al., 1974). 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 (Ar and Rahn, 1980; Booth and Rahn, 1990; Zicus et al., 2004). 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 (Ar and Rahn, 1980; Barott, 1937; Romijn and Roos, 1938). Gas exchange contributes to the rate of water loss, and can be measured across the eggshell as the water vapour conductance (G_{H2O}). Therefore, G_{H2O} 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 (Portugal et al., 2014; Romijn and Roos, 1938). Because all nutrients for embryonic development are deposited by the mother into the egg prior to laying, maintain suitable humidity levels for gas exchange and incubation temperature comprise the female's only physical control of the requirements for her offspring's embryonic development once the egg has been laid (Mortola, 2009).

The physical characteristics of the nest environment are known to be important determinants of G_{H2O}, and therefore G_{H2O} 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 (Igic et al., 2011). 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. 2014 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 (Jaeckle et al., 2012). Contrary to this expectation, they found that the G_{H2O} 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 (Portugal et al., 2014). The lower G_{H2O} 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 (Honza et al., 2007).

Here we test the hypothesis that a reduced eggshell G_{H2O} 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_{H2O}, 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*.

Materials and methods

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 table 2.1 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. 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's been shown that G_{H2O} of fresh eggs is not significantly different from museum specimens that have been stored over 50 years (Portugal et al., 2010).

The following host species were collected from the same location (corresponding parasites in brackets following species names) (see table 2.1): little bee-eaters (*Merops pusillus*) (parasitized by greater honeyguides), crested barbets (*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 parasitised 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*).

Table 2.1a Sample sizes, collection locations and parasite strategy for the seven species of brood parasites for which eggshell conductance (G_{H20}) was measured. N refers to the number of (*i*) whole eggs where G_{H20} 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 Methods). "High virulence" is where the parasite kills the hosts offspring, while "low virulence" refers to a strategy whereby the parasite does not directly kill the hosts 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	High virulence
Greater honeyguide (*Indicator indicator*)	3 whole eggs, 24 shell fragments from 4 eggs	Zambia	High virulence
Cuckoo finch (*Anomalospiza imberbis*)	6 whole eggs	Zambia	Low virulence
Pin-tailed whydah (*Vidua macroura*)	6 shell fragments from 3 eggs	Zambia	Low virulence
Purple indigobird (*Vidua purpurascens*)	5 shell fragments from 1 egg	Zambia	Low virulence
Common cuckoo (*Cuculus canorus*)	9 whole eggs 4 shell fragments from 1 egg	U.K. (NHM)	High virulence

Table 2.1b Sample sizes, and primary parasite for the 11 species of hosts for which eggshell conductance (G_{H20}) was measured. N refers to (*i*) the number of whole eggs (WE) where G_{H20} 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 6 shell fragments were used from this egg. Host eggs were collected from the same location as their respective parasites (see table 2.1a). Species marked with an asterisk were collected in Zambia, but were not hosts of parasites included in this study.

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 * (*Amandava subflava*)	6 whole eggs 9 fragments from 3 eggs	Jambandu indigobird (West Africa only)
Blue waxbill * (*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 * (*Ortygospiza fuscocrissa*)	1 fragment from 1 egg	Quailfinch indigobird (West Africa only)
Locust finch * (*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 * (*Lagonosticta senegala*)	2 fragments from 2 eggs	Village indigobird
Orange-winged pytilia * (*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

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_{H2O}. The G_{H2O} of the eggs was measured following the same standard protocol (Board and Scott, 1980; Portugal et al., 2014a; Portugal et al., 2014b) that was used by studies that were the source of comparative literature data, and researchers were blind to which species each egg came from during the initial process of measuring G_{H2O}. 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_{H2O}; (Taigen et al., 1978)). 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). The plastic covering the blow hole comprised, 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 (Portugal et al., 2014b). The glue was left to dry for 4 hours, before the eggs were weighed to the nearest 0.0001g (Sartorius 1265 ms, Göttingen, Germany) and placed in a desiccator (Camlab, Cambridge, U.K.) filled with self-indicating silica gel. The desiccator was housed in a constant temperature cabinet (Porkka, Hertfordshire, U.K.) to 25 ± 1°C. After 24 h, the eggs were weighed to the nearest 0.1 mg before being returned to the desiccators. The eggs were the weighed at the same time of day on three successive days to provide two values of 24-hour G_{H2O}, and a mean was taken. Any mass loss was assumed to be the result of water loss (Booth and Seymour, 1987; Portugal et al., 2010). Calculation of G_{H2O} was as previously described (Booth and Seymour, 1987; Maurer et al., 2011; Portugal et al., 2010). Briefly, the G_{H2O} of a shell can be calculated as:

$$G_{H_2O} = \frac{M_{H_2O}}{\Delta P_{H_2O}} \quad (1)$$

Where G_{H2O} = water vapour conductance (mg day^{-1} torr^{-1}), M_{H2O} = the rate of mass loss (mg day^{-1}), and ΔP_{H2O} = water vapour pressure difference across the shell (torr). Internal P_{H2O} was assumed to be 23.8 torr (water vapour pressure of saturation at the egg temperature, 23.8 torr at 25°C), and an external P_{H2O} of 0 torr, due to the desiccator atmosphere being close to zero humidity.

Fragment eggshell conductance measurements

Previously it's been demonstrated that eggshell fragments can be used to measure G_{H2O} across eggshells (Portugal et al., 2010; Portugal et al., 2014b). For eggs that had been broken in the field, or had cracks present, fragments were used to determine G_{H2O}. 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, U.K.) to aid transport and weighing, and to ensure the Eppendorf was upright at all times. Once the glue had dried, the eggshell fragments were checked to ensure the fragment was adhered securely, before superglue (RS Components, Corby, U.K.) was applied to the underside of the fragment, 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. All other aspects of G_{H2O} measurement and analyses were identical to the whole-egg protocol.

All G_{H2O} species values extracted from literature were measured using the same methods to our study and hence are comparable.

Life-history and ecological data

A total of 43 species was used for whole egg analyses, and 36 species for fragment analyses. 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 (Del Hoyo et al., 1992- 2010), and cross referenced with Birds of the Western Palearctic (Southern and Cramp, 1978). In addition, supplementary data were obtained from family and species-specific monographs, and field guides to nests.

We restricted the number of life history traits included to those which have been found to have a significant effect on G_{H2O} (Portugal et al., 2014b). 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. Body mass of adult birds was taken as a mean for both sexes, primarily from (Dunning, 2007). Breeding latitude was compiled from data tabulated by (Orme et al., 2006).

Phylogenetic methods and analyses

R statistical software was used to conduct all statistical analysis and production of figures (R statistical software Rv3.3.2) through the Integrated Development Environment 'R Studio' Values of G_{H2O} 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, where both whole and fragmented eggs values were available. The number of eggs and eggshell fragments measured per species can be found in tables 2.1a and 2.1b.

Since species are not statistically independent, we modelled G_{H2O} while taking into account their shared phylogenetic history (Freckleton et al., 2002; Pagel, 1999; Paradis et al., 2004). 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. (2006). For each set of trees, a maximum clade credibility tree was produced using the function maxCladeCred from the R package phangorn (Schliep et al.,

2017; Schliep, 2011), and these trees were used for subsequent phylogenetically informed analysis (Figure 2.1).

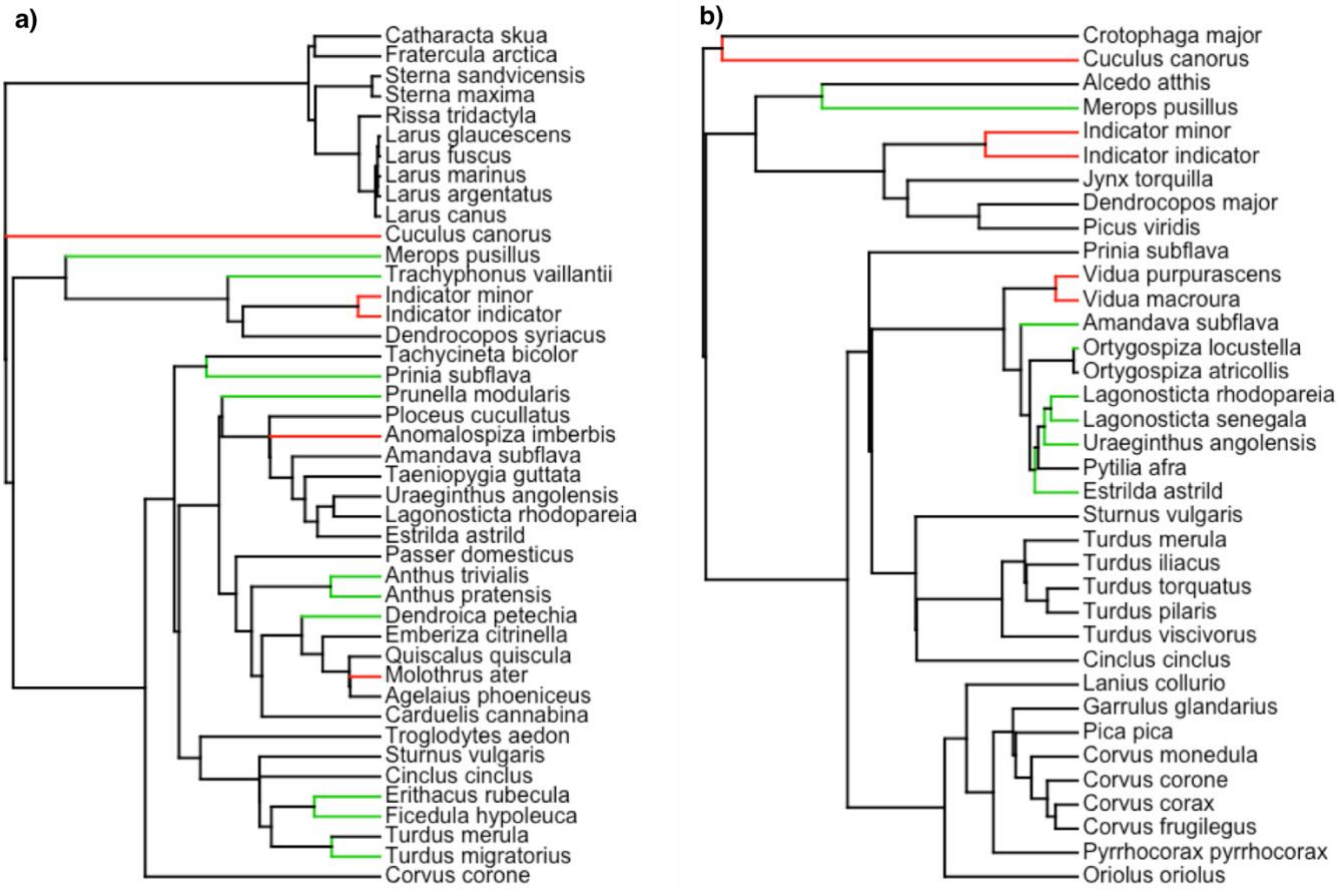

Figure 2.1. Phylogenetic trees with representatives for (*a*) whole egg analysis and (*b*) shell fragment analysis. Red lines indicate brood parasitic species, and green lines indicate frequent hosts of parasites in each subset. Trees generated using a backbone tree from Ericson et al. 2006.

We estimated Pagel's λ for G_{H2O} on each tree, using the function phylosig (from the R package Phytools (Revell, 2012, 2010). 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 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 (Pagel, 1999).

We constructed phylogenetic generalised least squares models (pgls) using the pgls command in the package caper (Orme, 2013). These models incorporate the expected similarity between sister taxa by producing a co-variance matrix of how they are expected to co-vary 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_{H2O}, 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, since brood-parasitic species tend to have thicker eggshells than expected for their size (Igic et al. 2011, Spottiswoode 2010). Despite this pattern in brood parasites, adult body mass and fresh egg weight were highly correlated with eggshell thickness (explaining > 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 table 2.2 (a = models for whole eggs, b= models for shell fragment data). 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 (Bartoń, 2009). Plots and phylogenetic trees produced using R packages ggplot2 (Wickham, 2009) and phytools respectively. A Welch two sample *t*-test was used to initially compare G_{H2O} between parasites and common hosts (host spp. In (a), with the addition of eight host species from the literature; however, due 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 this data did not change the significance of the models (supplementary analysis 2.1).

Table 2.2 Model support table (AICc) for the top-ranked PGLS models (model weight >0.05) of G_{H2O} which contribute to the average models. Estimates for parameters are provided to indicate inclusion in respective models. Model weight are estimate across entire set of 32 models and sum to 1. (a) displays top models for whole egg analysis, and (b) top models for shell fragment analysis.

(a)

Model #	Intercept	Parasite status	Shell thickness	Nest type	Wet parent	Mean breeding latitude	Parasite status/Shell thickness	AICc	δAICc	model weight	logLik
1	0.06	-0.71		1.34		0.01		83.50		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

(b)

Model #	Intercept	Parasite status	Shell thickness	Nest type	Wet parent	Mean breeding latitude	Parasite status/Shell	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

Results

Comparison of G_{H2O} in parasites and their respective hosts

Brood parasites had a significantly lower G_{H2O} when compared in a pairwise manner to common host species. For whole eggs, G_{H2O} (mean ± S.E.M) was 0.76 ± 0.12 mg day^{-1} torr^{-1} for brood parasite species (n=5) and 1.34 ± 0.20 mg day^{-1} torr^{-1} for host species (n=16; $t_{13.9}$ = 2.39, p = 0.031). For eggshell fragments, G_{H2O} was 0.39 ± 0.05 mg day^{-1} torr^{-1} for brood parasite species (n=5) and 0.74 ± 0.06 mg day^{-1} torr^{-1} for host species (n=10; $t_{7.8}$ = 3.98, p = 0.004) (Figure 2.2).

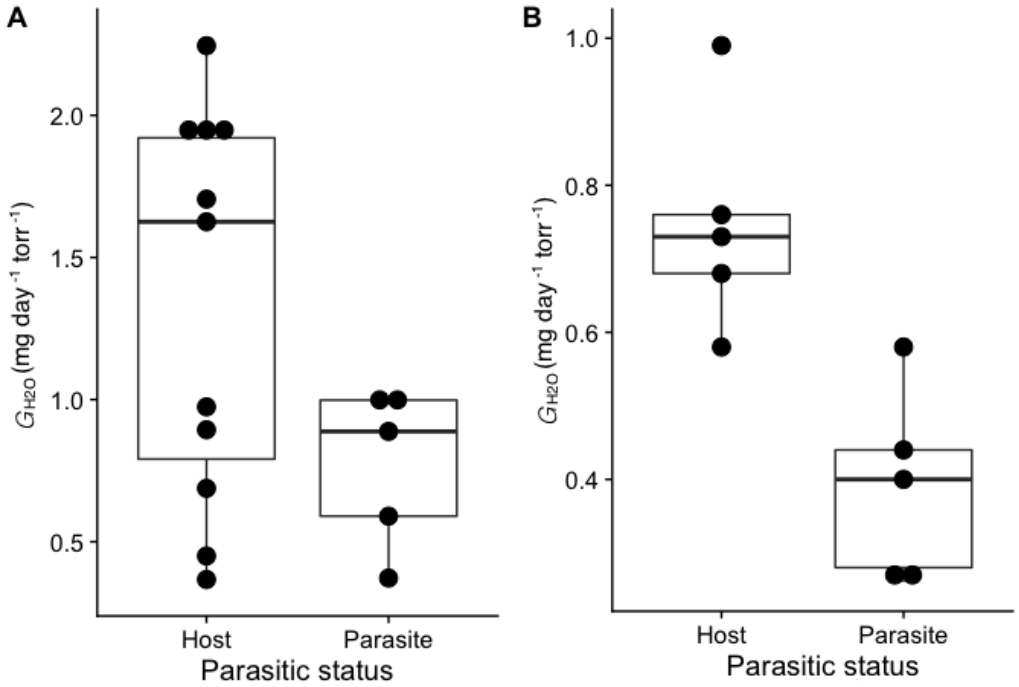

Figure 2.2 Non-mass corrected mean G_{H2O} (mg day^{-1} torr^{-1}) of avian brood parasites and common hosts. For whole eggs (A), five parasitic and 11 host species were compared, and for shell fragments (B), five parasitic and five hosts species were compared. Host species had significantly higher in G_{H2O} both cases (t-test; A: $t_{13.9}$ = 2.39, p = 0.03, B: $t_{7.8}$ = 3.98, p = 0.004).

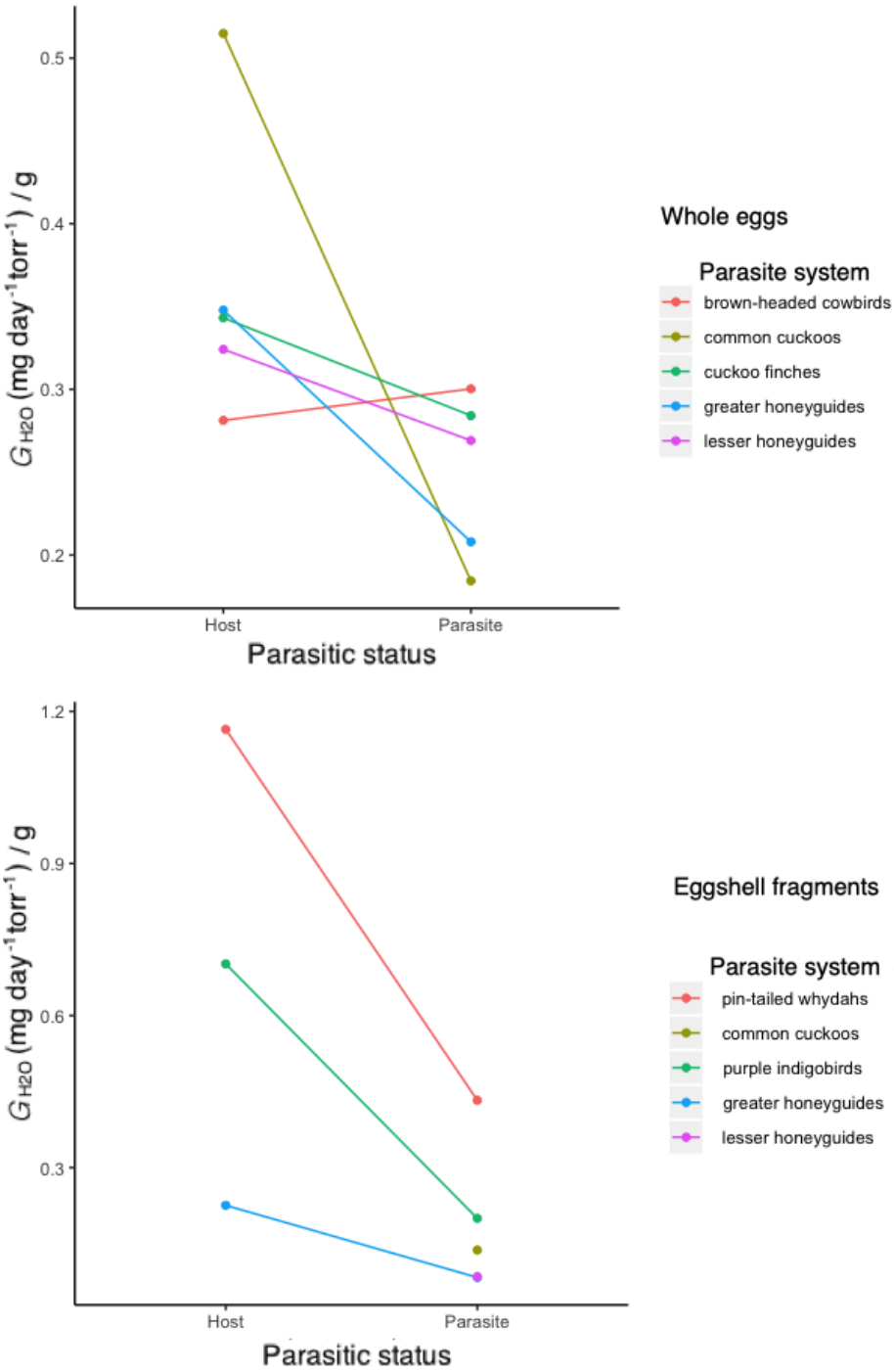

Figure 2.3 Top: G_{H20} (mg day^{-1} torr^{-1}) corrected by egg weight (g) for whole eggs. Brood parasites and respective host species connected by coloured lines. Note: Average G_{H20} of several hosts (n=5) of the common cuckoo is presented in the host category. Bottom: G_{H20} (mg day^{-1} torr^{-1}) corrected by egg weight (g) for shell fragments. Brood parasites and respective hosts are linked by coloured lines. Note: No G_{H2O} 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 cuckoos in the top panel.

Due to potentially confounding effects of egg size on G_{H2O}, 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_{H2O} 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 2.3). No statistically significant difference was found in G_{H2O} or mass corrected G_{H2O} between high virulence and low virulence brood parasites ($p > 0.05$ for whole eggs and eggshell fragments), although there was a strong trend for greater G_{H2O} in the eggs of low virulence parasites (Table 2.1a, Figure 2.4). However due to the small sample size (high virulence brood parasites: $n = 3$, low virulence brood parasites: $n = 2$), a non-significant *p*-value may be the result of insufficient power for this analysis.

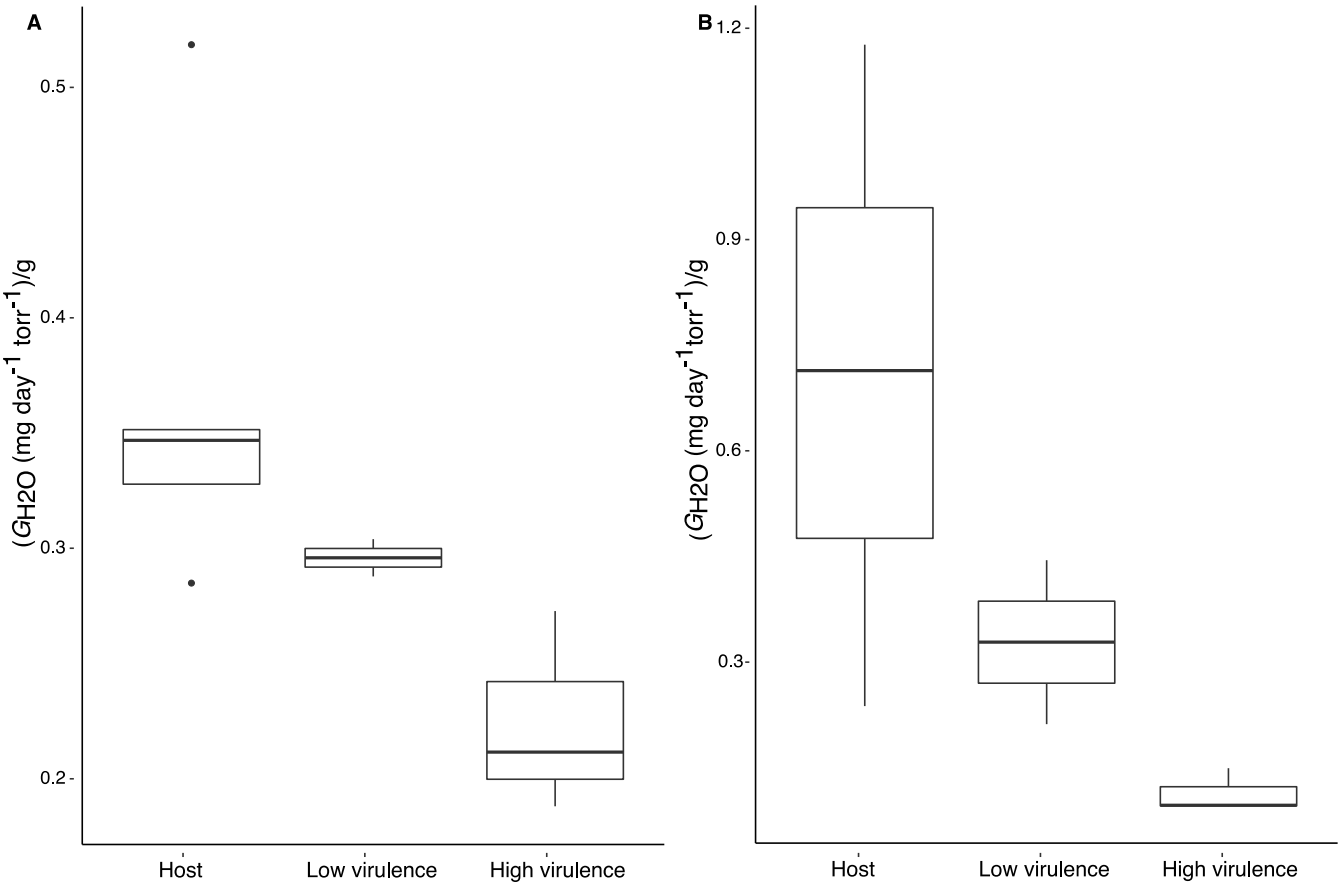

Figure 2.4 (A) Mean G_{H20} (mg day^{-1} torr^{-1}) corrected by egg weight (g) for eggshell fragments. Three hosts species compared to with low virulence brood parasites and three high virulence brood parasites. (B) Mean G_{H20} (mg day^{-1} torr^{-1}) corrected by egg weight(g) for whole eggs. Five host species compared with two low virulence brood parasites and three high virulence brood parasites. There was no significant difference was between high virulence and low virulent species in either case (Whole eggs: $t_{2.38} = 2.70$; $p = 0.09$, Eggshell fragment: $t_{1.56} = 1.82$; $p = 0.31$)

Phylogenetic signal

Pagel's lambda for G_{H2O} 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_{H2O}, 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.

Whole egg conductance

For whole eggs, the average model (Table 2a) of the pgls analysis for mean G_{H2O} retained the predictors parasitic status (binary), eggshell thickness (μm), 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_{H2O} nor improved the fit of the models to the data. Parasitic status explained a significant amount of variance in G_{H2O} ($z = 2.24$, $p = 0.025$), with parasitic species displaying lower G_{H2O} than would be expected given their phylogenetic position ($n = 5$ of 43). Nest type also significantly predicted mean G_{H2O} ($z = 1.96$, $p = 0.050$), with shallow and scrape style nests having a lower G_{H2O} than cup nests. However despite being retained in the average model based on AICc, 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, since 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.

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_{H2O} (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_{H2O} 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).

Discussion

Consistent with findings for common cuckoos (Portugal et al., 2014a), the eggs of brood parasite species in the present study generally had lower G_{H2O} than expected for their size and phylogenetic position. All species of brood parasites had lower G_{H2O} than their hosts, with the exception of brown-headed cowbirds, whose mean G_{H2O} was higher than the host species to which it was compared. However, brown-headed cowbirds are extreme generalists (Davies and Quinn, 2000; Krüger, 2007) that parasitise a large variety of host species, and as such the single host species for which we had G_{H2O} 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_{H2O} 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_{H2O} was notably greater for egg fragments than whole-egg analyses. This is likely due to the eggshell fragments comprising areas primarily from the equator region of the shell, which may exacerbate the difference between hosts and parasites (Portugal et al., 2010).

A lack of fine tuning of brood parasite G_{H2O} to their hosts' nest environment?

Brood parasites all shared lower than expected eggshell G_{H2O} 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_{H2O} is finely tuned to the nest environment to ensure optimal water loss (see Portugal et al., 2014b and

references therein). How then are parasitic eggs able to develop successfully under the nest conditions of their respective hosts? Traditionally, species which deviate from the expected allometric relationships between egg mass and G_{H2O} (see (Ar et al., 1974)) 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 (Ar and Rahn, 1985; Sotherland et al., 1984). 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_{H2O} to minimise water loss through the shell (Arad et al., 1988; Carey, 1980). The eggs of these 'extreme nesters' typically exhibit an increase in G_{H2O} to ensure that the optimal amount of water is lost during the incubation period, despite these extreme conditions.

Eggshell G_{H2O} is largely considered to be species-specific (Ar et al., 1974), and proposed to be genetically controlled (Sotherland et al., 1979). Among brood parasites, there is little variation within species G_{H2O} (Portugal et al., 2014a, present study), suggesting G_{H2O} 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 (Spottiswoode, 2010; Spottiswoode and Stevens, 2012). Future studies might specifically compare G_{H2O} of different cuckoo gentes, or of other brood parasites comprising multiple lineages of host specialists, such as honeyguides (Spottiswoode et al., 2011).

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 million years ago (Jetz et al., 2012), suggesting that their shared physiological trait of a low G_{H2O} 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_{H2O} is to brood parasites.

One possible adaptive explanation for low G_{H2O} 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., 2006 established that eggs with lower conductance of H_2O, O_2 and CO_2 experienced reduced heart rates and improved embryo survival, compared to eggs with higher conductance (both relative to a mean species level G_{H2O}). Given the increased effort required for most species of brood parasite to hatch from an egg of greater structural strength (Honza et al., 2001), 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. Wilmore et al., 1995). 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_{H2O}, 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 (Christensen et al., 2006, 1993). In addition, results are contradictory, with an earlier study showing no such relationship (Christensen et al., 2005). 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_{H2O}. 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 to that of their hosts. Interestingly, we did not detect a statistically significant difference in G_{H2O} between brood parasitic species which could be considered high virulence (that is, evict and/or kill the young of the host) versus low virulence parasites (that is, outcompete host young, not necessarily fatally; see Table 2.1a; Figure 2.4), suggesting that reduced G_{H2O} 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_{H2O} is specifically adapted to the energetic demands of being a highly virulent brood parasite would be enlightening.

Trade-off between shell hardness and G_{H2O}

The hypothesis above proposes an adaptive explanation for the surprisingly low G_{H20} of brood-parasitic eggshells. However, depending on the mechanisms underlying variation in G_{H20}, a brood-parasitic lifestyle may also impose constraints on G_{H20}, even if a low G_{H20} is itself not adaptive. The need for brood parasites to maintain hard eggshells might impose a strong constraint on G_{H2O}, that may partially explain why it is surprisingly low: if high G_{H2O} 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_{H2O} even were it adaptive for other reasons. This hypothesis could be readily testable using a combination of biomechanical and physiological tests on eggshells.

Is G_{H2O} 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_{H2O} measurements are generally representative of G_{H2O} at the onset of incubation. However, G_{H2O} may not be consistent throughout development. Two possible mechanisms could generate changes in G_{H2O} as incubation proceeds; we will consider each in turn.

First, G_{H2O} may increase over 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 (Spottiswoode, 2010; Spottiswoode and Colebrook-Robjent, 2007). 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_{H2O} during later development (Igic et al., 2017). If so, then G_{H2O} measured in freshly-laid eggs is not necessarily representative of the incubation period as a whole (Portugal et al., 2014a), 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_{H2O} 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_{H2O} is unclear, and recent studies suggest it is likely to be more complex than G_{H2O} simply increasing when an eggshell thins. Moreover, it is unclear whether parasitic eggshells do thin more rapidly: Igic et al. (2017) established that the degree of eggshell thinning experienced by common cuckoo eggs was similar to that of their hosts.

Second, G_{H2O} 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 (Booth and Seymour, 1987). Any such changes in pore geometry may trade off against the continued requirements for structural hardness, as discussed above (Igic et al., 2017). This trade-off may be exacerbated in common cuckoos, which have furcated eggshell pores that might open into more pathways for diffusion as the inner mammillary layer erodes, potentially at the cost of weakening the shell's structural integrity (Board and Scott, 1980).

Conclusion

We found that brood parasites have lower G_{H2O} than their phylogenetic position and egg size would predict. Moreover, other than brown-headed cowbirds, all had lower G_{H2O} 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 co-evolutionary arms-race between hosts and parasites may drive a trait in a direction counter to what would otherwise be optimal under certain environmental conditions.

Chapter 2 Supplementary Materials

<u>Supplementary analysis 2.1</u> Additional analysis with exclusions of small sample sizes

In order to address the issue of small sample sizes, the analyses were rerun excluding species were only a single egg was available. In the case of the PGLS model of eggshell conductance, only the African quailfinches was excluded, as only a single sufficiently large shell fragment was available. This did not change the significance of the factors included in the average model (see model output below).

As requested by the reviewer we have reperformed the analysis with the exclusion of these species. Two species were removed from the whole egg dataset and six from the fragment dataset. This had minimal effect on the results of the comparative analysis between host and parasite G_{H2O}. However due to the increased 'weight' of the cowbird and host pair (for whom the trend was reversed) in the model, parasite status was just above significant. When the cowbird was also removed there was once again a significant effect of parasite status on G_{H2O} (p= 0.02). (see below)

1. **<u>pgls model excluding African quailfinches</u>**

a) Model support table (AICc) for the top-ranked PGLS models (model weight >0.05) of G_{H2O} which contribute to the average model.

Component models:

Model #	df	logLik	AICc	Delta AICc	weight
1	5	-12.11	36.3	0	0.25
2	3	-14.86	36.49	0.19	0.23
3	4	-14.05	37.44	1.14	0.14
4	6	-11.26	37.52	1.22	0.14
5	2	-16.6	37.58	1.28	0.13
6	5	-12.99	38.05	1.75	0.11

b) Model output of conditional averaged PGLS model predicting GH2O of eggshells fragments.

	Estimate	Std. Error	z value	Pr(>|z|)	
(Intercept)	-0.56623	0.304086	1.862	0.0626	.
parasite.staty	-1.60934	0.724134	2.222	0.0263	*
Shell_thickness_um	-4.265344	1.789727	2.383	0.0172	*
Wet.parentyes	-0.551965	0.30914	1.785	0.0742	.
parasite.staty:Shell_thickness_um	16.385773	7.804396	2.1	0.0358	*
Breeding.latitude	-0.004312	0.003467	1.244	0.2135	

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

c) Importance weighting and number of containing models of predictor variables in pgls analysis

Variable	Shell_thickness	Wet.parent	parasite.stat	parasite.stat * Shell_thickness	Breeding.latitude
Importance	1.00	0.76	0.50	0.50	0.39
N containing models	6	4	3	3	3

2. Host-parasite comparison

Fragment samples with 6 species with n=1 removed

G_{H2O} (not corrected by mass)

Welch Two Sample t-test

G_{H2O} by host-parasite status

t = 2.8706, df = 4.7112, p-value = 0.03748
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
0.03041374 - 0.66280250
sample estimates:

mean in group Host	*mean in group Parasite*
0.7666081	*0.4200000*

G_{H2O} /g egg weight

Welch Two Sample t-test

G_{H2O} / g by host- parasite status

t = 3.8519, df = 4.934, p-value = 0.01229
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
0.2186354 - 1.1066137
sample estimates:

mean in group Host	*mean in group Parasite*
0.8629638	*0.2003392*

Whole egg analysis with 2 species with n=1 removed

G_{H2O} (not corrected by weight)

Welch Two Sample t-test

G_{H2O} by host-parasite status

t = 2.0495, df = 12.803, p-value = 0.06148
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
-0.02840023 - 1.04722600
sample estimates:

mean in group Host	*mean in group Parasite*
1.2790794	*0.7696665*

G_{H2O} /g egg weight

Welch Two Sample t-test

GH2O by host-parasite status

t = 3.5334, df = 10.832, p-value = 0.004796
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
0.09397622 - 0.40600808
sample estimates:

mean in group Host	*mean in group Parasite*
0.4991607	*0.2491686*

G_{H2O} (not corrected by weight), with brown-headed cowbird and host also removed

Welch Two Sample t-test

G_{H2O} *by host-parasite status*

t = 2.5362, df = 10.959, p-value = 0.02774
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
0.0868561 - 1.2313763
sample estimates:

mean in group Host	*mean in group Parasite*
1.3711993	*0.7120831*

CHAPTER 3. More frequent embryonic movement during development in avian brood parasites: Preparation for the demands of early parasitic life?

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Abstract

Movement of the growing embryo is essential for musculoskeletal development in vertebrates. However, variation in embryo movement as a mechanism for altering physiology has rarely been studied. Avian brood parasites – species which use hosts to rear their offspring – exhibit exceptional behaviours and extreme feats of strength in early life. The thicker eggshell of brood parasites makes hatching more strenuous, and, in highly virulent species, the newly hatched parasite must kill or evict multiple eggs and/or nestmates which are often larger than itself. This is a muscularly demanding and energetically costly activity, yet little research has focused on how brood parasite embryonic development may be adapted to this challenge. We hypothesised that an increase in embryonic muscle movement could allow brood parasites to develop the required musculature for these early-life demands of being a parasite. As such, we investigated whether brood parasites differed in the rate of embryo movement over the course of incubation compared to both their hosts, and closely related species of the parasite. Embryo movement was measured repeatedly over the incubation period for five species of brood parasites and five of their respective hosts, as well as nine non-parasitic species for phylogenetic comparison. The rate of embryo movement was compared between parasites and non-parasites using a phylogenetically controlled analysis to account for shared ancestry. We found that the rate of embryo movement increased over incubation for all species, but crucially that the brood parasites had a significantly steeper rate of increase in muscular activity than non-parasitic species. In pairwise comparisons, three of the five species of parasites, including two species with highly virulent chick behaviours, exhibited a greater increase in embryo movement compared to their host. However, we also found similar patterns in less virulent species of brood parasites, indicating embryo activity may have more general adaptions for parasitism. This is consistent with our hypothesis that increased embryo movement may be responsible for the development of stronger muscles and skeletal elements required for the arduous physical tasks of hatching from thicker eggshells coupled with the demands of removing or competing with host young. This is a novel result showing correlation between embryonic activity and reproductive strategy in birds that appears to have evolved convergently across brood parasite lineages.

Introduction

Much of an organism's lifetime physiology is determined during the embryonic stage of development (Durant et al., 2013; Ricklefs, 2006). This period of early development is fundamental for species that exhibit extreme physiologies, behaviours, or life histories to achieve the necessary traits they need to thrive and reproduce in their respective environment (Gou et al., 2007; Podrabsky et al., 2007; Wearing et al., 2017). Obligate brood parasites follow a rare alternative, non-parental reproductive strategy whereby they lay their eggs in the nests of other species (hosts) to raise their progeny. This strategy requires specialised physiological and behavioural adaptations in their young to survive in the host nest upon hatching. Brood parasitism has been observed in insects and fish, but is most prevalent in birds (Blažek et al., 2018; Field, 1992; Thorogood et al., 2019). Obligate avian brood parasitism has evolved independently at least seven times among birds (Payne, 2005), and specialisations to this reproductive strategy have arisen convergently in these lineages (Birkhead et al., 2011; Brooker and Brooker, 1991; Spottiswoode and Koorevaar, 2012). These specialisations include adaptations of brood parasite eggs and embryonic development, such as earlier hatching and faster growth (Birkhead et al., 2011; Croston and Hauber, 2010), thicker and stronger eggshells (Antonov et al., 2012; Igic et al., 2011; Picman, 1997) and reduced water vapour conductance across the shell (McClelland et al., 2019). The commonality of reduced eggshell conductance is counter-intuitive to the observed faster development of these species, but has been proposed to facilitate greater aerobic fitness post-hatching (Christensen et al., 2006; McClelland et al., 2019). Brood parasites vary in their level of virulence (Kilner, 2005), with highly virulent species, such as common cuckoos (*Cuculus canorus)*, removing or destroying host eggs or nestmates in order to monopolise the resources provided by their foster parents (Honza et al., 2007; Kilner, 2005). Other, less virulent species, such as the brown-headed cowbirds (*Molothrus ater*) use physical size advantage and behavioural exaggeration to outcompete host nestmates to receive biasedly greater provisions from host parents (Hauber, 2003; Kilner, 2005). The physical demands of high virulence require additional adaptations to brood parasite behaviour and morphology which have likewise arisen convergently across species, for example, the bill hook exhibited in hatchling of honeyguides (*Indicatoridae*) and striped cuckoos (*Tapera naevia*) which serves as a weaponised appendage for stabbing host nestmates (Antonov et al., 2012; Spottiswoode and Koorevaar, 2012). Despite these observations, little

is known about how the unique chick physiology of brood parasites is achieved over a relatively short ontogenetic time in the egg, and how embryonic behaviour might shape this.

Movement is essential for successful embryonic development across vertebrates, regardless of whether development takes place internally in the womb or externally in an egg (Müller, 2003; Pitsillides, 2006). Embryonic movement (EM) shapes the development of an animal's musculoskeletal system, and ranges from sporadic twitching of muscle tissue in the early stages of development, to coordinated motions akin to walking or flying closer to hatching (Heywood et al., 2005; Nowlan et al., 2010). The importance of EM in humans has been well established, with aberrations from normal activity in the womb linked to multiple developmental disorders (Hammond and Donnenfeld, 1995), a lack of EM in human foetuses, known as fetal akinesia, is particularly associated with retarded or altered skeletogenesis (Verbruggen et al., 2018). While EM has been acknowledged as vital in embryogenesis, most of the focus has been on identifying and mitigating the molecular causes for the lack of movement, while less focus has been given to understanding directly how and why EM affects the embryo's form. Moreover, most investigations have centred on human or mouse model systems, and very little is known about the function or natural variation of avian species. What we do understand of this process in birds comes from a limited number of studies on captive species, primarily chickens (*Gallus gallus domesticus*).

Birds are useful models for understanding EM because it is possible to manipulate EM within the egg and observe the effects on the musculoskeletal system of developing embryos. In chickens, EM of embryos has been manipulated pharmaceutically to induce paralysis or hyperactivity, with striking consequences on development (Germiller et al., 1998; Pitsillides, 2006; Pollard et al., 2017). Paralysed chicks, for example, show pathological effects such as malformation or fusion of cartilaginous joints, reduced muscle tone and stunted growth of the long and craniofacial bones (Hall and Herring, 1990; Hosseini and Hogg, 1991; Müller, 2003). Experimentally manipulated hyperactivity has been shown to increase the number of primary muscle fibres in embryos, which is a key factor determining potential for post-natal muscle growth (Pitsillides, 2006). As is seen in adult animals, embryonic 'exercise' causes muscle to become larger (Felsenthal and Zelzer, 2017; Lemke and Schnorrer, 2017). This presents a possible mechanism by which animals which exhibit exceptional strength in early life, such as brood parasites that must hatch from thick eggshells and/or eject or kill host chicks, might achieve the necessary musculature. In turn, the exact mechanism, that permits the young

of these species to perform these pre- and post-hatching feats is not understood. One such mechanism by which the required strength could be achieved is a greater density of muscle fibres. The chicks of common cuckoos have been shown to have a denser number of muscle fibres per square millimetre in their *mucsculus complexus*, the hatching muscle of their necks (Honza et al., 2015) and this is speculated to be an adaption to their need to hatch from significantly stronger eggshells and may also be linked to their eviction requirements of the parasite chick. This is an example of how a trait associated with brood parasitism – the evolution thicker eggshells – has produced selective pressure on the embryo physiology of the parasite (Honza et al., 2015; Igic et al., 2011). Given the evidence that muscle development is shaped by embryo activity in avian species, increased embryo movement provides a plausible mechanism by which a denser *musculus complexus* could be achieved by common cuckoos.

Beyond its essential role in optimal embryonic development, EM has also been shown to shape the evolution of form and function of the vertebrate skeleton; the forces generated by the muscles during EM can affect epigenetic changes to limb structure and orientation (Botelho et al., 2015a; Danos and Staab, 2010; Vargas et al., 2017). For example, epigenetic changes have been hypothesised to act as a factor in the evolution of toe orientations in birds; Botelho et al., (2014) showed that zygodactyl toe orientation (two digits facing back, two forward) in Psittaformes is produced by muscle action on the forming foot during early development, whereas absence of muscular activity in the embryos of zebra finches (*Taeniopygia guttata*), results in an anisodactyl orientation that is characteristic of passerines. The evolution of these, and other toe orientation in birds, has been integral for them to conquer a large diversity of ecological niches by facilitating perching, grasping, and numerous types of locomotion (Botelho et al., 2015a, 2015b; Vargas et al., 2017). As such, EM likely represents a channel for greater phenotypic plasticity to arise in birds and other vertebrates. Therefore, this may present an explanation for the extreme early-life physiologies that have evolved convergently across brood parasites or may have produced physical traits predisposing certain avian lineages to evolving brood parasitism.

The role of EM in the development of obligate brood parasites has not previously been investigated, despite there being abundant evidence that brood parasites have specialised egg structure and content, and developmental traits which contribute to their successful parasitic reproductive strategy (Davies, 2011). Brood parasites face challenging conditions in their early life. Even before emerging from the egg, these species require greater strength and stamina to

hatch from an eggshell that is structurally stronger (an adaption to prevent ejection or breakage) requiring more pecks to interior surface, over a longer period, to successfully hatch (Honza et al., 2015, 2001; Yoon, 2013). After hatching, in order to successfully fledge from the host's nest, brood-parasitic young must ensure that they receive the bulk of the food provisioned (Hauber and Moskát, 2008; Kilner et al., 2004), by earlier hatching followed by either out-competing or killing host young (Bortolato et al., 2019; Lichtenstein and Sealy, 1998; Soler, 2014). These strategies are often physically strenuous and requires a level of strength, coordination and energy expenditure that is not usually seen in the young of altricial offspring (Blom and Lilja, 2004; Starck, 1993). Eviction of the host's eggs or chicks by common cuckoos, for example, has been shown to be costly in terms of growth and oxidative stress (Anderson et al., 2009; Hargitai et al., 2012). Cuckoo chicks are often required to lift a mass equal or greater to themselves during the eviction process (Grim et al., 2009b). This feat can be expected to exert a significant strain on the skeleton of the newly hatched chick, and potentially cause skeletal damage, if this is not compensated for by increased muscular support or denser/more ossified bones. Altricial offspring hatch in an underdeveloped state, with much of their tissue not fully developed, along with having an unossified skeletons and low muscle mass (Blom and Lilja, 2004; Starck and Ricklefs, 1998). Precocial species, conversely, hatch with their skeletons more ossified and the muscles of the locomotory limbs highly developed (Dial and Carrier, 2012; Starck and Ricklefs, 1998). Obligate brood parasites, with the exception of black-headed ducks (*Heteronetta atricapilla*), are categorised as altricial lineages (Dearborn et al., 2009). However, the ability of brood parasite hatchlings to evict, kill, or out-compete host young belies this definition, as these behaviours would be difficult or impossible with a typical altricial physiology. An increase in embryonic movement in brood parasites could provide a mechanism to develop a stronger musculoskeletal system to perform these activities.

Brood parasitism is not phylogenetically conserved across birds, and has evolved independently in several different bird families (Soler, 2017). However, many avian brood parasites exhibit commonalities in both their adult and chick physiologies and behaviours (Davies, 2011; Krüger, 2007). Embryo movement could provide mechanical stimulation for the development of a stronger musculoskeletal system supporting a parasitic lifestyle, via the mechanisms proposed above, resulting in this convergence between distantly related parasitic species. This may particularly be the case in virulent parasite species whose physical demands

in early life are exceptional. In this study, we aimed to measure the rate of EM over the course of incubation across the full avian phylogeny of brood parasites, their hosts, and their non-parasitic relatives. Here, we test the hypothesis that brood parasites should exhibit a higher degree of embryo movement during their development in order to achieve the strength needed for the demands of their early life, both the effort of hatching from a thick eggshell and the tasks of killing or out-competing host young. We further predict that if increased EM is adaptive for hatchling strength, then highly virulent species will show a more pronounced increase in embryo movement due to the physical exertion required of the young parasite to kill the young of the host.

Methods

Embryo movement quantification

Embryonic movement (EM) was measured using a portable digital egg monitor ("Egg Buddy™", Avitronic Services, Abbotskerswell, Devon, UK) originally designed for monitoring the viability of embryos in avian breeding at both the commercial and domestic settings. The use of the EggBuddy for biological research was validated by Pollard et al. (2016), and has been used by several studies to monitor embryo development and heart-rate in both birds and reptiles (Angilletta et al., 2013; Sheldon et al., 2018). To quantify the frequency of EM, the egg is placed on a rubber cup inside the egg monitor chamber. The monitor transmits a beam of infrared light through the egg and detects any disruption to the beam caused either by movement of the embryo, or the contraction of blood vessels in response to a heartbeat (Pollard et al., 2016). In early incubation, heartrate is detectable before muscle twitching becomes evident. However, as the size and activity of the embryo increases, the heartrate measure becomes unreliable due to the increased muscular movements of the embryo (Pollard et al., 2016), and as such was not recorded.

As a standard protocol, the egg was placed into the chamber of the monitor immediately after removal from the nest or incubator and allowed to acclimatise in the darkened interior for approximately 30 seconds. Longer acclimation periods were not performed to prevent the egg from excessive cooling. Embryo movement was displayed in real-time on the screen of the egg monitor in the form of a moving bird icon, and a 60-second video of the screen was recorded immediately following acclimation and analysed at a later date. The egg was positioned with the long axis of the egg roughly perpendicular to the laser beam, with slight adjustments made to the angle if movement was not initially detected. If no movement was detected after this, no measurement was made. The number of EMs, measured as movements of the bird icon up or down, were counted from playback of the video recordings at 0.5 x speed.

General field methods overview

Embryo movement was measured in the eggs of 14 species, including 5 parasitic species and their hosts (species list in Table 3.1). Host nests were monitored in-situ at several field locations (detailed in **Appendix 1**). Nests of the host or focal (non-parasitic relatives of parasite) species were located and visited frequently during the early egg laying stage to detect brood parasitism. Eggs were marked with pencil or marker upon completion of the clutch for later identification. When a nest was parasitized, it was not disturbed for the first two days of incubation so as not to interfere with natural egg rejection or acceptance by host parents. Eggs were visited from the second or third day of incubation, depending on species, and measurements of embryo movement were taken for the parasite egg and then a randomly selected host egg. The measurements were taken close to the nest to minimise the time that eggs were out of the nest, and eggs were out of the nest no longer than 10 minutes in total. The same host and parasite eggs were then measured again every second day until hatching. Repeat measures were not obtained for some eggs due to clutch loss from predation, host rejection, or other natural factors. The feasibility of estimating exact incubation start dates varied with species and field site, and therefore we sometimes needed to estimate embryo age by candling the egg (Figure 3.1) and assigning a stage system (Hemmings and Birkhead, 2016; Lokemoen and Koford, 1996; Spottiswoode and Colebrook-Robjent, 2007) (see **Appendix item 2**).

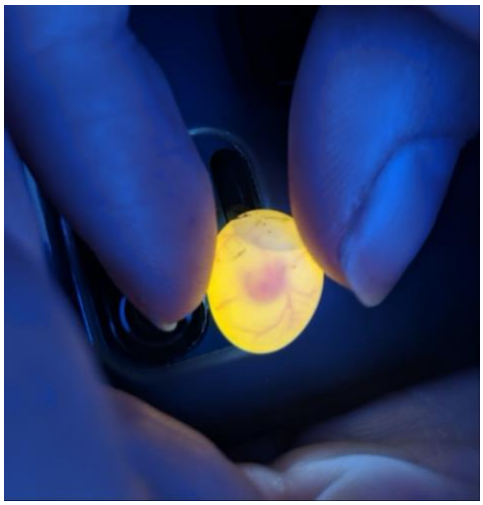

Figure 3.1 Egg candling of a common waxbill (*Estrilda astrild*) egg. The size and shape and level of development of the embryo can be seen by shining light through the egg.

Statistical methods

All statistical analyses were conducted in R (R statistical software Rv3.3.2, R Core Team) using the frontend 'R Studio' (R Studio team 2020). EMR (embryo movement rate) was defined as the number of movements per minute recorded by the egg monitor. Measurements at stage 1 that recorded 0 EMR were excluded from analysis, as false zeros were possible due to the small size of the embryo.

We used phylogenetically-controlled analysis for our comparison of EMR between these 14 species, as species cannot be considered statistically independent due to shared ancestry (Freckleton et al., 2002; Guigueno et al., 2019). The inclusion of two species of non-parasitic cuckoos (white-browed coucals and black coucals) provided within-group phylogenetic control for common cuckoos, as the latter are more distantly related to their hosts than the other paired-species (host-parasite) in these analyses (Krüger and Davies, 2002; Sorenson and Payne, 2001). The honeyguides (Indicatoridae) are a sister group to the barbets (Lybidae) which are hosts to lesser honeyguides, and hence this host provided a suitable comparison. The phylogenetic relatedness of our focal species was constructed and downloaded from the open tree of life and using the 'rotl' package (Michonneau et al., 2016) in R v. 3.3.2 (Figure 3.2). Using this phylogenetic tree, we constructed phylogenetically informed mixed models (PMM) (Garamszegi, 2014) to compare the rate of EMR per stage between all species using the package 'sommer' R v. 4.0 (Covarrubias-Pazaran, 2018). The phylogenetic element of this model allowed us to separate the percentage of variance in EMR that is potentially explained by phylogeny, from any variance that could be attributed to parasitic lifestyle, or other life-history factors. The phylogenetic signal of the trait (EMR) was calculated as the percentage of variance explained by phylogeny as a proportion of the total variance in EMR and is presented as H^2. This value is comparable to Pagel's lambda in other analyses (Hadfield and Nakagawa, 2010). The 'emtrends' function using the package 'emmeans' R v. 1.4.6 (Lenth, 2020) was applied to the PMM to compare the slope of increase in EMR over incubation stage in parasites and non-parasites.

PMMs were constructed with EMR as a response variable and a combination of incubation stage, parasitic status, fresh egg mass, breeding latitude and mean incubation length as predictor variables. Akaike's information criterion (AIC) scores of these models were then compared to determine the best fitting model to explain the data, where the best fitting model

was at least 2 AIC points lower than the next lowest AIC. Neither mean incubation length nor mean breeding latitude of species (values taken from Orme et al., 2006) were retained in the final model as neither were statistically significant and did not improve the fit of the model by >2ΔAIC. Egg mass significantly improved the fit of the model by more than 2 AIC points, and was retained in the final model, but was not statistically significant (1.17 ± 0.94, t = -1.24, p = 0.26). Egg identity was included as a random variable in all models to account for repeated measurements from the same egg at different incubation stages. Similarly, nest identity was included as a random variable to account for eggs which were sampled from the same host nest. The model with the best fit for predicting EMR in these species included fresh egg mass and the interaction of parasitic status and incubation stage as fixed factors, and egg identity and nest identity as random factors.

Species-to-species comparisons were also undertaken using separate linear mixed models (LMM) (using the lmer function in the package 'lmerTest') (Kuznetsova et al., 2017) to examine potential differences between each parasite species and their respective hosts. Common cuckoos, great reed warblers and both coucal species were compared in a single linear mixed model, and post-hoc testing was used to compare species to each other. As with the prior analyses, egg and nest identity was included as a random effect to account for repeated measurements from the same eggs or clutch. Species identity and the interaction between parasitic status and incubation stage were included as predictor variables. As with the phylogenetic models, species breeding latitude was not found to be a significant or informative predictor for EMR and was therefore dropped from the final model.

Results

Using a non-invasive method to measure embryonic muscle twitching, we recorded embryonic movement rate (EMR) as the number of embryo movements per minute, repeatedly measured over the period of incubation, in 437 eggs from 14 species of birds, including five host-parasite systems from three continents. Incubation period was divided into five stages to standardize embryonic development (**Appendix Item 2** and Supplementary Table S3.1) and egg size was accounted for in the analyses. While egg size improved the fit of the model, it did not significantly predict EMR (see statistical methods). After controlling for phylogenetic relatedness (Figure 3.2), we found that brood parasites had a significantly higher overall rate of increase in EMR over the course of incubation (slope of interaction between parasite status and incubation stage) compared to non-parasitic species (phylogenetically-controlled mixed model (PMM), slope ± SE = 7.28 ± 1.85, t = 3.94, p = 0.002, Figure 3.3). Phylogeny explained a small percentage of the observed variance in EMR (H^2 = 0.17 ± SE 0.09), indicating that EMR is not strongly predicted by species position within the phylogeny (i.e., species relatedness). This supports the hypothesis that reproductive strategy (parasitic vs. parental) is the main determinant of EMR over the course of incubation, as opposed to phylogenetic relatedness. Across all species, EMR significantly increased with incubation stage (PMM, estimate ± SE = 16.11 ± 1.08, t = 14.89, p < 0.001, Figure 3.3).

When we compared individual species pairs of hosts and parasites, linear mixed models (LMMs) showed differences between most brood parasite species and their hosts, in the rate of increase in EMR over the incubation period. For instance, common cuckoos had a significantly greater increase in EMR across incubation compared to their hosts, great reed warblers (*Acrocephalus arundinaceus)* (slope ± SE = -7.03 ± 2.66, t_{832} = -2.64, p = 0.008, Figure 3.4), and also compared to one of the two non-parasitic cuckoo species recorded, white-browed coucals (*Centropus superciliosus*) (slope ± SE =12.36 ± 5.64, t_{735} = -2.19, p = 0.03, Figure 3.4), but not the other, African black coucals (*Centropus grillii*) (slope ± SE = 9.25 ± 5.98, t_{765} = 1.55, p = 0.12, Figure 3.4). This suggests that the demands of hatching and virulence in common cuckoos may have driven their relatively high EMR.

Similarly, lesser honeyguides (*Indicator minor*) increased their EMR over incubation at a significantly higher rate than their hosts, black-collared barbets (*Lybius torquatus*) (LMM, slope ± SE = 15.36 ± 7.10, t_{189} = 2.16, p = 0.03). The increase in EMR of lesser honeyguides

was also significantly higher than that of the congeneric, greater honeyguides (*Indicator indicator*) (slope ± SE = 17.81 ± 8.67, t_{182} = 2.05, p = 0.041). Unlike the lesser honeyguides and their hosts, the slope of increase of EMR in greater honeyguides did not differ significantly from that of their hosts, little bee-eaters (*Merops pusillus*) (slope ± SE = 2.91 ± 7.67, t_{191} = 0.38, p = 0.70), which themselves had a relatively high EMR.

Of the two low virulence parasites measured, brown-headed cowbirds (*Molothrus ater*) exhibited a significantly steeper slope of increase of EMR over incubation than their hosts, prothonotary warblers *(Protonotaria citrea)* (LMM, slope ± SE = -12.95 ± 5.90, t_{88} = 2.20, p = 0.03). However, stage 1 cowbirds also had an EMR that was lower than correspondingly aged prothonotary warbler embryos, resulting in the steep slope of increase seen in cowbird eggs (Table 3.1). The other low virulence species, pin-tailed whydahs (*Vidua macroura*), did not significantly differ in the slope of EMR increase compared to their hosts, common waxbills (*Estrilda astrild*) (LMM, slope ± SE = 10.90 ± 8.11 t_{99} = 1.34, p = 0.18). Overall, among parasitic species, we did not find a significant difference between high virulence and low virulence species (LMM, slope ± SE = 6.29 ± 4.26, t_{486} = 1.48, p = 0.14; Figure 3.5, Table 3.1 indicates which parasite species are categorised as high or low virulence). The mean embryonic movement rate (EMR) of each species of parasite and host at each stage of incubation is shown in Table 3.1.

Table 3.1 Mean rate of embryo movement (EMR) per minute and standard errors at each incubation stage (1–5), for parasitic species and their hosts. Parasites are in red. Designation of high virulence or low virulence of parasite species based on Kilner 2005.

Species	**Stage 1 (EMR, mean ± SE)**	**Stage 2 (EMR, mean ± SE)**	**Stage 3 (EMR, mean ± SE)**	**Stage 4 (EMR, mean ± SE)**	**Stage 5 (EMR, mean ± SE)**
Common cuckoos (high virulence)	39 ± 6.7	55.9 ± 4.2	83.0 ± 3.8	111.6 ± 4.4	129.8 ± 9.0
Great reed warblers	35.1 ± 4.4	61.5 ± 4.2	76.3 ± 4.1	92.5 ± 4.3	98.1 ± 9.4
Lesser honeyguides (high virulence)	24.5 ± 3.5	65.8 ± 7.8	82 ± 13.1	101.2 ± 15.9	148 ± 25.4
Black-collared barbets	40.5 ± 9.4	73.8 ± 11.5	73.8 ± 8.43	81.3 ± 6.9	98.0 ± 10.5
Greater honeyguides (high virulence)	76.6 ± 29.7	52.6 ± 13.9	70.7 ± 16.4	88.3 ± 16.8	100.4 ± 16.8
Little bee-eaters	64.2 ± 12	74.0 ± 10.3	70.7 ± 9.1	97.8 ± 13.0	135.0 ± 18.9
Brown-headed cowbirds (low virulence)	39.1 ± 7.8	49.8 ± 12.8	53.3 ± 6.3	88.6 ± 12.8	123.8 ± 16.9
Prothonotary warblers	56.1 ± 13.6	54.0 ± 7.0	104.3 ± 12.2	88.4 ± 13.8	54.5 ± 18.3
Pin-tailed whydahs (low virulence)	NA	50.0 ± 25.6	62.2 ± 12.1	42.7 ± 8.1	84.0 ± 25.6
Common waxbills	6.0 ± SE 1.5	43.7 ± 12.1	59.9 ± 8.3	69.0 ± 10.7	81.3 ± SE 4.6

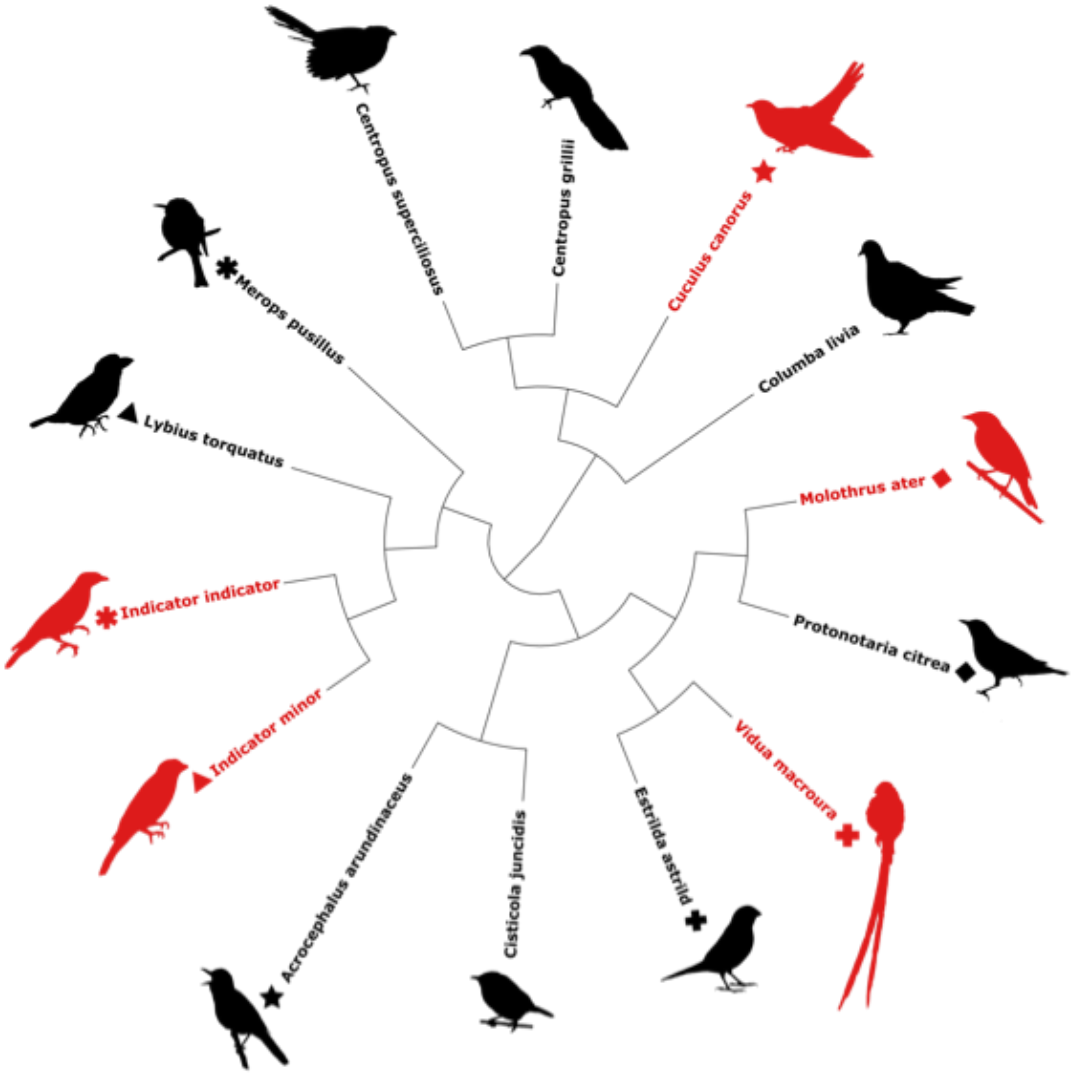

Figure 3.2 Phylogenetic tree showing the species in the phylogenetically informed mixed model. Species in red are brood parasites. Symbol shapes match brood parasites to species they parasitise. Constructed from the "Tree of life database" using the R package 'rotl' (Michonneau et al. 2016). Branch-lengths set at 1.

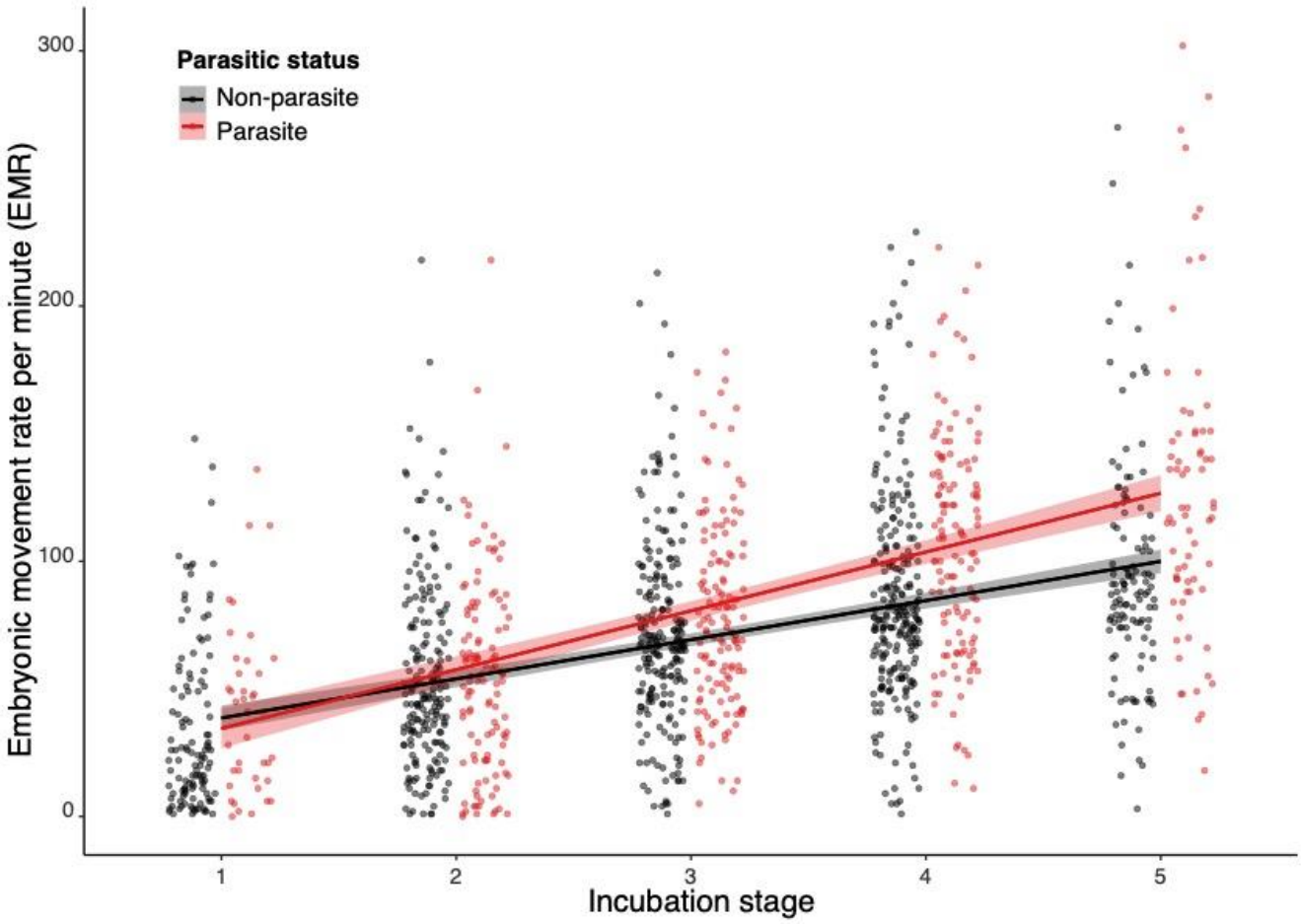

Figure 3.3 Rate of embryo movement over the course of incubation for all parasitic species (red) and all non-parasitic (black) species combined. Shading indicates standard error.

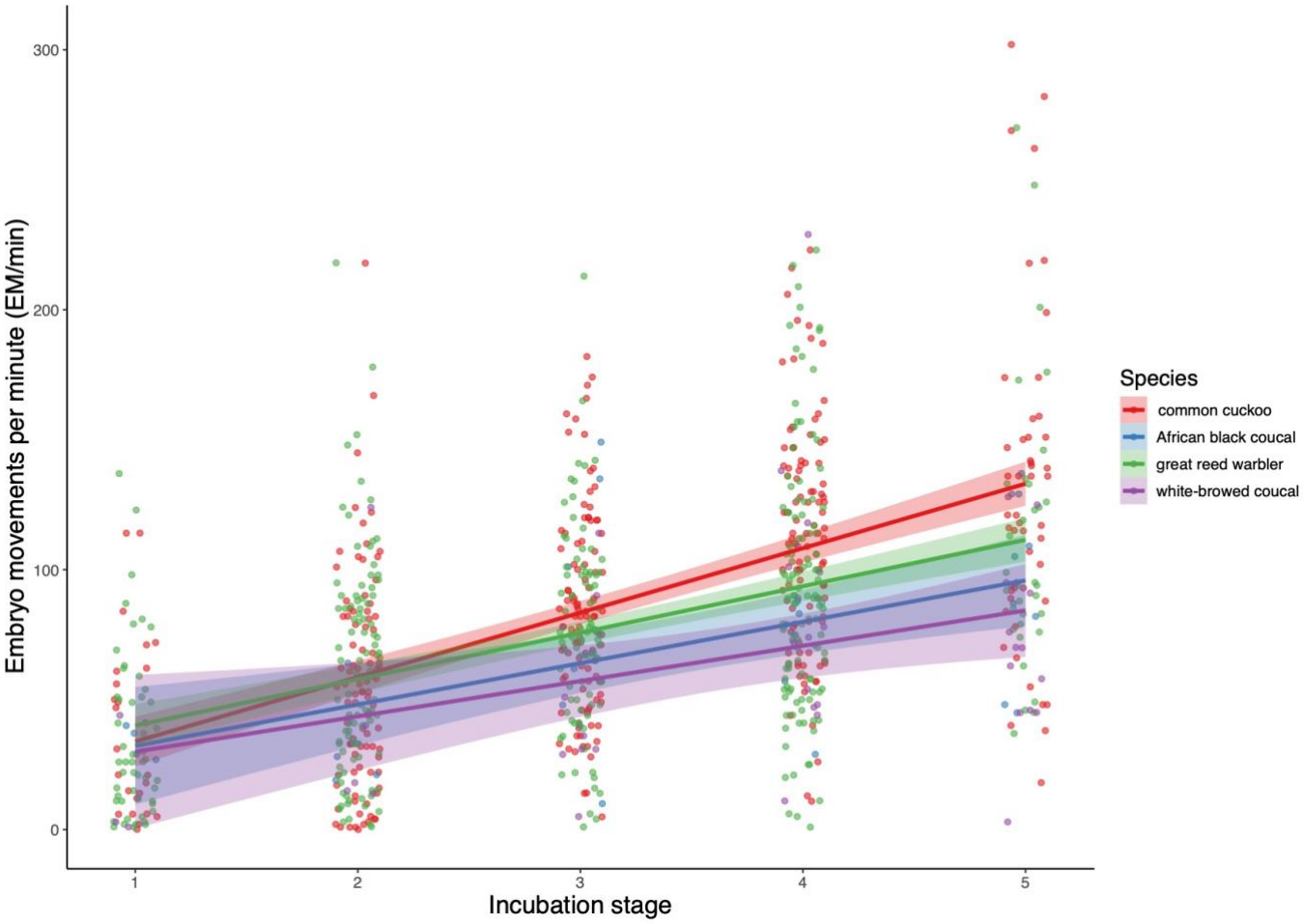

Figure 3.4 Rate of embryo movement over the course of incubation of common cuckoos and their hosts (great reed warbler) and relatives, white-browed coucals and African black coucals. Shading indicates standard error.

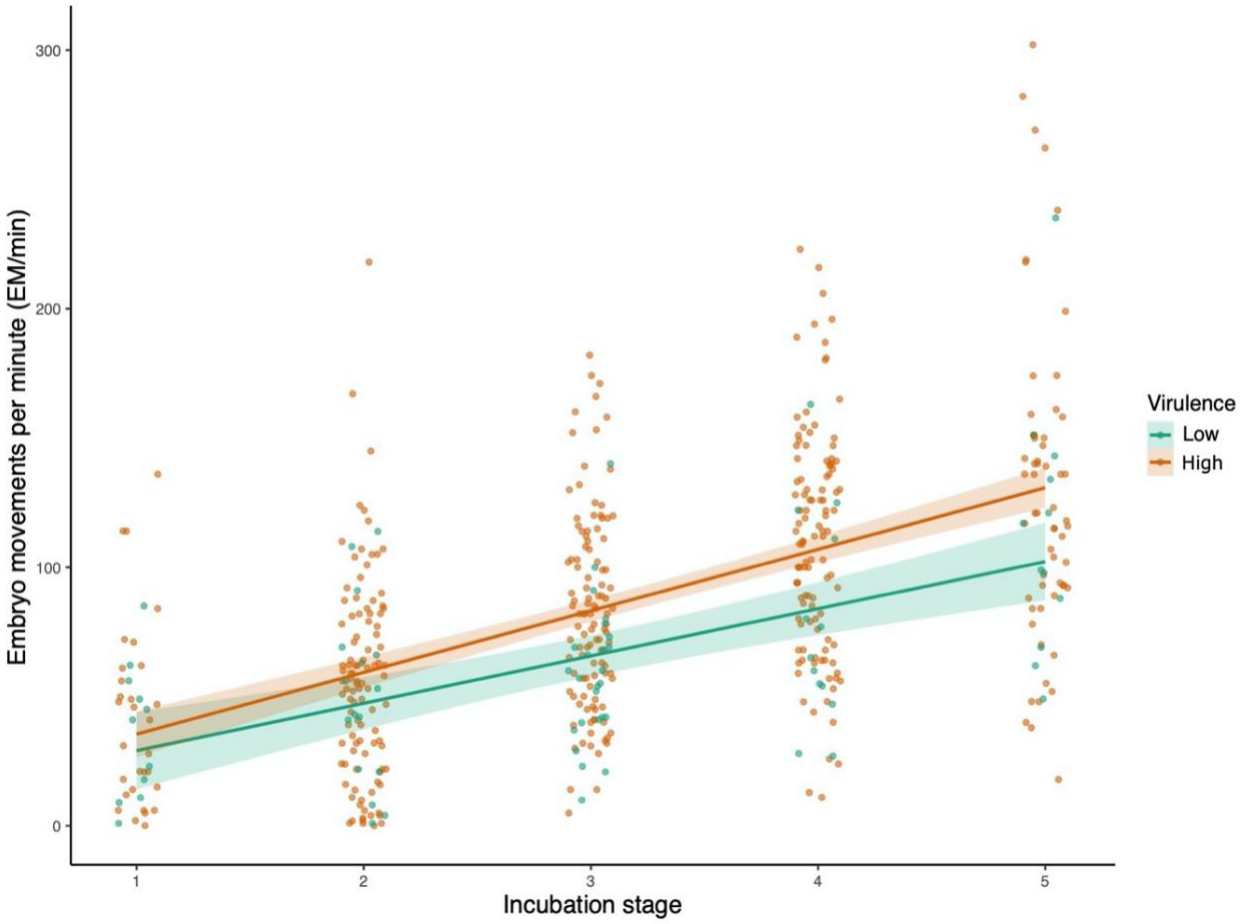

Figure 3.5 Non-significant difference between high virulence brood parasite species and low virulence species. High virulence species (that actively kill host young) comprised common cuckoos, and greater and lesser honeyguides. Low virulence species comprised brown-headed cowbirds and pin-tailed whydahs. Shading indicates standard error.

Discussion

Brood-parasitic species displayed a significantly higher degree of embryonic muscle movement over the course of incubation, in comparison to both their host species and other non-parasitic species. While there was variation between species in the rates of increase in movement over the incubation period, most brood parasite species were found to increase their movement, over time, at a significantly steeper slope compared to that of their hosts or related non-parasitic species. This effect was particularly evident in the later stages of their incubation, where the parasites exhibited especially high EM rates. In particular, common cuckoos, lesser honeyguides and brown-headed cowbirds demonstrated exceptionally high rates of embryo movement near the end of the incubation period. These findings are consistent with our hypothesis that embryonic movement may evolve in response to the demands of brood parasitism on both the pre-hatched embryo and the newly hatched chick. These species of brood parasites represent four of the seven known evolutions of brood parasitism in birds (Payne, 2005; Sorenson and Payne, 2002), suggesting that this embryonic adaptation to a brood-parasitic lifestyle is likely explained through convergent evolution, as has been proposed for reduced conductance across the eggshell (McClelland et al., 2019).

Strength requirements of nestling brood parasites

Brood parasitism impose selection for greater strength both while still in the egg and during the early post-hatch periods. With regards to pre-hatching requirements, increased EM in brood parasites fits with our hypothesis that EM may be adaptive to the tasks of hatching from exceptionally strong eggs. Studies have shown that brood parasites lay eggs with structurally stronger shells, presumably as a defence against breakage during rapid laying or host puncture (Brooker and Brooker, 1991; Igic et al., 2011; Picman, 1997). However, stronger eggshells have been shown to require adaptions to both the embryos physiologically and behaviourally to hatch successfully (Honza et al., 2015, 2001; Yoon, 2013). As such, it follows that an increase in EM throughout incubation, and particularly in late incubation, could facilitate the development of the musculature and stamina required to break out of a significantly stronger eggshell (Felsenthal and Zelzer, 2017). The strength of this selective pressure may also account for the lack of significant difference we found between highly virulent and less virulent parasite

species, as thicker eggshells has been shown to be a commonality across brood parasites regardless of virulence, however the benefits of increased EM for hatching and for the work involved with eviction are unlikely to be mutually exclusive.

Post hatching demands are more variable across parasite species. For example, eviction behaviour of common cuckoos is likely to impose substantial strain on the musculoskeletal system, as the nestling needs to lift heavy eggs or chicks over the barrier of the nest wall which it needs to climb up to evict, a distance that can equal twice their standing height (Honza et al., 2007). This feat often needs to be repeated several times for each host egg and nestmate within a short space of time, often against the steep wall of the host nest (Hargitai et al., 2012). Common cuckoos had a significantly higher rate of EM both compared to their host and compared to at least one of the non-parasitic cuckoo species measured – white-browed coucals – suggesting increased EM to be specifically associated with the parasitic lifestyle of common cuckoos. However, common cuckoos did not have a significantly higher rate than African black coucals, though this may be due to the relatively low sample size for this species.

Honeyguides also kill host nestmates shortly after hatching, in this case by biting and shaking them vigorously (Spottiswoode and Koorevaar, 2012); however, the demands of killing host young differ between greater and lesser honeyguides. Due to maternal egg puncturing behaviour of greater honeyguide females, few host eggs hatch, thus reducing the demands on the parasite chick to remove their competition (Spottiswoode et al., 2011; Spottiswoode and Koorevaar, 2012). Moreover, the host young are a fraction of the size of the greater honeyguide chick. In contrast, lesser honeyguides parasitize black-collared barbets whose nestlings are approximately twice the mass of the lesser honeyguide chicks (Short and Horne, 2001). Furthermore, adult lesser honeyguides don't puncture host eggs, likely due to aggressive behaviour of the adult barbet hosts limiting the parasites time in the nest when laying. As a result, lesser honeyguide young must themselves kill the full clutch of barbet young, often as many as four chicks (Spottiswoode and Colebrook-Robjent, 2007). The observed difference in EM between lesser honeyguides and greater honeyguides therefore likely mirrors the comparatively lower muscular and metabolic demands on greater honeyguide chicks. That these two honeyguide species are of the same genus (Benz et al., 2006) makes this difference particularly striking, as it suggests that EM has the potential to evolve rapidly in response to differences in host behaviour and morphology.

Role of virulence

Contrary to our predictions, we did not find a significant overall difference between brood parasite species that exhibit highly virulent behaviours (i.e. killing host young), compared to those with lower virulence (i.e. outcompeting but not actively killing host young). Brown-headed cowbirds, which we considered low virulence, had significantly higher rates of EM compared to their hosts, the prothonotary warblers. This suggests EM may give an advantage to brood parasite nestlings that is unrelated to the killing of host young. Brown-headed cowbirds, like many other brood parasites, perform intensive begging displays to solicit extra food from parents (Dearborn, 1998; Hauber, 2003). While there is mixed evidence as to whether begging is an energetically expensive activity for nestlings (Kilner, 2001; McCarty, 1996), the intensity and duration of begging, and the height of the begging posture influence food allocation (Kilner, 2002; Leonard and Horn, 1996; Lichtenstein and Sealy, 1998). Nestmate-tolerant brood parasites have been shown to beg for longer periods and with greater success than their host nest-mates (Dearborn et al., 2009; Soler et al., 1999), and therefore muscle development stimulated by EM could be beneficial to acquire the endurance necessary to achieve and maintain exaggerated begging postures and behaviours for long periods of time.

Mechanisms of regulation of EM

We propose that increased EM is an important factor in the embryonic development of brood parasites. What is less clear is how EM is controlled, both at the onset and over the incubation period. The pattern that a species position in the phylogeny did not significantly predict the rate of EM implies that it is under selection in these species hence is not evolving in a neutral (Brownian motion driven) manner; however, molecular work such as genome-wide association studies would need to be undertaken to definitively determine the genes involved or whether EM constitutes an epigenetic source of variation on embryo development depending on how EM is regulated (Frésard et al., 2013; Müller, 2003). There is evidence that environmental factors such as light exposure and temperature can influence EM of avian embryos (Bursian, 1964; Reed and Clark, 2011), so nest environment may play a role in affecting EM. However, were the immediate nest environment the sole factor determining EM, we would not expect to observe such distinct differences between brood parasites and the eggs of their hosts who share the same nest environment. For example, the hosts of brown-headed cowbirds studied here and those of both honeyguide species are cavity nesters, providing an environment where light and

temperature are relatively stable (Amat-Valero et al., 2014) and yet some consistent differences in EM were still detected between hosts and parasite eggs. A potential factor could be the thermal properties of parasite eggs, which due to their thicker shell have been shown to retain heat for longer periods during incubation breaks (Yang et al., 2018), which would influence any temperature-mediated activity of the embryo (Hammond et al., 2007).

Another potential mechanism controlling embryo movement *in-ovo* is hormonal regulation. There is evidence that maternally deposited androgens in the egg effect the embryo growth and early life behaviour of birds, although their role in EM has not been studied to our knowledge (Gil, 2003; Groothuis and Schwabl, 2008). Yolk testosterone has been shown to increase the size of the hatching muscle in red-winged blackbirds (*Agelaius phoeniceus*), but the mechanism of this increase is unclear (Lipar and Ketterson, 2000). However, multiple studies have indicated that brood parasites do not have consistently higher levels testosterone or other androgens in their yolk (Hahn et al., 2005; Hargitai et al., 2010; Hauber and Pilz, 2003; Török et al., 2004).

Certain reproductive traits exhibited by brood parasites, such 48-h laying intervals in cuckoos, have been suggested to have facilitated a transition towards reproducing parasitically (Spottiswoode et al., 2012). Whether a higher rate of EM was a precursor to brood parasitism in such a way or has evolved in conjunction with, or after, brood parasitism is difficult to determine, particularly given how little is known about what modulates EM. Our comparison between cuckoos and their close relatives, the coucals, gave mixed results whereby common cuckoos were higher than one but not the other species of coucal. Therefore, it is unclear whether high EM evolved early in this family and was subsequently lost in at least one species, or whether selection separately increased EM in two or more species in the cuckoo family tree before or after the development of parasitism. The extensive variation in strategies in the cuckoo family, from classical biparental systems, to communal breeding and male-only care to facultative and obligate brood parasitism (Payne, 2005), further obscures attempts to untangle the role of reproductive strategy in this group, and may be enlightened by further study on other branches of the lineage.

Convergent selection pressures for increased embryo movement

Eviction behaviour in common cuckoos and chick-killing in honeyguides have evolved independently as methods of removing competition with host young (Kilner, 2005). This would suggest that any similarity in EM between these species has, likewise, arisen in a similar convergent manner. Enhanced musculoskeletal development stimulated by EM could benefit brood parasite offspring in many ways and may have subsequently been co-opted for the purpose of host-siblicide. Many muscle complexes have multiple functions for which rate of development can be optimised. The *musculus complexus* in the neck of birds is important for the process of hatching and has been shown to be enlarged in the necks of common cuckoos (Honza et al., 2001). However, this muscle complex is also important for begging behaviour as it regulates dorsal flexion of the neck and coordination of head movement, and thereby effects competitive interactions between nestlings (Lipar and Ketterson, 2000). Whether the *musculus complexus* is used in the process of egg eviction or nest-mate killing has not been investigated, although from the postures and motions involved in these behaviours it seems plausible. Therefore, evolution of EM rates to increase this and other muscles in the developing chick could potentially benefit both high virulence and low virulence brood parasites in numerous ways during their early life, and their respective parasitic strategies.

There are likely to be costs associated with higher rates of EM during development. Increasing EM is associated with an increase in aerobic respiration and metabolism which will deplete the energy reserves of the egg more quickly (Hammond et al., 2007; Krischek et al., 2016). Consequently, there are assumed costs associated with EM as well as benefits, and how the energy stores of the egg are used must be optimised for the embryo's development.

While significant differences between species were detected, we also found a high level of intraspecific variation in EM rate across the different incubation stages, and this was particularly evident in later incubation. Increased within-stage variation in the later stages of incubation is possibly due to the influence of circadian rhythms; more developed avian embryos have cycles of activity and rest over the course of the day (Hamburger et al., 1965). While these activity patterns have been measured in domestic chicken eggs, little is known about these cycles in other species. Although we were unable to account for this in the present study, whether brood parasite embryos differ from host embryos in the frequency or length of their wakeful active periods, would be an interesting future avenue of research, particularly

given that environmental cues which typically regulate circadian rhythms are shared with the host's eggs.

Conclusion

The activities of nestling brood parasites are extraordinary not just from a behavioural perspective, but also demonstrates their exceptional physical abilities about which we know relatively little. Here we have shown that the behaviour of the embryo during development might shape the physiology of these birds and could be a key factor in their success as parasites. The pattern we found of increased EM in distantly related brood parasite species, yet not in many of their non-parasite relatives, suggests that this has evolved convergently to deal with the similar challenges they face as young parasites; and EM may even be fine-tuned to differences in host ecology, as the differences between congeneric greater honeyguides and lesser honeyguides suggest. As well as its relevance for the study of avian parasites, these findings suggest that embryo movement may be a generally overlooked process in the evolution of the diverse forms and functions we see in birds.

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Chapter 3 Supplementary Material

Supplementary Table S3.1 Number of days after start of incubation that correspond to incubation stage for each species studied. 'Unk' means this information is unknown. "-" represents no data collected for these stages. "Inferred" means embryo stage was estimated based on proportion of incubation period completed as a proportion of mean incubation period for that species, and informed by published resources on development patterns in altricial species [**Appendix item 2**.]. "Field observation" means embryo stage was determined by candling in the field (see **Appendix item 2**). Sample size shows number of eggs from which EMR was measurements were attained during at least one incubation stage.

Species	**Stage 1**	**Stage 2**	**Stage 3**	**Stage 4**	**Stage 5**	**Method of egg staging**	**Sample size (*n*)**
Great reed warblers (*Acrocephalus arundinaceus)*	3,4	5,6	7,8	9,10,11	12,13,14	field observation	95
African black coucals (*Centropus grilli*)	7,8	9,10	11,12,	13,14	15,16	Inferred	30
White-browed coucals (*Centropus superciliosus*)	7,8	9,10	11,12	13,14	15,16	inferred	39
Zitting cisticolas (*Cisticola juncidis)*	1,2	3,4	5,6	7,8	9,10	field observation	17

Common cuckoos (*Cuculus canorus*)	2,3	4,5	6,7,	8,9,10	11,12	field observation	68
Common waxbills (*Estrilda astrild*)	2,3	4,5	6,7	8,9	10,11	field observation	22
Greater honeyguides (*Indicator indicator)*	unk	unk	unk	unk	unk	field observation	13
Lesser honeyguides (*Indicator minor*)	unk	unk	unk	unk	unk	field observation	13
Little bee-eaters (*Merops pusillus*)	unk	unk	unk	unk	unk	field observation	38
Black-collared barbets (*Lybius torquatus*)	unk	unk	unk	unk	unk	field observation	58
Brown-headed cowbirds (*Molothrus ater*)	2,3	4	5, 6	7,8	9,10	inferred	17
Prothonotary warblers (*Protonotaria citrea*)	3,4	5,6	7,8	9,10	11,12	inferred	14
Pin-tailed whydahs (*Vidua macroura)*	-	3,4	5,6	7,8	9,10	field observation	13
Domestic pigeons (*Columba livia*)	3,4,5	6,7,8,9	10,11,12,13	14,15,16,17	18,19,20	field observation	9

CHAPTER 4. How much calcium to shell out? Eggshell calcium carbonate content is greater in birds with thinner shells, larger clutches, and longer lifespans

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Abstract

The avian eggshell is a bio-ceramic structure that protects the embryo. It is composed almost entirely of calcium carbonate and a small organic component. An optimal amount of calcium carbonate in the eggshell is essential for the embryo's development, yet how the ratio of calcium carbonate to organic matter varies between species has not been investigated. Calcium is a limiting resource for most birds, so its investment in their eggs should be optimised to a bird's life history. We measured relative calcium carbonate content of eggshells in 222 bird species and tested hypotheses for how this trait has evolved with these specie's life history strategies and other traits of their respective egg physiologies. We found that; 1) eggshell calcium carbonate content was positively correlated with species' having thinner eggshells, and smaller than expected eggs relative to incubating parental mass, 2) species with small mean clutch sizes had lower calcium carbonate content in their eggshells, and 3) for species with larger clutch sizes, eggshell calcium carbonate content was negatively correlated with their mean lifespan. The pattern of lower eggshell calcium carbonate in longer-lived, larger clutched, birds suggests that calcium provision to the eggshell has long term costs for the individual.

Introduction

Life history theory explains what determines when, how, and to what extent reproduction should occur for an organism to optimise its individual fitness (Stearns, 2000). A key aspect of these reproductive strategies is investment in individual reproductive bouts versus self-maintenance, and the spreading of investment over multiple reproduction attempts (Stearns, 2000; Williams, 1966). The avian eggshell, as an extension of both a bird's phenotype and its life history, is under the influence of strong selective factors, since embryonic development and reproductive success are highly dependent on the optimal functionality of the eggshell (Birchard and Deeming, 2009; Carey, 1983). Birds' eggshells have evolved many specific adaptations in their composition and structure for ensuring successful embryonic development across different life histories, nest environments, and climatic conditions (Attard and Portugal, 2021; D'Alba et al., 2021). Egg production provides a critical example of life history theory in action as the investment into an egg and/or clutch will greatly influence the quality of that offspring, but conversely, will reduce the parent's resources for both immediate self-maintenance and future reproductive investment (Erikstad et al., 1998). This trade-off has been explored in the context of egg contents (Williams, 1994), such as androgen deposition in the yolk (Merrill et al., 2019; Tschirren et al., 2009) and pigment deposition in the shell matrix (Hodges et al., 2020), yet the production of the eggshell itself and its composition has not been considered within the same framework.

The avian eggshell performs multiple functions to enable and facilitate embryonic development. The eggshell provides a rigid armour to protect the developing embryo from mechanical damage and acts as a physical barrier to microbial infection (Hincke et al., 2012). Moreover, the eggshell controls the appropriate exchange of heat, water, and respiratory gases with the immediate nest environment (Ar et al., 1974), while also providing a reservoir of calcium and other trace minerals for absorption by the developing embryo (Igic et al., 2017; Österström et al., 2013). Simultaneously, pigment deposited on the outer surface can play an important role in varied behaviours, such as crypsis, thermoregulation and sexual signalling (Cassey et al., 2011; Maurer et al., 2012; Romanoff and Romanoff, 1949). The evolution and adaptations of the eggshell has allowed birds to breed in almost all terrestrial environments and habitats globally (Hauber, 2014). A key component of this success has been the presence of calcium carbonate in the eggshell in the form of calcite (Carey, 1983; Packard and Packard,

1980). How calcite crystals form to produce the structure of the eggshell has been rigorously studied (Dauphin et al., 2018; Nys et al., 2010), and the detrimental impacts of calcium deficiency on reproduction are well established (Graveland and Drent, 1997; Reynolds, 2001). Despite this, the quantity of calcium carbonate in the shell has rarely been considered as an evolved trait in bird species (but see Igic et al., 2011), even though broad-scale macro-ecological studies have found global patterns in egg shape (Stoddard et al., 2019), egg size (Figuerola and Green, 2006; Martin et al., 2006) and shell pigmentation (Cassey et al., 2010; Kilner, 2006; Wisocki et al., 2020).

Eggshells are sophisticated bio-ceramic structures consisting of a calcium-based mineral structure interwoven with an organic protein matrix (Hernández-Hernández et al., 2008; Hincke et al., 2012; Polat and Sayan, 2020). Calcium carbonate is believed to make up approximately 98% of the eggshell for most bird species (Reynolds and Perrins, 2010; Romanoff and Romanoff, 1949), though the variation across species has not been previously explored. An appropriate amount of calcium carbonate deposited in the eggshell is essential for the embryo to develop correctly, as incomplete calcification of the shell can lead to overly large pores and desiccation, while excess calcium can lead to severely reduced gas exchange (Nyholm and Myhrberg, 1977; Reynolds and Perrins, 2010). Insufficient calcium in the shell can also cause the embryo to become hypocalcemic resulting in retarded growth, or in extreme cases, death (Dunn and Boone, 1977, 1976).

Here we investigate the macro-phylogenetic patterns present in eggshell calcium carbonate content across a large number of diverse avian species, and investigate the relationship between eggshell calcium carbonate to organic component ratio and a species' life history traits. Many life history traits can be expected to impose constraints or trade-offs in the amount of calcium allocated to the eggshell. Calcite or its isoforms cannot be stored to any significant amounts in most avian bodies (Pahl et al., 1997; Reynolds and Perrins, 2010), though cyclic osteoporosis can provide a portion of the calcium for egg formation in some species (Larison et al., 2001). As such, this mineral must be obtained from the mother's diet during egg formation (Reynolds and Perrins, 2010). Acquiring sufficient calcium for egg production for many species requires behavioural adaptations such as diet switching, and/or strenuous foraging beyond their normal requirements, and outside their normal ranges potentially increasing inter-territorial disputes (Monaghan and Nager, 1997; Wilkin et al., 2009). It is assumed that the greater the number of eggs produced, the less calcium available to be provisioned to each (Patten, 2007).

The structure of the shell is under differing selective pressures to optimize strength, gas exchange, and hatchability (Attard and Portugal, 2021; Birchard and Deeming, 2009), among other factors, each of which might cause contradicting directional selection on the eggshell calcium carbonate content. We considered a number of pertinent life history traits where there is evidence of selection on other aspects of egg physiology and formulated 10 key hypotheses and predictions with respect to eggshell calcium content in 222 species (Table 4.1). These hypotheses were subdivided based on the framework of Tinbergen's four questions to address variation in eggshell calcium carbonate content between species from a mechanistic, proximate perspective (mechanism and ontogeny) and from a broader adaptive, evolutionary perspective (adaptation and phylogeny) (Bateson and Laland, 2013; Tinbergen, 1963). The goal of our novel investigation into macro-evolutionary patterns of a key eggshell trait was to explore new associations between eggshell content and avian life history, phylogeny and physiology.

Table 4.1 Hypotheses and predictions with supporting rationale, of how eggshell calcium carbonate content in birds relates to life history strategies and eggshell characteristics. Hypothesis are divided based on Tinbergen's four question structure.

Level of question/ prediction	Hypothesis	Prediction	Rationale and / or proposed mechanism
Mechanism	1) Thicker eggshells are achieved through greater deposition of calcite but not matrix during layer formation resulting in higher relative calcium carbonate content of thicker eggshells.	Species with eggs that have thicker shells also produce shells with higher calcium carbonate content compared to species with thin-shelled eggs.	The crystalline structure of the shell is believed to be controlled primarily by the organic matrix, which modulates the deposition of calcium from the uterine fluid (Nys et al., 1991; Rodríguez-Navarro et al., 2015). Selection for thicker eggshell could increase the binding of calcite crystals to the organic matrix during shell formation.
	2) Calcium carbonate content of eggshells will be influenced by diet.	Species with diets that are normally higher in calcium invest more calcium in their eggshells.	The majority of calcium needed for egg production must be obtained from their diet during egg formation (Reynolds and Perrins, 2010).
	3) Eggshell pigmentation has evolved to compensate for lower calcium carbonate content.	Pigmented eggshells contain less calcium carbonate than immaculate eggshells.	In great tits (*Passer major*) and Eurasian sparrowhawks (*Accipiter nisus*) calcium stress and eggshell thinning has been correlated with more pigmented eggshells, suggesting protoporphyrin pigment might be used to strengthen eggs in compensation for lacking calcium (Cherry and Gosler, 2010; Gosler et al., 2011). However, in another species (black-headed gulls; *Larus ridibundus*) the correlation between pigmentation and shell thinning was found to be weak (Maurer et al., 2011).
	4) Species eggshell calcium carbonate content will be adjusted to their breeding latitude as a result	Species breeding at higher latitudes (further from the equator) will have a	Multiple egg traits are known to vary latitudinally both at an inter- and intra-species level, believed to be a response to variation in temperature and solar radiation (Boyer et al., 2010; Gómez et al., 2018; Wisocki et al., 2020).There is evidence that thicker eggshell can

	of calcium availability and selection for thicker shells in colder climates.	higher calcium carbonate content in their eggs.	retain heat longer, which may benefit species breeding at colder latitudes (Yang et al., 2018), which lead to greater calcium carbonate content in theses eggs. Additionally, calcium availability in the environment is known to increase in higher latitudes (Patten, 2007).
Ontogeny/Proximate	5) Precocial species deposit more calcium overall into their eggshell in order to supply the higher demand for embryonic growth without compromising the integrity of the eggshell through excessive thinning.	Eggshell calcium carbonate content is higher in species with precocial modes of development.	Nestlings of precocial species hatch in a more developed state than those of altricial species, in particular, they have a more ossified skeleton and muscles, and larger brains (Karlsson and Lilja, 2008). This requires greater sequestration of calcium during development, which is supplied by a greater number of mammillary tips of the eggshell (Karlsson and Lilja, 2008; Österström et al., 2013).
	6) Incubation period influences calcium carbonate content.	Species with longer incubation periods will have more calcium carbonate in their eggshell.	Longer incubation period requires less porous eggshells to prevent excessive water loss, and as a result may have denser eggshell produced through greater calcite crystal deposition (Rahn and Ar, 1974). Zimmermann and Hipfner, (2007) show an evolutionary relationship between eggshell porosity and incubation length in Alcidae species.
Adaptation/ Ultimate	7) Calcium carbonate content will be influenced by reproductive investment (clutch size).	Calcium carbonate content decreases with increasing clutch size	(Patten, 2007) suggested that the evolution of clutch size is influenced by the availability of calcium in the breeding habitat. This would suggest a strong correlation between clutch size and eggshell calcium content
	8) A species lifespan influences calcium carbonate content per egg.	Lifespan is negatively correlated with calcium carbonate content.	If calcium foraging is an expensive activity, longer lived species might invest less calcium in eggs per clutch in order to conserve energy for future reproductive attempts compared to species which only have the opportunity to breed few times over their short lifespan. There is evidence that lifespan influences egg size and clutch size in birds (Blackburn, 1991).

	9) Eggshell calcium carbonate content is higher in species with eggs that are smaller than predicted for the weight of the incubating parents.	Calcium carbonate content will be predicted by the residual difference between fresh egg weight (as a proxy for egg size) and adult body mass.	Egg traits such as the size, shape, and thickness of eggs has evolved in tight concert with adult body mass, as the egg needs to be able to support the weight of the parent during incubation, yet remain thin enough to allow the chick to hatch (Birchard and Deeming, 2009; Juang et al., 2017). Smaller eggs experience a greater force per unit area of the shell from the weight of the incubating parent and as such could require a higher calcium carbonate content to compensate.
Phylogeny/Ultimate	10) A large component of variation in eggshell calcium carbonate content is correlated with species phylogenetic position.	Calcium carbonate content has a phylogenetic signal close to, but less than, 1 (Pagel's λ) (Pagel, 1999).	Many eggshell characteristics have been shown to strongly covary with phylogenetic relatedness in birds (Brulez et al., 2016; Cassey et al., 2011; Portugal et al., 2014), as such we expect eggshell calcium carbonate content to be similarly correlated to phylogeny.

Methods

Calcium carbonate content (ash) of eggs

All eggshells were obtained from the Destructible Collection at The Natural History Museum Tring, a unique resource containing blown eggs mainly of European breeding birds, identified to species levels but otherwise too data-poor to allow admission to the museum's main collection (Cassey et al., 2010). Due to limitations of the information available for this (destructible) subset of the collection, we did not have specific details about where eggs were collected or the clutch size they were taken from. Eggs were assumed to be freshly laid at collection, due to the small size of blow holes. A small blow hole suggests no substantial embryo was present, as the liquid egg content could be extruded through this narrow opening. All eggshells were cut in half vertically (from sharp to blunt pole) using a diamond-tipped dentist drill (Milnes Bros., Surrey, UK). One half of each egg was weighed on a precision electronic balance (Sartorius, Göttingen, Germany), before being put in an oven at 60 °C to dry to a constant mass. To assess this, all shell halves were weighed individually twice daily, between 09:00-10:00 and 16:00-17:00, until no change in mass was detected for four consecutive weighing sessions, at which point they were considered 'dry'. Following this, each shell half was placed in a small ceramic crucible and weighed with this container. The crucibles with the dry shell were then placed into a muffle furnace (AAF 1100; Carbolite, Hope, UK) for 30 hours at 650 °C to burn off the organic component of the shell. Immediately after removal from the furnace, each crucible with the shell ash was placed in a desiccator to cool down without absorbing moisture from the air before being weighed again. Calcium carbonate content was calculated as the ash mass of the shell half, as a percentage of the dry mass of the shell half. Other inorganic minerals that occur in trace amounts alongside calcium carbonate in the eggshell (e.g., phosphorous and magnesium) where not considered separately as they occur in extremely small quantities (<0.1% of the eggshell) (Clunies et al., 1992; Itoh and Hatano, 1964).

Life history and physical egg traits

Life history and ecological data were gathered primarily from the Handbook of the Birds of the World Volumes 1–13 (Del Hoyo, J.; Elliot, S.A. & Sargatal, 1992), and cross-referenced with Birds of the Western Palearctic (Southern and Cramp, 1978). Body mass of adult birds was taken as a mean of the mass of both sexes, primarily from the Handbook of Avian Body Masses (Dunning, 2007). Residual variation in egg size was calculated as the residual variance of each species from the predicted values of a linear correlation between $\log_{10}$ corrected body mass and $\log_{10}$ corrected fresh egg mass. Lifespan was extracted from (Storchová and Hořák, 2018), and mean breeding latitude was calculated from (Orme et al., 2006). Clutch size data were collected as mean number of eggs but subsequently divided into two categories, with species producing either a single egg or two eggs per clutch categorised as 'small', and all other species categorised as 'large'. This is due to an unequal distribution of clutch sizes in the data (Supplementary Figure S3.1) and preliminary results supporting a categorical rather than a continuous effect of clutch size. Species mean eggshell thickness values were extracted from (Maurer et al., 2012).

Statistical analyses

All statistical analyses were conducted in R statistical software Rv3.3.2 (R Core Team 2020) through the Integrated Development Environment 'R Studio' (R Studio Team 2020). A phylogenetic tree was constructed for the 222 species included in this study from the Open Tree of Life project, using the R package 'rotl' (Michonneau et al., 2016), which constructs a tree using multiple taxonomies as a backbone. The strength of the phylogenetic signal (Pagel's λ) in calcium percentage of the eggshells, was estimated on the mean values for each species, using the 'phylosig' function in the R package 'Phytools' (Revell, 2012). The r package 'caper' (Orme, 2013) was used to construct phylogenetically informed least squares models (PGLS) using the constructed phylogenetic tree. In these models we were able to include phylogeny and Pagel's λ as a covariance matrix, thereby accounting for phylogenetic non-independence of the residual error in the response variable (calcium carbonate content). Pagel's λ was assigned by maximum likelihood in all models (Freckleton et al., 2002).

Calcium carbonate percentage was first arcsine transformed to account for the proportional nature of the data, and then $\log_{10}$ transformed to account for a non-normal distribution

(Supplementary Figure S3.2). This was our response value in the subsequent models and tested against life history and physiological traits as predictors. PGLS models require a single response value per species, as such mean calcium carbonate content was determined for each species. To test our hypotheses, candidate PGLS models (Revell, 2010) were constructed with combinations of the following predictors: log eggshell thickness (mm), residual egg size variance relative to adult body mass (g), precociality – assigned categorically by whether or not eyes are open at hatching (precocial/altricial), mean clutch size (small (≤ 2) or large (*2.5 to 16*)), mean incubation period (days), species mean breeding latitude (degrees), species diet (omnivore or carnivore, no herbivores were available in the dataset), mean lifespan (years), and whether eggs are pigmented or immaculate (yes/no). Several two-way interactions were also included in PGLS models, listed here (*denotes interaction): log eggshell thickness*calcium diet, log eggshell thickness*precociality, log eggshell thickness*clutch size, lifespan*clutch size, clutch size*precociality, mean incubation period*clutch size, mean incubation period*precociality, and lifespan*precociality.

These candidate models were ranked based on Akaike Information Criterion values corrected for small sample sizes (AICc), and model averaging was applied to all models (n=3) which could not be rejected based on having an AICc score within 2 points of the lowest AICc valued model (Burnham and Anderson, 2004). The R software package 'MuMIn' was used for model selection and averaging (Bartoń, 2009). The averaged model produced contained only the predictors: log eggshell thickness, residual egg mass, lifespan, clutch size, latitude, and the interaction between lifespan and clutch size (Supplementary Table S4.1).

PGLS models can only compare mean calcium content value (Mundry, 2014) and do not account for intraspecies variability; to account for this we further constructed a phylogenetically informed multivariate mixed model (PMM) (Brommer et al., 2019), which included all measurements per species (samples per species varied between N = 1 and 5), tested against the predictors of the averaged PGLS model list above. The PMM was fitted with the package 'sommer' R v. 4.0 (Covarrubias-Pazaran, 2018), using the same phylogenetic tree described above. The phylogenetic tree (Figure 4.1) was visualised using 'ggtree' package (Yu, 2020).

Results

Our final PMM (containing predictors identified by model averaging of PGLS candidate models, Supplementary Table S4.1) contained the predictors log shell thickness and residual egg size variance, as well as key life history traits of clutch size, lifespan, the interaction between clutch size and lifespan, and mean breeding latitude. All other predictors and interactions were not retained in the averaged model set of PGLS models, indicating these variables neither improved the fit of the model nor were significant predictors of eggshell calcium content, and as such were not included in the PMM. There was an effect of phylogeny on mean eggshell calcium carbonate content with an intermediate Pagel's λ value of 0.82, which was significantly different from both zero and one ($p > 0.005$, 95 % CI: (0.686, 0.906), suggesting close relatives were correlated in the values of eggshell calcium content, though less than would be seen under a strict Brownian motion model of evolution.

Calcium carbonate content was negatively correlated with eggshell thickness (estimate = -0.04, SE ± 0.006, $t = 6.86$, $p < 0.005$), after accounting for phylogeny, with thicker eggshells having a lower calcium carbonate content (as a percentage of dried shell mass) than thinner eggshells (Figure 4.1). There was a significant effect of residual variation in adult body mass relative to egg mass on eggshell calcium carbonate content (estimate = -0.01, SE ± 0.005, $t = 2.46$, p= 0.01), indicating that species with eggs that were larger than expected for their adult body mass had a higher eggshell calcium carbonate content. Calcium carbonate content was also predicted by clutch size, with species with smaller clutches having lower eggshell calcium carbonate content (estimate = -0.02, SE ± 0.007, $t = 2.85$, p= 0.004). Additionally, there was an interaction between clutch size and lifespan on calcium carbonate content (Figure 4.2): among species with a clutch size over two eggs, calcium carbonate content of eggs decreased with increased lifespan, however, this effect was not evident in species with less than an average of 2.5 eggs per clutch (interaction, estimate = 0.0007, SE ± 0.0002, $t = 3.13$, $p = 0.002$). There was also a pattern of lower eggshell calcium carbonate content at higher breeding latitudes (estimate = 0.0001, SE ± 0.00005, $t = 2.63$, $p = 0.012$). Lifespan alone was not a not significant predictor of eggshell calcium carbonate content ($p = 0.99$) outside of the interaction with clutch size. The high value of phylogenetic signal (H^2=0.80 ± 0.04) of the PMM (accounting for intraspecific variation) was consistent with the high Pagels λ value found for mean calcium carbonate content.

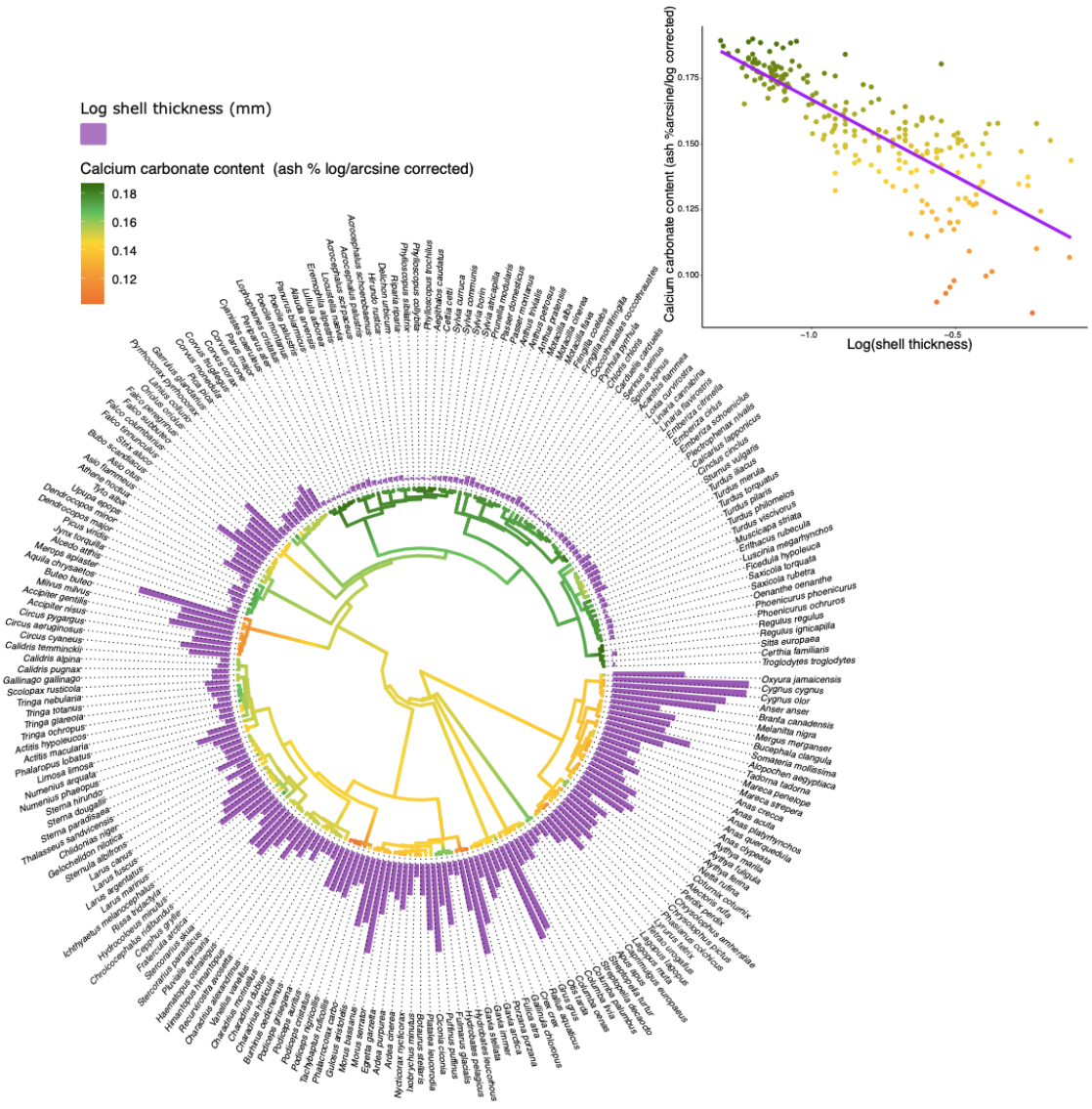

Figure 4.1 Phylogenetic tree of mean eggshell calcium carbonate content (ash % of dry eggshell mass) of species eggs. Phylogenetic tree of all included species (n=222) generated from the open tree of life (Michonneau et al., 2016). Branch colour represents ancestral reconstruction of eggshell calcium content (log Arcsine of eggshell calcium %) with green representing higher calcium carbonate content and orange representing a lower content. Purple bars display log eggshell thickness (mm) of each species. Inset graph: calcium carbonate content (ash % of dry eggshell mass) predicted by (log) eggshell thickness.

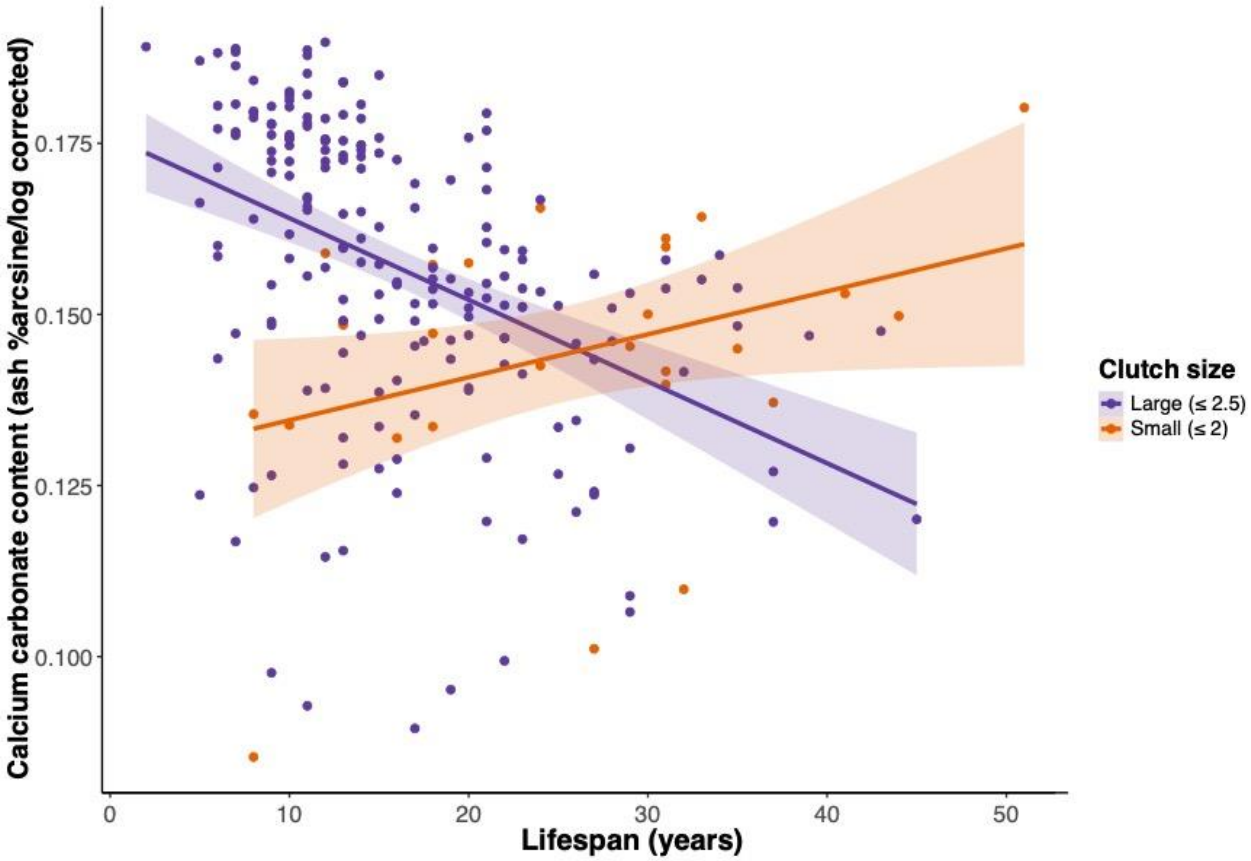

Figure 4.2 Mean carbonate calcium content (ash % of dry eggshell mass) of species eggs in relation to lifespan and clutch size (eggs/nest). Mean eggshell calcium carbonate content of 222 species (Log – Arcsine transformed) calculated from 817 eggs, showing ash % decreases with increasing lifespan in species with large sized clutches, but not species with small clutches (t = 3.13, p = 0.002). The regression lines are representative of linear regression, not corrected for phylogenetic relatedness.

Discussion

Our results support several of the proposed hypotheses, such as a species lifespan and clutch size dictating its eggshell calcium investment, while also showing an interesting negative correlation with eggshell thickness, which was opposite to our predictions (Supplementary Table S4.2). We found that differences observed in eggshell calcium carbonate content covary with a combination of physiological traits (eggshell thickness, and egg mass) and life history traits (lifespan, clutch size and breeding latitude). We found a phylogenetic signal in the variation in eggshell calcium carbonate content between species that was stronger than would be expected if this trait was evolving neutrally (Brownian-motion model of evolution) (Pagel 1999), meaning that closely related species were more similar to one another than distantly related species, as a result of shared ancestry (Freckleton et al., 2002). This would suggest that calcium carbonate content is under strong genetic control, as is the case for other known calcium-related eggshells properties such as calcite crystal size and organisation (Dunn et al., 2012).

Some of our results – lower eggshell calcium carbonate content in longer lived, large clutched species – indicate that the allocation of calcium in avian eggshell production is likely to be a feature of life history evolution to maximize lifetime fitness. These findings complement current understandings of life history evolution (Stearns, 2000), assuming calcium deposition in eggshells is costly to the female (Monaghan and Nager, 1997; Reynolds and Perrins, 2010). Species with shorter lifespans are likely to have fewer opportunities to reproduce and, as such, are more likely to invest heavily in the few broods that they do produce (Stearns, 2000). In contrast, long-lived species may reserve energy and resources for future reproduction at the expense of their current reproductive effort (Erikstad et al., 1998). Excess calcium is not known to be stored in the body long-term in most birds, meaning that current investment of calcium into a brood is unlikely to significantly impact future calcium availability (Reynolds and Perrins, 2010). However, the investment of calcium into a clutch of eggs may have other costs to future reproduction. The calcium needed for egg production must be acquired from the environment within a brief window prior to egg laying, in order to increase circulating calcium (Monaghan et al., 1998; Pahl et al., 1997; Reynolds and Perrins, 2010). This requires strenuous foraging, often for food sources that differ from the usual diet or requiring extraterritorial excursions, which increases energy expenditure and predation risk for the female (Monaghan

and Nager, 1997; Reynolds et al., 2004; Wilkin et al., 2009). The extent of calcium-targeted foraging can have an impact on body condition and, therefore, probability of survival to the next breeding season (Mänd and Tilgar, 2003). Females of many bird species are believed to be osteoporotic during egg laying as a result of calcium sequestration from medullary bones (Reynolds and Perrins, 2010), especially where dietary calcium is limited (Larison et al., 2001), resulting in higher susceptibility to skeletal fractures (Whitehead and Fleming, 2000). Reducing the calcium carbonate content of eggshells might, therefore, present a trade-off between producing eggs with a strong shell and bountiful calcium supplies for the embryo, or optimizing lifetime reproductive output by producing many clutches of eggs with sufficient but less than ideal eggshell calcium carbonate content.

For bird species with small clutches (one or two eggs), there was no statistical effect of lifespan on eggshell calcium carbonate content. Overall, species with small clutch sizes had lower calcium carbonate content per eggshell than other birds. Investment strategies of species producing such 'micro-clutches' might differ from the investment strategies predicted in larger clutched birds (Brockelman, 1975; Jetz et al., 2008). One theory of clutch size evolution is that greater risk of predation selects for smaller clutches (Slagsvold, 1982; Fontaine and Martin, 2006). As small clutches are associated with species under high predation risk (Styrsky et al., 2005; Fontaine and Martin, 2006), it would be strategic to reduce the calcium carbonate content of these eggs, in addition to reducing clutch size, in favour of survival and conserving body condition for future reproductive attempts by the female. This would especially be the case if calcium foraging increases the risk of adult mortality by increasing predation risk, as has been proposed but not tested (Monaghan and Nager, 1997).

There is a global gradient of increasing environmental calcium availability with higher latitude which is thought to have influenced the evolution of bigger clutches at higher latitudes (Patten, 2007). We expected to see higher calcium carbonate content in eggs of birds breeding at higher latitudes due to this greater availability, and potential selection for denser shells in colder climates. However, contrary to this, we found a decrease in proportional calcium carbonate content in eggshells of species breeding at higher latitudes. As this study composed primarily species breeding in the northern hemisphere, increasing latitude corresponded to greater distance from the equator. Although this does not correspond with global calcium availability patterns, or our rationale regarding temperature, there are many other factors that vary latitudinal such as climate and food availability (Gaston, 2000; La Sorte et al., 2014), and as

such it is difficult to identify the root cause of latitudinal variation. Additionally, the present study relied on mean breeding latitudes of these species, as detailed information on collection location did not exist for these eggs. As such, we were unable to account for intraspecific variation in latitude. Future studies should consider intraspecific variation and compare high latitude, temperate, species to those endemic to the tropics where environmental calcium availability is dramatically lower (Patten, 2007).

In addition to correlations with life history traits, there was a strong negative pattern between species eggshell thickness and eggshell calcium carbonate content. This is likely to be linked to the strength requirements of the eggshell, which needs to be finely balanced between being strong enough to support the body mass of the incubating parent while also remaining breakable from the inside for the chick to hatch (Ar et al., 1979; Birchard and Deeming, 2009). Eggshell strength increases with eggshell thickness (Ar et al., 1979), although other factors such as egg shape or calcite crystal size and orientation also influence strength (Juang et al., 2017; Athanasiadou et al., 2018; Soler et al., 2019). However, our results indicate that the increased strength with increasing thickness may not be achieved through greater calcium carbonate deposition, but rather a thicker eggshell may achieve this greater strength via alternative mechanisms. The eggshell is formed by the precipitation of calcium carbonate from the uterine fluid to form calcite crystals on the surface of the egg membrane (Hincke et al., 2012). The formation of these crystals, particularly the unit size of each crystal and how they orientate to and interlock with each other, is controlled by the organic component of the eggshell (Athanasiadou et al., 2018; Hincke et al., 2012). Moreover, this is highly heritable (Dunn et al., 2012) and largely determines the strength of the shell (Panheleux et al., 1999; Soler et al., 2019). An increase in osteopontin, a major component of the organic portion of the shell, leads to smaller crystal units in the nanostructure of the shell which increases the overall hardness of the material (Athanasiadou et al., 2018; Chien et al., 2008). Additionally the binding of osteopontin to calcite crystals during formation increases fracture resistance (Gautron et al., 2021). The observed lower calcium carbonate content in thicker shelled eggs indicates a greater organic component which could strengthen the shell in such a manner (Athanasiadou et al., 2018). Further investigation into how calcium carbonate content directly correlates with fracture resistance would be useful to elucidate this. Lower calcium carbonate in thicker eggshells may be a constraint of other required properties of the shell, such as flexibility and stiffness, which will vary with allometric scaling and thickness (Juang et al., 2017). Conversely, thinner eggshell might require more calcium carbonate formed into denser

calcite crystals to be strong enough to protect the egg. Eggshell thickness and egg size are strongly and positively correlated (Birchard and Deeming, 2009; Hahn et al., 2017); as a result it is feasible that in smaller eggs, an increase in thickness would increase the required interior breaking force (difficulty for the chick to hatch) to a greater extent than for larger eggs, due to shape and allometry (Ar et al., 1979; Hahn et al., 2017; Juang et al., 2017). As such, smaller eggs may achieve strength through denser calcium carbonate deposition while remaining thin enough for the developed chick to hatch. Further investigation into the role of calcium carbonate content on the structural properties of eggshells would be beneficial to our understanding of how this trait has evolved. Potentially, this association between eggshell thickness, calcium carbonate and size could explain the low eggshell calcium carbonate content seen in small clutches, since eggshells of single egg clutches tend to be larger and hence thicker shelled (Ar et al., 1979, 1974; Birchard and Deeming, 2009; Hahn et al., 2017).

There is a consistent scaling relationship between egg size, eggshell thickness, and the body mass of the incubating parents (Ar et al., 1979; Birchard and Deeming, 2009). We found that eggshell calcium carbonate content decreases as species' residual body mass (body mass relative to egg mass) increases. As such, species with eggs that are small relative to the size of the incubating parent have a lower calcium carbonate content in their eggshells, which would suggest that the shell's ability to support the mass of the incubating parent is not increased with calcium carbonate content. This agrees with the above discussion that a greater organic component could imbue greater strength to eggshells by regulating the organisation of calcite crystals (Chien et al., 2008; Athanasiadou et al., 2018). Additionally, high calcium carbonate content in eggs that are larger than predicted for a species body size would likely represent a substantial investment. Body mass is tightly correlated with skeletal mass in birds (Martin-Silverstone et al., 2015) and will likewise affect the potential quantity of circulating blood calcium that can be maintained during egg production, thereby increasing the rate at which calcium must be obtained during the period of shell formation (Reynolds et al., 2004; de Matos, 2008; Reynolds and Perrins, 2010). This is relevant to our understanding of the costs of egg production and how it effects investment strategies across avian families.

Our findings change the understanding of how avian species allocate mineral resources to their eggs and how this connects with their life history investment strategies. Calcium in eggs has long been acknowledged as an important factor for reproductive success, however, the association with lifespan should make us reconsider the investment costs involved. Along with

the strong phylogenetic signal, this suggest that species are under selection to optimise individual per egg calcium allocation for maximum lifetime reproductive success. This contradicts previous suggestions that calcium allocation to eggshells does not apply a long term cost to breeding females (Williams, 2005). These findings highlight how little we know about the costs associated with calcium acquisition, and what the benefits are to eggshell's structural integrity of a higher or lower calcium carbonate content. Additionally, it is not yet known what genetic factors control calcium allocation during eggshell formation and how flexible this trait is within a species under different conditions. Eggshell thinning as a result of environmental pollution (Lundholm, 1997; Burnett et al., 2013), but also reduced environmental calcium availability (Graveland et al., 1994; Hernández et al., 2018), has had severe detrimental effects on bird populations. Greater understanding of the optimal eggshell composition for a species' reproductive biology and life-history would enable us to better assist breeding programs for endangered birds. The specialisation in shape and microstructure of eggshells has evolved these vessels to be highly optimised for embryo development given a species specificities (Portugal et al., 2014; Stoddard et al., 2019), and these results show how eggshell calcium content has likewise evolved to complement avian life histories.

Acknowledgements

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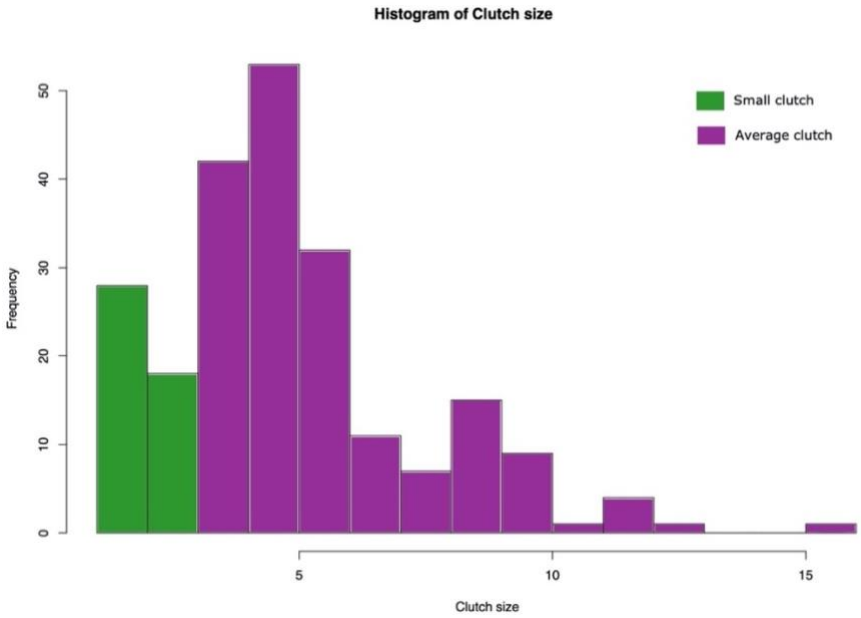
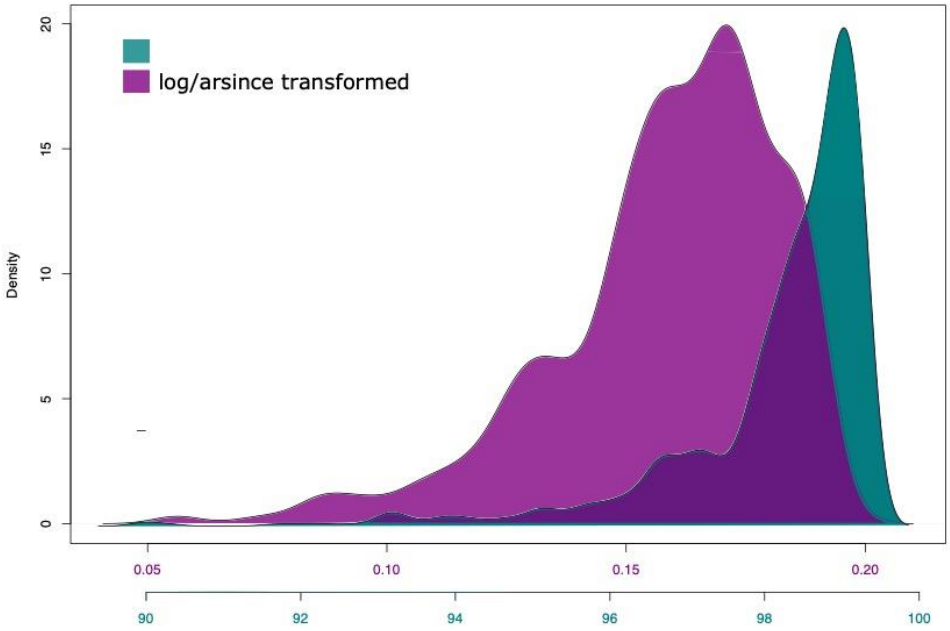
Chapter 4 Supplementary Material



Supplementary Figure S4.1 Distribution of mean clutch sizes of 222 bird species. Categorised as 'small' (≤2, green) or 'large' (2.5 and over, purple).



Supplementary Figure S4.2 Distribution of calcium content values before (blue) and after (purple) transformation (log and arcsine).

Supplementary Table S4.1. Results of model averaging of top fitting PGLS models (Δ AIC < 2)

Component models:

	df	logLik	AICc	delta	weight
123456	7	467.29	-920.06	0.00	0.47
12456	6	465.89	-919.38	0.68	0.33
12345	6	465.36	-918.32	1.73	0.20

Term codes:

Log.Shell.Thickness	clutch.cat	Latitude	Lifespan
1	2	3	4

Clutch size:Lifespan	residual egg mass
5	6

Model-averaged coefficients:
(full average)

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	2.741e-01	2.279e-02	12.027	< 2e-16 ***
Log eggshell thickness	-1.098e-01	1.482e-02	7.410.	< 2e-16 ***
Residual egg mass	2.280e-04	1.734e-04	1.315	0.18867
Lifespan	3.426e-05	3.616e-04	0.095	0.92452
Clutch size	-4.677e-02	2.054e-02	2.277	0.02277 *
Latitude	-1.802e-04	1.835e-04	0.982	0.32594
Clutch size * Lifespan	1.931e-03	7.171e-04	2.693	0.00708 **

(conditional average)

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	2.741e-01	2.279e-02	12.027	< 2e-16 ***
Log eggshell thickness	-1.098e-01	1.482e-02	7.410	< 2e-16 ***
Residual egg mass	2.839e-04	1.469e-04	1.933	0.05329 .
Lifespan	3.426e-05	3.616e-04	0.095	0.92452
Clutch size	-4.677e-02	2.054e-02	2.277	0.02277 *
Latitude	-2.707e-04	1.615e-04	1.677	0.09358 .
Clutch size *Lifespan	1.931e-03	7.171e-04	2.693	0.00708 **

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Supplementary Table S4.2 Support or refute of suggested hypothesis by this study. Supported hypothesis coloured in green, refuted hypothesis coloured in orange.

Hypothesis	Supported?	Details
1. Thicker eggshells are achieved through greater deposition of calcite but not matrix during layer formation resulting in higher relative calcium content of thicker shelled eggs.	No, inverse to prediction	Calcium content was inversely proportional to thickness of the eggshell, indicating a greater percentage of the eggshell is composed of organic materials in these species.
2. Calcium content of eggshells will be influenced by diet.	No	There was no significant difference between omnivorous and carnivorous species in their respective eggshell calcium content.
3. Eggshell pigmentation has evolved for a complementary structural function in the shell to compensate for lower calcium content.	No	There was no significant difference between species with immaculate or pigmented eggshell.
4. Species eggshell calcium content will be adjusted to their breeding latitude as a result of calcium availability and selection for thicker shells in colder climates.	No, inverse to prediction	Species with a breeding latitude further from the equator has lower calcium content in their eggshells.
5. Precocial species deposit more calcium overall into their eggshell in order to supply the higher demand for embryonic growth without compromising the integrity of the eggshell through excessive thinning.	No	There was no significant difference between species classified as precocial or altricial.

6. Incubation period influences calcium content.	No	Incubation period was not correlated with eggshell calcium content.
7. Calcium content will be influenced by reproductive investment strategy (clutch size).	Yes	Species with small clutches (1 or 2 eggs) had lower calcium carbonate content in their eggshells.
8. A species lifespan influences calcium carbonate content per egg.	yes	For large clutched species, eggshell calcium carbonate content per egg decreased with increasing lifespan.
9. Eggshell calcium content is higher in species with eggs that are smaller than predicted for the mass of the incubating parents.	Yes	Calcium content was positively correlated with a larger discrepancy between adult body mass and egg size, whereby species with smaller eggs than predicted for the mass of the adult bird had greater calcium content in their eggshells.
10. A large component of variation in eggshell calcium content is correlated with species phylogenetic position.	Yes	There was a strong phylogenetic signal in pattern of calcium content among species.

CHAPTER 5. Eggshell composition and surface properties in avian brood parasites compared to non-parasitic species

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Abstract

Obligate brood parasites exhibit multiple adaptations in their eggs to allow them to deceive hosts and optimise development in the hosts nest. The structure and composition of the eggshell is essential for the protection and growth of the embryo, and as such, must be adapted to the challenges faced by parasite eggs in the host's nest. Here we investigate whether the eggshells of brood parasites exhibit specialisation in their surface structure properties and calcium carbonate composition to the demands of a parasitic lifestyle. We measured eggshell properties (wettability, roughness, and calcium carbonate composition) in the eggs of a phylogenetically and geographically diverse range of brood parasites, their hosts, and close relatives of the parasites. Within a phylogenetically-controlled framework, we found no significant differences in wettability or roughness between parasitic species and non-parasitic species, nor were parasite eggs more similar to their hosts in wettability than to the eggs of randomly assigned non-host species. A significant difference, however, was found in the surface roughness between the maculated and non-maculated regions of cuckoo finch (*Anomalospiza imberbis*) eggs, whereas such a difference was not observed between these regions in the eggs of their hosts, tawny-flanked prinias (*Prinia subflava*). Calcium carbonate content of brood parasite eggs was not significantly different from that of non-parasite eggs. The lack of significant differences between parasites and non-parasite species, including hosts, in the measured structural traits suggests general adaptations for embryo development in eggshell properties outweigh any influence of a parasitic lifestyle.

Introduction

Calcified eggshells are ubiquitous amongst all living birds and provide numerous essential functions for embryo health and development (Board, 1982; Hahn et al., 2017). The shell protects the embryo from mechanical, solar, and microbial damage (Carey, 1983; Fecheyr-Lippens et al., 2015; D'Alba et al., 2017), modulates gas exchange (Portugal et al., 2014a; Attard and Portugal, 2021) with the environment, and provides calcium for tissue development (de Matos, 2008; Orłowski and Hałupka, 2015b). The microstructure and outer topography of the eggshell is a highly labile trait that has evolved many variations across bird lineages to meet specific demands of the embryo based on their nest environment and parental incubation strategies (Igic et al., 2015a; D'Alba et al., 2016, 2017;). As such, characteristics of the egg and nest have evolved in close concert in most birds (Nagy et al., 2019).

However, approximately 1% of extant bird species are obligate brood parasites, meaning they do not raise their own young, and instead deposit their eggs in the nests of host species (Davies, 2011; Krüger and Pauli, 2017). These birds have no subsequent impact over the development of their eggs after laying and have no capacity to influence the external conditions the egg experiences during incubation. As such, producing a suitably resistant eggshell for survival in a host's nest can be assumed to be under strong selection (Picman and Pribil, 1997; Antonov et al., 2012). For example, while many brood parasites specialise on a single host species, and hence a single nest type, others are generalists that parasitise hosts breeding in a wide variety of nest types and habitat, from cups and domed nests to tree cavities and burrows, requiring their eggs to be adapted to these very different conditions (Antonson et al., 2020; Feeney et al., 2012; Moksnes and ØSkaft, 1995). Selection for egg size, egg shape and eggshell pattern mimicry of host eggshells by avian brood parasites has been intensively studied (Attard et al., 2017; Spottiswoode, 2013; Stoddard and Hauber, 2017; Stoddard and Stevens, 2011), yet less attention has been given to the mechanical adaptations of the parasite eggs, which if the host does not reject the egg, will be essential in determining its survival to hatching. Such mechanical adaptations include thicker eggshells with increased microhardness to protect against puncture ejection (Igic et al., 2011; Picman, 1989) or breakage during rapid laying (Soler and Martínez, 2000). Certain eggshell traits may also reflect specific physiological adaptations to achieve a parasitic lifestyle. For example, low water vapour conductance relative

to their host eggs has been reported in several avian brood parasites, and has potential to increase hatchling aerobic fitness of parasitic chicks (McClelland et al., 2019, **Chapter 2**).

A further challenge for the eggs of brood parasites is the risk of infection; it has been shown that both host and parasite eggs in parasitised nests have a higher microbial load and greater risk of trans-pore infection than eggs in the nests of a non-parasitised species (Geltsch et al., 2018; Soler et al., 2011). Higher microbial loads in parasitised nests are commonly attributed to microbes introduced by the laying parasite in addition to those already present in the host nest's microbiome, or from decomposition of host eggs broken by the parasite during laying (Hahn and Reisen, 2011; Soler et al., 2015, 2011). As well as greater microbial quantities of the parasitised nest, a greater density of microbes were found on the eggshell surface of the eggs of European magpies (*Pica pica*) compared to the eggs of parasitic great spotted cuckoos (*Clamator gladarius*) in the same nest (Soler et al., 2011). This distinction in bacterial load between host and parasite egg suggest that parasite eggs possess surface properties that work to reduce the adhesion of microbes to their shells, as documented in other bird species, potentially as an adaptation to the greater diversity of potentially harmful bacteria in parasitised nests (Wellman-Labadie et al., 2008; D'Alba et al., 2014). Whether surface structures of brood parasite eggs have evolved to minimise microbial infection and other challenges of host nest conditions has not been investigated previously.

In this study, we investigate several physical eggshell traits of avian brood parasites and their hosts to determine which eggshell characteristics have allowed their unique reproductive lifestyle to succeed by overcoming various challenges faced within the hosts nest environment. One of these eggshell characteristics is wettability of the shell, i.e., whether eggshells are hydrophobic (water repelling) or hydrophilic (water-attracting). Birds under particularly high risk of infection, such as compost-nesting malleefowl (*Leipoa ocellata*), have evolved an extremely water-repellent eggshell surfaces composed of nanosphere-type structures (D'Alba et al., 2014; Grellet-Tinner et al., 2017). These nanospheres cause water to 'bead up' rather than spread out over the shell surface, and in doing so, are thought to potentially trap harmful microbes in these water droplets, preventing the formation of biofilms (D'Alba et al., 2017). Eggshell surface wettability also influences the exchange of respiratory gases and water vapour through the eggshell pores (Board, 1981). A suitable and controlled exchange of gases is essential for the embryo; a vital component of this process is the prevention of pores being blocked by water or dirt (Board, 1982). Hydrophobic eggshells are believed to capture dirt and

other contaminants in surface water droplets, isolating debris and preventing the clogging of pore cavities (Igic et al., 2015a). The wettability of many biological surfaces, including eggshells, is largely controlled by chemical and physical properties, the latter being dictated by micro- and nano-level variations in surface roughness (Neinhuis and Barthlott, 1997; D'Alba et al., 2016). For this reason, eggshell roughness is expected to be a functionally important eggshell-surface property (Attard and Portugal, 2021), however large-scale comparisons have rarely been made between avian species. Eggshell surface roughness and wettability of brood parasites could potentially allow parasite eggs to achieve low rates of infection in host nests where microbial loads are high.

While the surface properties of avian eggshells provide the first line of defence against microbial threats, on a different scale the composition of the shell is important for protecting the embryo from mechanical damage. The quantity and structure of the calcium carbonate crystals that form the bulk of the shell (~85-99%) has a significant impact on the mechanical properties of the eggshell (Nys et al., 2004; Athanasiadou et al., 2018). However, calcium is an expensive mineral for many bird species to acquire from their diet (Reynolds et al., 2004) and as such their investment in eggshell formation must weigh this cost against the requirements of the egg. McClelland et al. (2021) (**Chapter 4**) demonstrated that the ratio of calcium carbonate to organic proteins varies between species and is partially explained by their life-history and investment strategies. For avian brood parasites, egg production represents almost the entirety of energetic cost incurred during reproduction for these species. This is in contrast to other species where investment in egg production is believed to be constrained by rearing costs (Lack, 1947; Monaghan and Nager, 1997; Monaghan et al., 1998). As such, the 'clutch size' of brood parasites is not constrained by the number of chicks they can raise, and therefore, they can invest more energy in laying substantially more eggs. For example, brown-headed cowbirds (*Molothrus ater*) are believed to lay as many 40 eggs per season (Davies and Quinn, 2000; Reetz, 2008), whereas a similar-sized congener species with parental care might be expected to raise two clutches of four eggs, at most, per year (Böhning-Gaese et al., 2000). To lay this substantial number of eggs requires a huge investment of calcium from the cowbird female; if each egg contains approximately 0.25 g of calcium, then 40 eggs will require about 10 grams of calcium, which is as much as five times as much calcium as the adult's own skeleton (Prange et al., 1979; Dunning, 2007). Females of some brood parasites (e.g., common cuckoos (*Cuculus canorus*) and brown-headed cowbirds) ingest one of the hosts' eggs while laying their own egg to replace it (Davies, 2011; Peer et al., 2018). Not only does this maintain

the same number of eggs after the nest is parasitised, but also allows the females calcium levels to be replenished before producing her next egg (Sealy, 1992; Peer, 2006). However, as brood parasites have a short breeding period, laying a large number of eggs might instead favour adaptations to eggshell structure and composition to reduce their calcium content. A previous comparative study on eggshell composition across a phylogenetically wide diversity of birds, showed that thicker shelled species (which is a common trait in brood parasites (Picman 1989), actually had a lower calcium carbonate content than thinner shelled species (McClelland et al., 2021, **Chapter 4**).

In this study, we aim to identify whether eggshell surface properties (wettability and surface topography) and calcium carbonate composition of brood parasites represent specialized adaptations to a parasitic lifestyle. We predict that either 1) the eggshell surface topography and wettability of brood parasites will be similar to their hosts eggs due to selection to match their host nest environment, or 2) the eggshell surface of brood parasites will be rougher and thus more hydrophobic than those of their host/s, to outcompete these eggs in the microbially dense and diverse nest environment of a parasitised nest. Furthermore, if physical adaptations of the shell cuticle microstructures are responsible for wettability of these eggs, we expect to see a positive correlation between the eggshell surface roughness and its degree of water-repellence. Regarding eggshell composition, we expect parasitic eggs will have lower calcium carbonate content (as a proportion of the whole shell) than their hosts as a result of their thicker eggshells (based on McClelland et al. 2021, **Chapter 4**), and a potentially greater organic component to increase eggshell structural hardness.

Additionally, we investigate whether deposition of dark pigmentation spots on the eggshell of a mimetic brood parasite, cuckoo finches (*Anomalospiza imberbis*), alters the surface roughness of the eggshell, and if so, whether it is similar to the roughness of the maculated regions of their hosts, tawny-flanked prinias (*Prinia subflava*). Eggshell maculation is primarily produced by the pigment protoporphyrin (Duval et al., 2016) and is either laid down superficially on the outer surface of the cuticle and/or embedded within the outer layers of the eggshell (Kilner, 2006; Brulez et al., 2014). Pigmentation occurring as superficial layers of the eggshell could potentially influence the surface properties of eggs in these regions. The vertical depth of protoporphyrin on the eggshell has not been accessed in brood parasites relative to

their hosts eggs. However, differences in surface roughness of maculation and non-maculated eggshell regions between brood parasites and their hosts would indicate variation in the location of the pigment, and suggest the mimicry is only superficial.

Methods

Overview of samples, sources, and preparation

Eggshell surface wettability (i.e., hydrophobicity or hydrophilicity), roughness, and eggshell calcium carbonate content were measured in eggshells of brood parasites and their respective hosts, and in a number of species which were neither parasites nor typical parasite hosts, to assess the general phylogenetic spread of these parameters. Measurements were taken from either whole eggs or eggshell fragments which were sourced from the field or museums. Where possible, wettability and roughness measurements were taken from the equatorial region of the eggshell, as this region has lower curvature than the poles of the egg and curvature can affect the measure of wettability.

Most eggshells (n eggs =104) were obtained from the destructive collection (Class II) of the Natural History Museum collection at Tring, where minimal clutch information was available (Maurer et al., 2012). Many of these specimens comprised fragments only, with no information on which eggshell region they represent. Eggshell fragments (n= 37) from Western Foundation of Vertebrate Zoology (WFVZ) in Camarillo (California, USA) were sampled from the equatorial region of the egg. Equatorial fragments were also sampled for all eggs collected during fieldwork in Choma region, Zambia (n=136) (collection site information in (McClelland et al., 2019; Spottiswoode and Stevens, 2012)). Samples were used firstly for profilometry analyses, followed by wettability measurements, then finally calcium carbonate content was extracted as this measurement is destructive. Eggs were stored in air-tight plastic containers padded with cotton wool, and the surface was cleaned gently with a cotton bud dipped in distilled water prior to analysis.

Non-contact optical profilometry (measurement of surface roughness using reflectance) and most wettability measurements were performed at the engineering department laboratory at the Open University (Milton Keynes, UK), between October 2019 and March 2020. The remaining wettability measurements were performed at the Department of Mechanical Engineering Sciences, University of Surrey, Guildford, Surrey, between October 2020 and January 2021. A change of location was required due to laboratory closure as a result of the Covid-19 pandemic. The same protocols were used at both locations.

Profilometry measurements of surface roughness

Measurements of surface roughness were obtained from 283 eggshells from 45 species, including 125 eggs from 12 parasite species. Shell fragments were placed on a glass slide with the outer shell surface facing upwards. For stability, a pressure-sensitive adhesive (Blu Tack, Bostik, UK) was used to secure the fragment in position. Whole eggs were balanced on the lid of a Falcon tube for stability when under the microscope, with the longitudinal-axis of the egg parallel with the bench. Eggshell samples were placed under a non-contact optical profilometer using a white light source interferometric microscope (Leica DCM 3D, Germany). We used an objective magnification of x20 to give a pixel resolution of 768 x 576 (636 x 477 um^2). Three to four non-overlapping locations on the fragment were selected and measured at 100 focal planes (totally a vertical depth of 100μm from the outermost surface) at a resolution of 1μm per scan. Two-dimensional (2D) and three-dimensional (3D) digital elevation models of each shell location were saved as separate image files (Figure 5.1).

SPIP (Scanning Probe Image Processor, SPIP version 4.4.3.0, Image Metrology, Hørsholm, Denmark) was used to process images and extract measurements of surface roughness, S_a, which was calculated from an average of images over four shell locations per egg. S_a was measured in nm and represents the arithmetic mean distance of each point from the mean focal plane of the sample. Due to the curved surface of the fragments a second-order polynomial function was applied to the plane correction (Green et al., 2015).

In addition, eggshell surface roughness was specifically measured on maculated (foreground 'spots') regions and non-maculated (background) regions of cuckoo finch (*Anomalospiza imberbis*) eggshells and those of their host, tawny-flanked prinias (*Prinia subflava*). The foreground of maculated eggshells was differentiated from the background through visual inspection on the view finder of the profilometer. The view finder produces a grey scale image, with darker coloured eggshell regions displayed in darker tones. In most cases, the spots were large enough that the pigment covered the entire field of view of the scan.
Pigmentation is not investigated in other species in this study as the eggs were either immaculate or had pigmentation patterns that were too diffuse across the shell surface to analyse separately.

Wettability measurements

Wettability measurements were taken on 148 eggshells from 39 species, including 47 eggs from 11 parasitic species. Wettability of the eggshell surface was determined using the contact angle (CA) of a sessile water droplet on the shell surface (hydrophilic = CA < 90°; hydrophobic = CA $\geq$ 90° and CA $\leq$ 150°; and superhydrophobic = CA > 150°) using a DSA 100 Drop Shape Analyzer (Krüss, Germany). Our protocols were based on D'Alba et al. (2014). The shell fragment was placed on a slide mounted in front of the analyser's highspeed camera, and a 4-9 μl drop of deionized water was deposited onto region of the shell exhibiting the lowest curvature, using a flat-pointed needle tip. The volume of the drop was estimated by Advance software (version 1.8-01) from Krüss (Germany) from the images recorded. The droplet was placed on the surface of the eggshell by lifting the stage until the droplet on the syringe tip contacted and adhered to the shell surface. The stage was then lowered away from the syringe. This method prevented spreading of the droplet that would be caused by the force of dropping from the syringe point onto the fragment surface. The droplet was imaged every second for the first 5 seconds immediately after contact with the shell surface. Further droplet spreading was minimal after 5 seconds. The Advance software was then used to apply a curved baseline to account for curvature of the eggshell surface using a Young-Laplace fit model and left and right angles of contact of the droplet were calculated at each time point and averaged (CA, Figure 5.2). The last recorded time point was taken for each fragment. Measurements were taken at room temperature. Humidity was not controlled but was within 40-70% relative humidity.

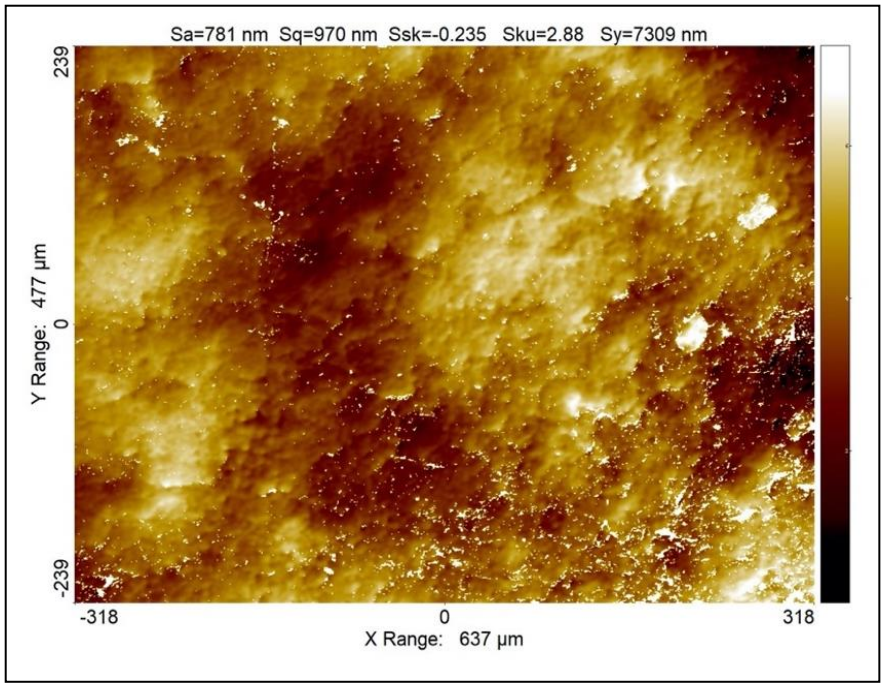

Figure 5.1 Profilometry 2D image of surface roughness of a background region of a cuckoo-finch (*Anomalospiza imberbis)* egg, showing higher surface points as light yellow and deeper points as darker brown. Image generated by SPIP software (Image Metrology, Denmark). Mean surface roughness (S_a) is shown along the top of the image.

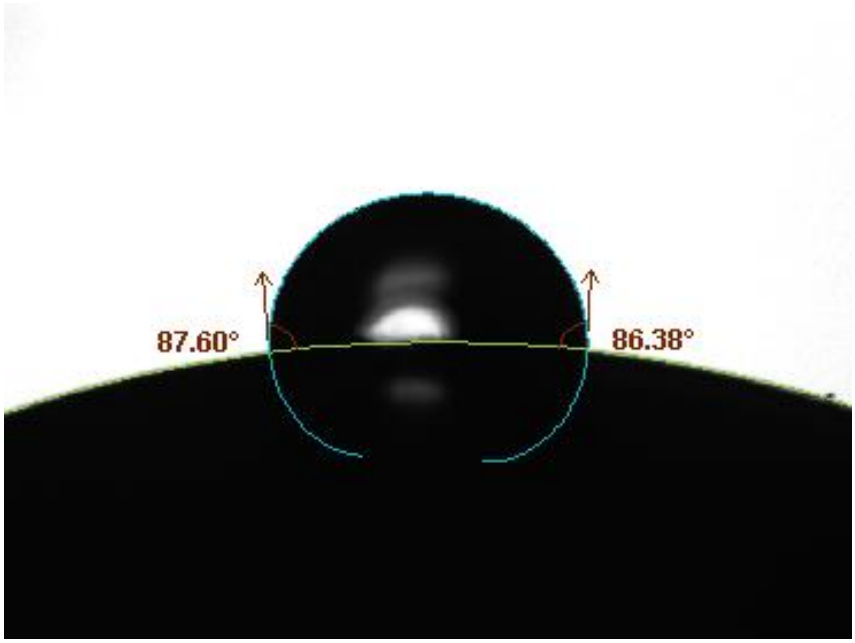

Figure 5.2 Sessile water droplet on the surface of a chestnut-winged cuckoo (*Clamator coromandus*) eggshell showing left and right water droplet contact angles. The green line illustrated the extrapolated curve of the surface and the blue lines illustrate the curve of the droplet. Image generated by Advance software (Krüss, Germany).

Calcium carbonate content

To determine the calcium carbonate content of eggshells, the same eggshells used for wettability experiments were 'ashed' in a muffle furnace (AAF 1100; Carbolite, Hope, UK). Shell fragments were placed in small (10ml) ceramic crucibles and weighed in grams on a precision balance (Sartorius 1265 MS, Gottingen, Germany) to 4 decimal places. Empty crucible mass was recorded prior. Eggshell fragments were then dried to a constant mass by being placed in an oven at 60 °C for 24 to 32 hours. Shell fragments were weighed twice daily, between 09:00-10:00 and 16:00-17:00, until no change in mass was detected for two consecutive weighing sessions, at which point they were considered 'dry'. The crucibles with the dry shell fragments were then placed into the muffle furnace for 18 hours at 500 °C to burn off the organic component of the shell. The crucible with the shell ash was placed in a glass desiccator immediately after removal from the furnace to cool down before being weighed again. The mass of the empty crucible was deducted to calculate dry weights of the shell fragment and ash. Calcium carbonate content was calculated as the ash mass of the shell fragment, as a percentage of the dry mass of the shell fragment. Other inorganic minerals that occur in trace amounts alongside calcium carbonate in the eggshell where not considered separately as they occur in extremely small quantities (e.g., <0.1% of the eggshell is comprised of phosphorous and magnesium) (Itoh and Hatano, 1964; Clunies et al., 1992).

Statistical methods

All statistical analyses were conducted in R statistical software version 3.6.3 (R Core Team 2020) using the frontend 'R Studio' (R Studio Team 2020). Figures were produced using the package "ggplot2" (Wickham, 2009).

- *Phylogenetically-controlled comparisons of surface properties between brood parasites and non-brood parasites.*

We constructed two separate phylogenetically-informed mixed models (PMM) to test whether parasitic species differed from non-parasitic species in either their eggshell roughness or their hydrophobicity using the R package 'sommer' Rv 4.0 (Covarrubias-Pazaran, 2018). This approach accounts for phylogeny, which is necessary as species are not statistically independent due to similarities which could be attributed to shared ancestry (Guigueno et al., 2019; Huey et al., 2019). A phylogenetic tree of all species included in this study (Figure 5.3) was produced using the R package "rotl" which accessed relatedness metrics from the online tree of life (Michonneau et al., 2016). Phylogenetic signal was calculated from PMM models by calculating what the proportion of total trait variance explained by the phylogeny and is stated as H^2. This measure of phylogenetic signal is equivalent to Pagel's lambda (Hadfield and Nakagawa, 2010).

A PMM was constructed with eggshell surface roughness (S_a*;* nm) as a response variable and parasitic status (parasite or non-parasite) as a predictor variable. Since S_a was measured on the same eggshell at multiple points where the fragment size allowed, individual egg identity was also controlled for as a random effect. A second PMM was constructed to compare hydrophobicity for eggshells where the response variable was CA and the predictor variable was parasitic status (parasite or non-parasite). Only a single CA was obtained per egg as contact angle is highly repeatable across the egg (Attard et al. 2021), so egg identity was not controlled for in this model. As *p* values are not generated as standard with this method, the *p* values of predictors in PMM models were calculated from a chi-squared test to compare between model containing the predictor and the same model without that predictor.

A linear mixed model was used to test whether eggshell surface roughness was a good predictor of eggshell wettability based on 67 eggs from 19 species where both values were obtained from the same eggshell fragment. Linear mixed models were preformed using the R packages

"lme4" (Bates et al., 2015) and "lmertest" (Kuznetsova et al., 2017), with species was included as a random effect.

- *Comparison of eggshell roughness on pigmented and non-pigmented shell location of brood parasites compared to their hosts.*

Eggshell foreground pigmentation was compared to eggshell background pigmentation of parasitic cuckoo finches and their hosts, tawny-flanked prinias (cuckoo finches: n = 18, tawny-flanked prinias: n = 4). Scans of foreground and background regions within each eggshell were completed separately, with between 3 and 10 scans per egg. A linear mixed model was constructed with eggshell roughness predicted by the interaction between egg species and whether that region was maculated (a spot) or non-maculated (background). Post-hoc Tukey honestly significant difference (HSD) comparisons were performed using the R package "emmeans" (Lenth, 2020) to assess whether roughness values were significantly different between foreground and background regions of each species.

- *Test of similarities in hydrophobicity between brood parasites and their hosts*

Permutation procedures (Attard et al., 2017) were used to determine whether brood parasites were more similar to their host in eggshell surface properties than would be expected by chance. These compared the differences in CA between either a brood parasite egg and an egg of their host species, or between a brood parasite egg and a randomly selected egg of a non-host species. We constructed 442 pairwise comparison of egg combinations (either parasite-host or parasite-random). Linear mixed models were then used to determine whether difference in CA between parasite-host eggs were significantly distinct from the differences in these values between parasite-random egg pairings. Species and egg identity were included as random factors, as the same parasite egg could be included in more than one pairing (random or host pairing). The difference in eggshell calcium carbonate content between brood parasites and non-parasites was tested using the same statistical methods as S_a and CA, as described above.

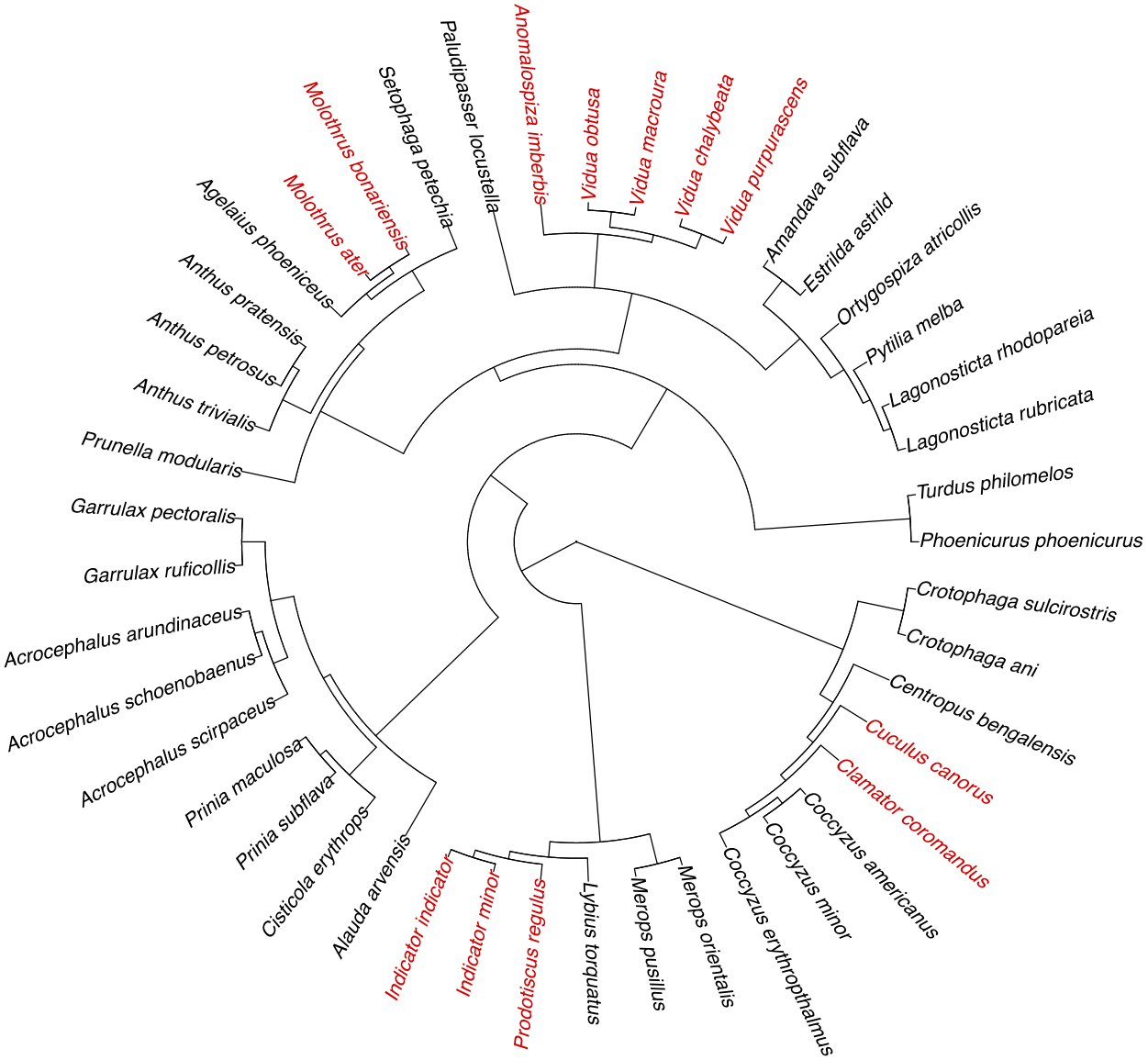

Figure 5.3 Phylogenetic tree generated from the online tree of life (Michonneau et al., 2016), representing all species sampled for eggshell roughness. All other measurements and analysis were performed on a subset of these species. Species labels in red text represent obligate brood parasites.

Results

Phylogenetically-controlled comparisons of surface properties between brood parasites and non-brood parasites.

Surface roughness ranged from 439 – 3987 nm in brood parasite eggs and 381- 3491 nm in host species (Figure 5.4a). However, the surface roughness of brood parasite eggs was not significantly different from eggs of non-parasitic species (PMM, Estimate = 26.82, SE ± 275.0, t = 0.09, p = 0.26, Figure 5.4a). There was a strong phylogenetic signal in the measurements of surface roughness (H^2 = 0.95±0.01). There was also no significant difference in wettability ($CA°_m$) between brood parasite and non-parasite eggs (PMM, Estimate =1.537, SE ± 2.61, t = 0.59, p = 0.79, Figure 5.4b). The phylogenetic signal for wettability was relatively low (H^2 = 0.03+ 0.07), suggesting life-history traits play a stronger role than phylogenetic relatedness in shaping eggshell wettability properties, in comparison to relatedness.

Across all species, S_a was not correlated with CA_m among the eggshells sampled based on Pearson's correlation coefficient (R^2 = -0.02, 95% CI [-0.35 0.13]), nor was S_a a suitable predictor of CA_m (LMM, Estimate = -0.0006, SE= 0.003, $t_{35.5}$=-0.197, p=0.845). The correlation between mean S_a and $CA°_m$ was low for both brood parasite eggs (R^2 = -0.07) and non-parasite eggs (R^2 = -0.003, Figure 5.5).

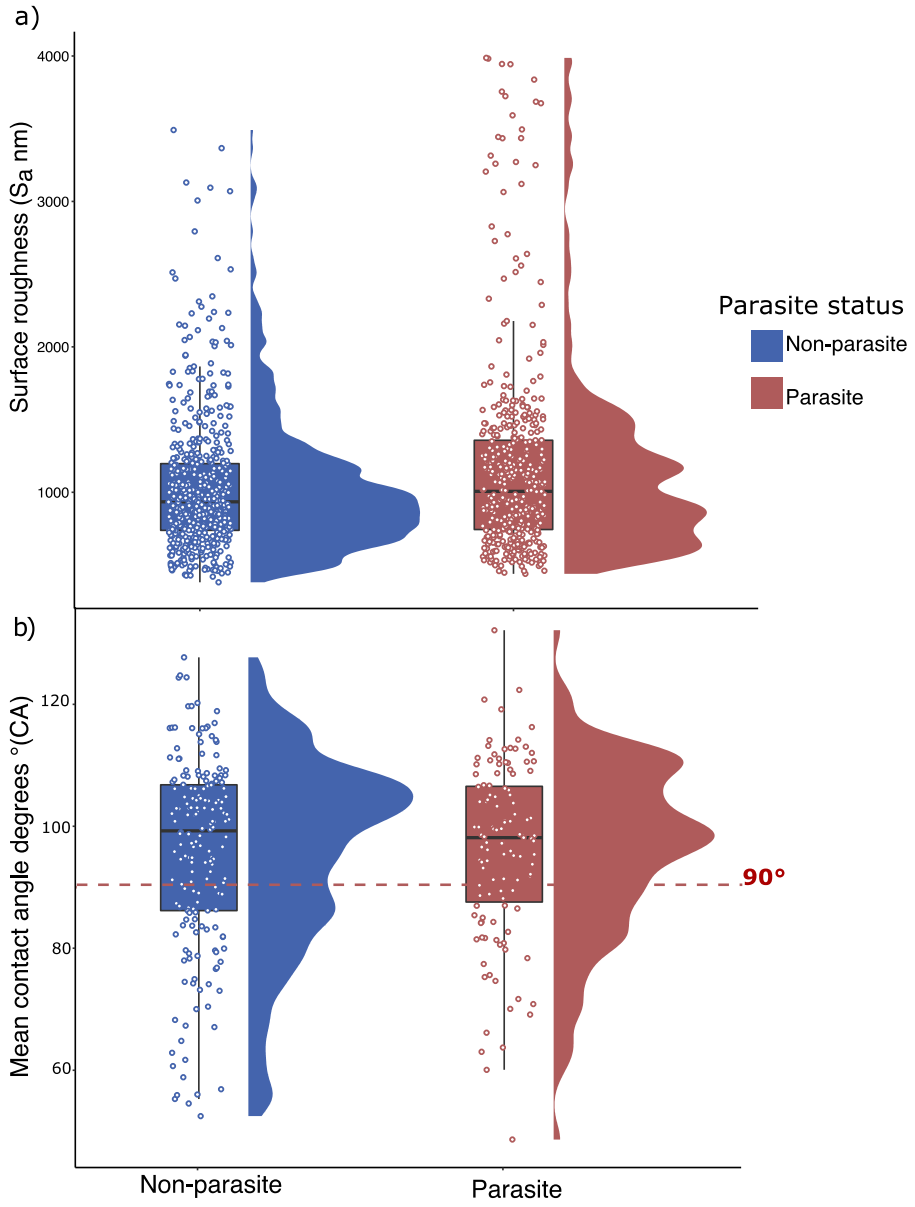

Figure 5.4 a) Surface roughness (S_a nm) of the eggshells of avian brood parasites (left, blue) and of non-parasite species (right, red). b) Mean surface contact angle of water droplet (CA) on the eggshell of avian brood parasites (left, blue) and of non-parasite species (right, red). Red dotted line at 90° demonstrates threshold between hydrophobic values above and hydrophilic values below. No significant difference was determined between parasite and non-parasite eggs for either trait (surface roughness: t = 0.09, p = 0.26; hydrophobicity: t = 0.59, p = 0.79). Boxplots show interquartile range and median value as a line, with whiskers encompassing values within to 1.5 times the interquartile value. *Distribution of data is shown in scatterplots (overlaid) and frequency plots (alongside each pairing).*

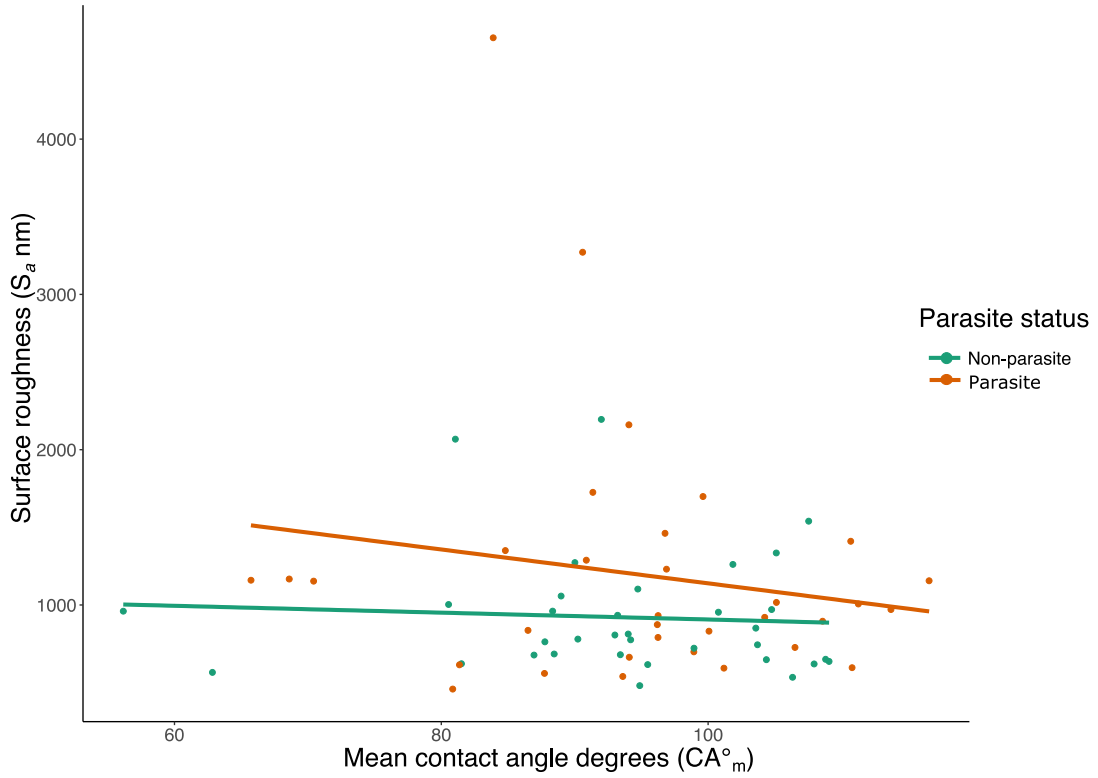

Figure 5.5 Association between surface roughness and mean contact angle in either parasites (orange) or non-parasite (green). R^2 for parasite eggs was -0.07, and R^2 for non-parasite eggs was -0.003.

Comparison of eggshell roughness on pigmented and non-pigmented shell location of brood parasites compared to their hosts.

Foreground (maculated) regions of cuckoo finch eggshell had a significantly greater surface roughness than background (non-maculated) regions (Estimate = -83.3, SE ± 25.6, t_{122} = -3.26, p = 0.014, Figure 5.6). However, the roughness of eggs of their hosts, tawny-flanked prinias, did not differ significantly between maculated and non-maculated regions (Estimate= -18.9, SE ± 43.9, t_{108} = -0.43, p = 0.67, Figure 5.6). Additionally, while the maculated regions of cuckoo finch and tawny-flanked prinia eggs were similar in roughness (Estimate = -95.4, SE ± 56.3, t_{30} = -1.69, p = 0.11), the non-maculated regions of cuckoo finch eggs were significantly smoother than the non-maculated regions of the prinia eggs (Estimate= -159.0, SE ± 60.2, t_{30} = -2.65, p = 0.011).

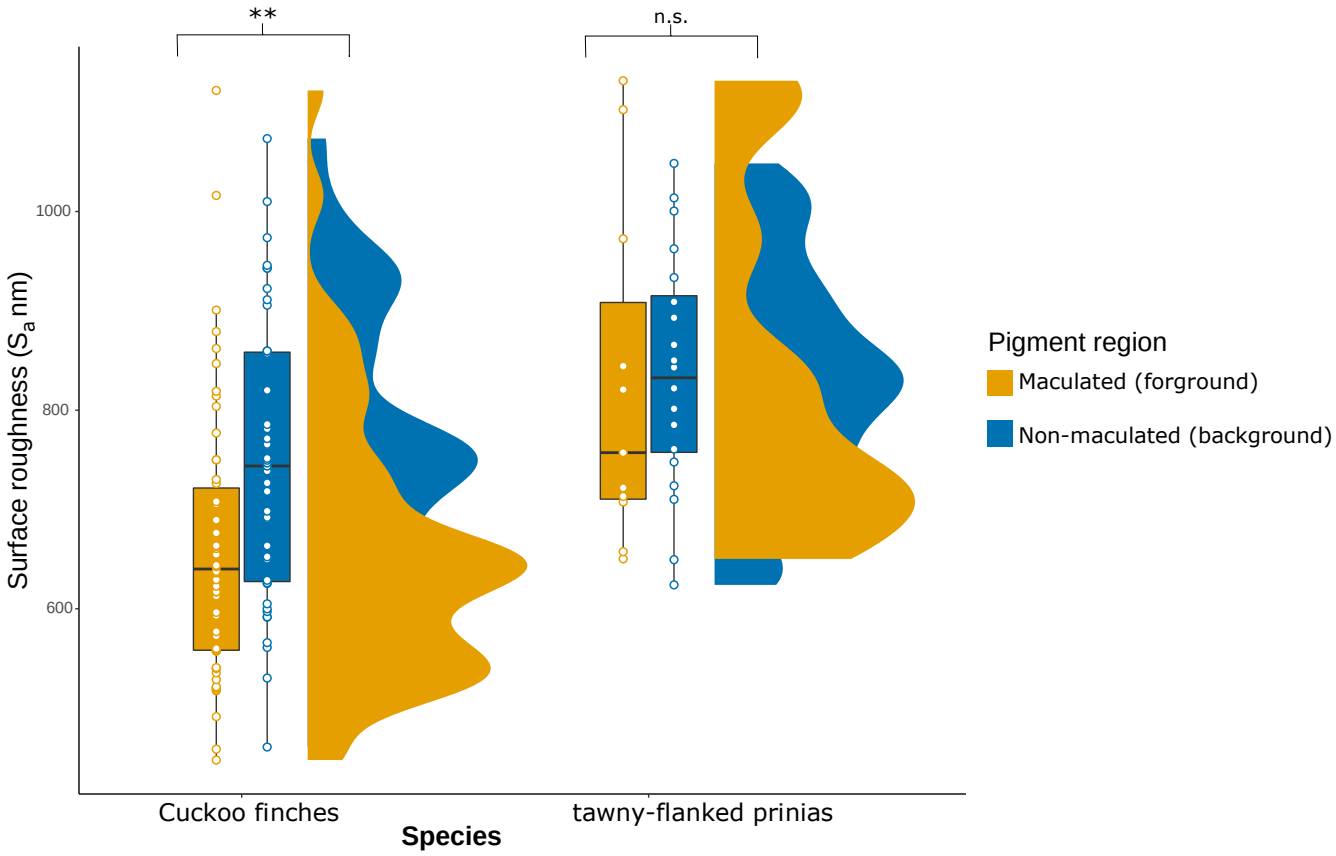

Figure 5.6 Differences in surface roughness (S_a nm) between maculated and non-maculated eggshell regions of the eggs of cuckoo finches and their hosts, tawny-flanked prinias. "**" signifies a significant difference with a p value < 0.005. "n.s." signifies no significant differences between groups. Boxplots show interquartile range and median value as a line, with whiskers encompassing values within to 1.5 times the interquartile value. Distribution of data is shown in scatterplots (overlaid) and frequency plots (alongside each pairing).

Comparison of hydrophobicity between brood parasites and their hosts

Eggshell hydrophobicity, measured as $CA°_m$, was no more similar between brood parasites and their hosts than between brood parasites and randomly assigned non-hosts species (Estimate = 0.46, SE ± 2.08, $t_{372} = 0.219$, $p = 0.827$, Figure 5.7).

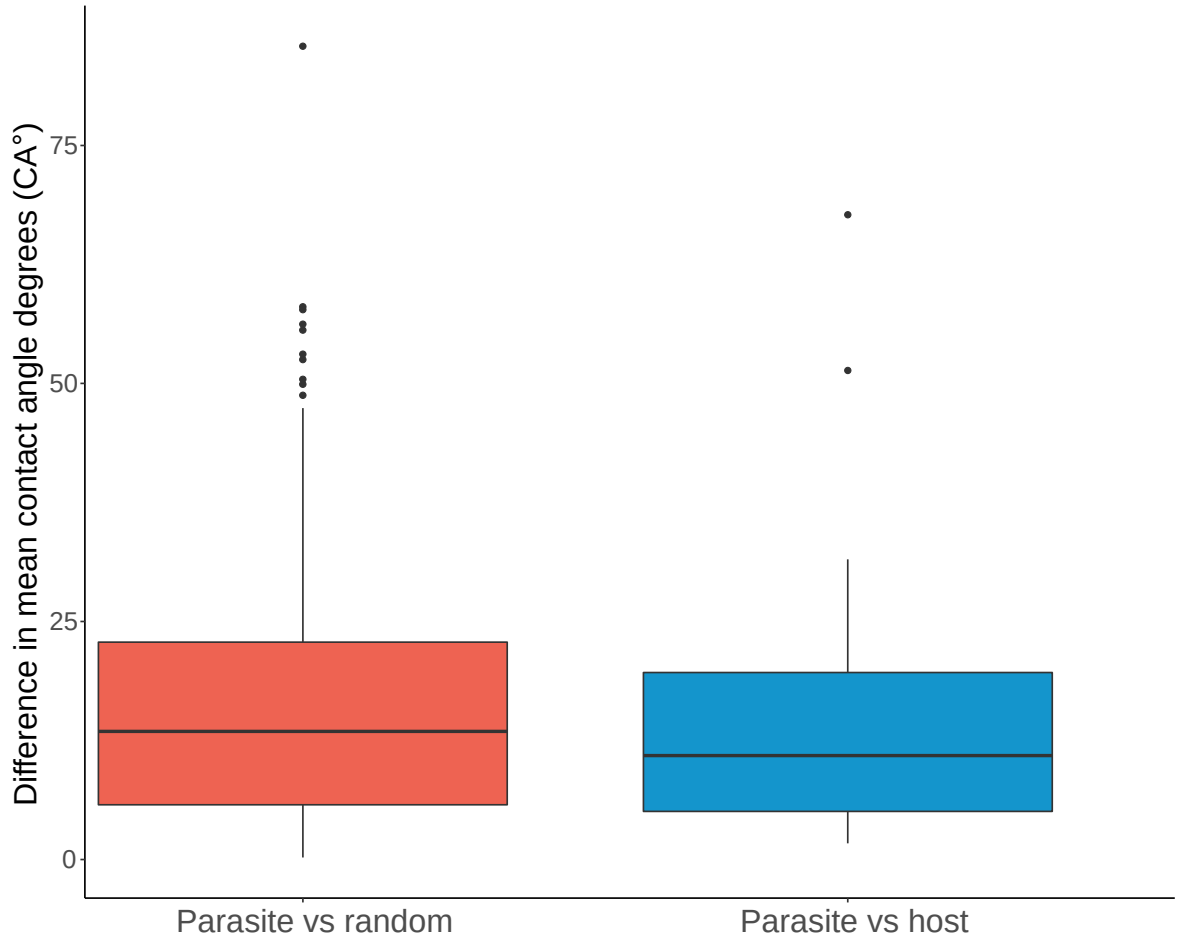

Figure 5.7 Differences in hydrophobicity (mean contact angle $CA°_m$) between brood parasites and their hosts (right, blue) were not different from differences between brood parasites and a randomly allocated non-host egg (left, red) ($t_{372} = 0.219$, $p = 0.827$). Boxplots show interquartile range and median value as a line, with whiskers encompassing values within to 1.5 times the interquartile value.

Calcium carbonate content

After accounting for phylogenetic relatedness, the calcium carbonate content of eggshell of avian brood parasites was not significantly different from that of non-parasitic species (estimate=0.013, SE ± 0.02, t = 0.5369, p = 0.62, Figure 5.8). The phylogenetic signal (H^2) was 0.88 ± 0.06.

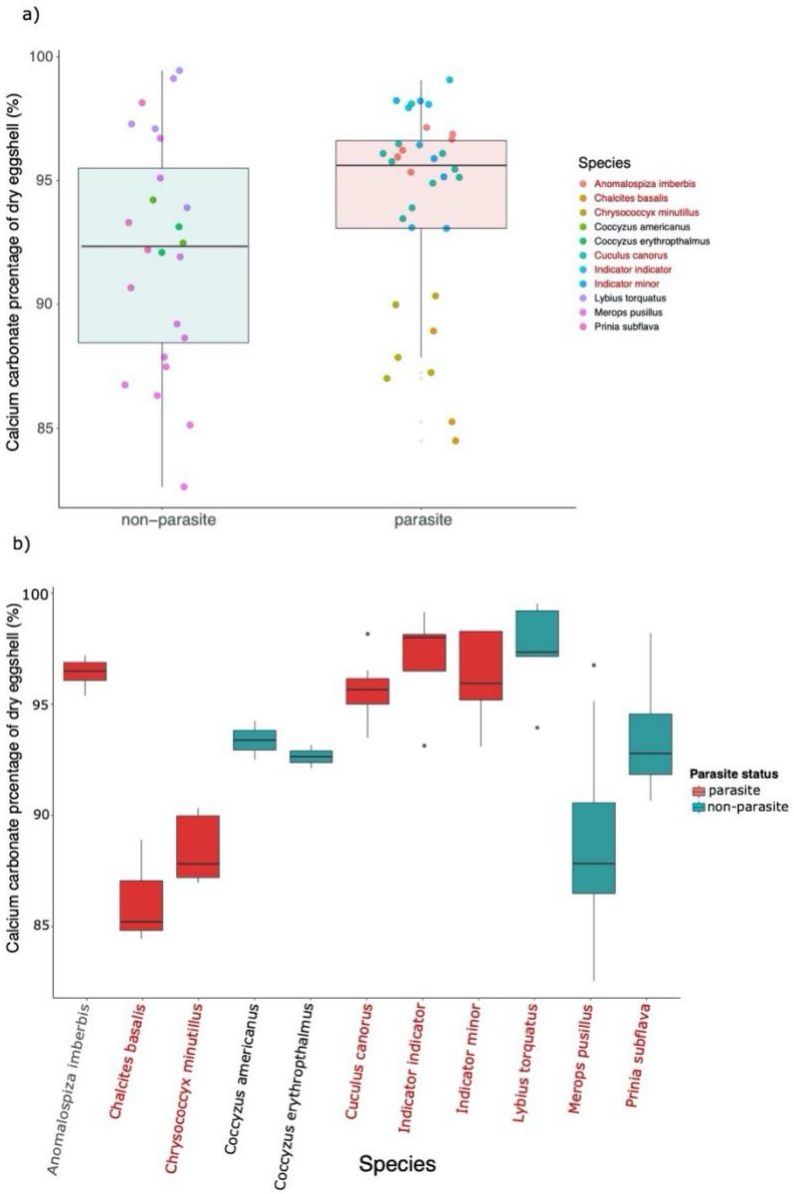

Figure 5.8 A) Calcium carbonate content of eggshell of brood parasite species compared to that of non-parasite species. Boxplots illustrate the group mean and interquartile range, and whiskers cover 1.5 times the interquartile range. Species of each measurement are differentiated by colour on the scatterplot. B) Calcium carbonate range for each species sampled, differentiated between parasites and non-parasites.

Discussion

The coevolutionary dynamics between parasites and their hosts have resulted in selection for a wide variety of adaptations to the eggs of both parties (Yom-Tov and Geffen, 2006; Spottiswoode, 2010). By comparing the mechanical and structural properties of brood parasite eggs to that of their close relatives and their hosts, we can further elucidate the associations between these physical eggshell traits and brood parasitism as a reproductive strategy. We found that, with the exception of differences in roughness of maculation on cuckoo finch eggs, brood parasites did not appear to display differences in their eggshell surface structures or calcium carbonate content compared to either their close relatives or their hosts. This lack of significant differences between host and parasite could imply that these traits are constrained by other factors more essential for embryo development, such as nest environment, which dominates any potential adaptation for parasitism.

We predicted that brood parasites would have more hydrophobic eggshells to decrease their vulnerability to microbial attack, as nests containing eggs of brood parasites have been shown to have higher infection loads. Contrary to this, we did not find a consistent trend in this direction; the eggs of brood parasites were not more hydrophobic. There are potentially a number of reasons which might explain why this heightened hydrophobicity was not observed. For one, evolutionary drivers of eggshell wettability are not mutually exclusive, and will be under other forms of selection which are shared by parasites and parental species alike, such as thermoregulation and crypsis (e.g., Attard et al 2021, *in press*). Adherence of water droplets to the surface of the shell will increase heat loss through evaporation (Monteith, 1981; Misyura, 2019). Maintaining a constant temperature is essential for optimal embryo development (Lourens et al., 2007; Reyna and Burggren, 2017), and as such the wettability of parasite eggshell might be constrained by this factor rather than adapted to microbial defence, or other features of a parasitic lifestyle (e.g., shell thickness). Eggshell roughness is, likewise, thought to influence heat loss from the egg, as rougher surfaces are thought to decrease heat transfer by disrupting the flow of air across surface (Maisuria, 2013; Attard et al. 2021 *in press*). Furthermore, eggshell roughness in brood parasites could also potentially influence the ability of the host to eject the parasite egg from its nest. If a host bird recognises a foreign egg, it may attempt to puncture it or grasp it in its beak to eject the egg (Moksnes et al., 1991; Underwood and Sealy, 2006; Rasmussen et al., 2009). The difficulty of removing the egg without damaging

their own eggs will influence their decision whether or not to attempt to eject the egg (Langmore et al., 2005; Peer et al., 2018). A smoother eggshell surface that provides less friction could potentially reduce the grip of the host beak during grasping ejection, thereby increasing the probability of breaking other eggs, and shifting the cost-benefit calculation of ejection. Similarly, some brood parasites are thought to have evolved thicker shells to increase the costs of puncture ejection by hosts (Spaw and Rohwer, 1987). However, we were unable to support this hypothesis based on the eggshell roughness data for the species we had available. It is possible that the ability of hosts to eject eggshell through puncturing them may negate any benefit to smoother eggs (Spottiswoode, 2010; Peer et al., 2018). This theory could potentially be tested further with behavioural experiments to measure the success of rejection by grasp-ejecting hosts with naturally, or artificially, smoother eggs.

Despite strong evidence from other biological materials, such as plant leaves or spider eggs (Yang et al., 2006; Wang et al., 2014; Makover et al., 2019), we did not find a strong correlation between eggshell roughness and wettability. This could suggest wettability is either additionally, or alternatively, determined by non-structural conditions of the shell surface, such as organic materials or coatings which could bind or repel water (e.g., cuticle lipid content) (D'Alba et al., 2016). This deserves further investigation on a larger species set. In particular, glycoprotein spheres which have been identified on the surface of some eggshells (Samiullah and Roberts, 2014) are both hydrophobic and antimicrobial (Board, 1982; Makover et al., 2019), and may function to protect the eggshells from microbial attack by an alternative mechanism to roughness mediated hydrophobicity.

We found that cuckoo finches, a brood parasite, had a striking difference in eggshell roughness between maculated regions and non-maculated regions of the egg, which was not the case in the eggs of tawny-flanked prinias, the host whose patterning they mimic. This would suggest that the deposition of pigment in cuckoo finches could be more superficial than their hosts. Brood parasite eggs have been shown to differ in pigment concentration from their host eggs despite similarities in eggshell patterning (Dainson et al., 2018), suggesting that selection for eggshell mimicry may have proceeded along differing evolutionary pathways to achieve the same egg appearance. The exhibited differences in surface roughness between cuckoo finches and their hosts complements this theory. The vertical distribution of the pigment in the cuticle and outer shell could be further investigated with cross-sectional imaging using scanning electron microscopy. In addition to its visual function, eggshell pigment is thought to

potentially have structural role that effects the strength of the eggshell (Cherry and Gosler, 2010; Sparks, 2011), including compensating for reduced calcium content (Gosler et al., 2005, 2011), however, this has been disputed (Maurer et al., 2011). Whether or not this is a factor that results in differences in the quantity or location of pigment deposition between parasites and hosts is not something that we can address with our data but warrants further investigation.

We did not find a difference in the proportion of calcium carbonate in parasite eggs compared to that of non-parasitic species. This suggests that, although parasites produce a large volume of eggs over the breeding season, they do not adaptively lower the calcium investment into each individual egg. Ingestion of host eggs, common in many parasite species, may be sufficient to compensate for the parasite's increased calcium demands (Davies, 2011). Additionally, many brood parasites exhibit longer laying intervals than parental species; 48 hours between eggs rather than 24 hours (Birkhead et al., 2011). This could be to allow them longer to acquire excess calcium from their diet, and/or to digest the calcium from the egg consumed during their last egg laying. These results fit with a previous study that found no difference in the calcium to carbon ratio (Ca:C) in each of the different eggshell layers of two brood parasites (common cuckoos and brown-headed cowbirds) compared to their hosts (Igic et al., 2011). However, Igic et al. (2011) confirmed parasitic species have significantly thicker and structurally harder eggshells and suggested alternatively that the organic matrices play a significant role in reinforcing the calcite structure of these thicker eggshells. This was supported by Soler et al. (2019) who showed that the increased hardness in the eggshells of greater spotted cuckoos (*Clamator glandarius*) is a product of their smaller, more randomly, orientated calcite crystals; a trait that is controlled by the organic matrix and increases hardness (Athanasiadou et al., 2018; Chien et al., 2008). The apparent lack of association between calcium carbonate content and thickness in brood parasite eggs would indicate that this trait may be integral to the eggshell function in a way that negates selective forces of parasitism. Igic et al. (2011) also compared Ca:C between several host-races (gens) of common cuckoos and found no significant differences; however, further research could address whether observed differences in thickness between cuckoo gens is achieved through a greater proportion of calcium carbonate.

Overall, these findings point towards a more conserved patterns of eggshell traits shared by brood parasites and their hosts. Although, brood parasite eggshells demonstrate many functional differences in their hardness, shape, and microbial defence, these do not translate to

adaptations to the structures investigated here. Future avenues of research might consider exploring chemical properties of brood parasite eggshell, particularly the cuticle, to elucidate how parasite eggshells function to enable development in host nests. This study investigated several structural traits of eggshell however there is much still to know about the structural properties of avian brood parasite eggshells.

Acknowledgements

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CHAPTER 6. Patterns of embryo metabolic rate in avian brood parasites: Highly virulent brood parasites exhibit a defined 'plateau' stage similar to precocial birds.

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Abstract

As a bird embryo grows and develops within the egg, metabolic rate increases. The patterns of how the rate of metabolism increase differs between species is linked to their development state at hatching. Brood parasites lay their eggs in the nest of other species to avoid the costs of parental care. Nearly all brood parasite chicks are altricial at hatching, yet many perform the physically demanding task of killing host progeny within days of emerging from the egg. Using flow-through respirometry *in-situ*, we investigated embryo metabolic rate in avian brood parasites which either kill host offspring (high virulence) or share the nest with host young (low virulence). High virulence brood parasite embryos reached a higher metabolic rate earlier in incubation compared to both non-parasitic species and low-virulence parasites, yet all of these groups had a similar metabolic rate shortly before hatching. The pattern of metabolic rate development in high virulence brood parasites showed a prominent 'plateau' approximately 80% into incubation which is similar to the pattern seen in precocial species. This suggests that high virulence brood parasite embryos use their energy supply differently over development compared to other altricial birds, and potentially this difference is a mechanism to facilitate the physical demands of nest-mate killing upon hatching.

Introduction

Brood parasitism is found in birds, insects and fish, where parental care is costly and can be stolen (Sato, 1986; Croston and Hauber, 2010; Manna and Hauber, 2016). Brood parasitism is comparatively more evident in birds, where the progeny of avian brood parasites develop in the nest of their respective host species, exhibiting numerous adaptations to exploit and maximise the parental care of their hosts (Davies and Quinn, 2000; Thorogood et al., 2019). Among birds, a brood parasitic strategy has evolved multiple times across different lineages, including multiple evolutions within the cuckoo family, and potentially first emerging around 20 million years ago in African finches, and in contrast as recently as 5 million years ago in North American passerines (Sorenson and Payne, 2001; Krüger and Pauli, 2017). There are approximately 100 extant bird species which are known to reproduce exclusively through brood parasitism, and these parasites exhibit many commonalities in their behavioural, physiological and anatomical adaptations to this reproductive strategy (Davies, 2011; Stevens, 2013). For example, thicker eggshells than would be expected for the size of their eggs is seen in many brood parasites, purportedly to prevent puncture ejection by hosts (Picman, 1989; Antonov et al., 2008). Similarly, shorter development periods to enable hatching earlier than the host young is seen in nearly all brood parasite species (Payne, 1977; Birkhead et al., 2011). However, how brood parasite embryo development is specialised to a shortened incubation period within a modified eggshell remains largely unknown.

Following this rapid development and early hatching, brood parasitic chicks face immediate demands on their physiology upon hatching, for which any delay in growth or underdevelopment would have significant detrimental impacts on the viability of the chick. For many parasitic species, the chick's survival is dependent on acting quickly to reduce the competition it faces from host young (Grim et al., 2009b), such as by evicting them from the nest or killing them *in situ*. Nestlings of common cuckoos (*Cuculus canorus*) which are prevented from or, are unable, to evict their host competition have a significantly lower chance of fledging (Rutila et al., 2002; Hauber and Moskát, 2008). However, killing the host young is likely to be energetically costly, requiring strenuous behaviour that can be considered remarkable for nestlings which hatch in a naked and altricial state (Anderson et al., 2009; Grim et al., 2009a). Common cuckoo nestlings remove competition by lifting the hosts eggs or young onto their back and pushing them out of the cup nest. Similarly, striped cuckoos (*Tapera naevia)* and honeyguide species (*Indicator sp*.) kill the host young by biting and shaking the

chicks with a specialised beak hook (Morton and Farabaugh, 1979; Spottiswoode and Koorevaar, 2012). Both of these behavioural types are aerobically demanding for the chick, requiring rest periods during and after each bout, and more food provisions are required to recover the costs and fuel the next bout (Hauber and Moskát, 2008; Anderson et al., 2009; Spottiswoode and Koorevaar, 2012). Moreover, these demanding early-life behaviours are also known to increases oxidative stress levels in the chick (Hargitai et al., 2012).

Early hatching and faster development in avian brood parasites has been attributed to multiple mechanisms, each theory garnering varying degrees of support. Among these possible explanations are larger yolks as energy stores (Török et al., 2004, but see Igic 2015), internal incubation of the egg prior to laying (Birkhead et al., 2011) and lower eggshell conductance to conserve energy stores (Portugal et al., 2014; McClelland et al., 2019;). However, while each of these adaptations likely contributes to the specialised development of brood parasites, these theories do not fully explain the speed of development, and the physical abilities of the newly hatched altricial chicks. Recently, it was shown that brood parasite embryos exhibit greater frequency of embryo movement, potentially as a mechanism to increase their muscle development (**Chapter 3**). While this study established that brood parasites increase their embryonic muscular activity during development, it is not clear whether this activity is accompanied by increased developmental energetic costs.

Thus, an unexplored development trait of brood parasites is their embryonic metabolic rate throughout the course of incubation. The metabolic rate of an organism is the amount of energy produced and expended by an animal during a defined time period, and where this energy is produced aerobically it can be measured by the consumption of 0_2 and production of $C0_2$ (Ricklefs et al., 1996). For avian embryos, this energy that powers metabolism must be obtained exclusively from the resources available in the egg, deposited by the female. Therefore, these resources are finite and must be appropriately rationed over the course of incubation (Mueller et al., 2015). The only external component of the metabolic process not provided by the egg at laying is the consistent exchange of oxygen and carbon dioxide through the eggshell pores (Mortola, 2009). The energy consumed by the embryo is spent on two key functions: growth and maintenance (Mortola and Cooney, 2008). As such, the metabolic rate of the embryo changes over the course of development as growth intensity fluctuates, and the cost of maintenance increases with increasing embryo mass (Vleck and Vleck, 1980; Dietz et al., 1998). The pattern of metabolic rate change over incubation has been mapped in several

species, and follows common patterns which depends on whether the nestling hatches in an altricial or precocial state (Vleck et al., 1979). This difference in metabolism between altricial and precocial developmental modes is not unexpected as embryo development is known to differ in multiple ways between precocial species (those hatching feathered, open-eyed, and capable of coordinated movement and thermoregulation) and altricial species (those hatching naked, blind and requiring external heating and parental care) (Starck and Ricklefs, 1998; Österström et al., 2013). Except for black-headed ducks (*Heteronetta atricapilla*), all avian brood parasites are considered altricial, based on the naked and dependent state of their hatchlings (Dearborn et al., 2009). Despite this, the activity undertaken by many newly-hatched brood parasites to remove their nest competitors would suggest coordination and strength more akin to that of precocial young (Starck, 1993; Starck and Ricklefs, 1998). Based on this, one could expect that the embryo development of brood parasites differs from the typical altricial mode, and in which case might emulate aspects of precocial development. The metabolic rate of brood parasite embryos could reflect this by sharing a pattern of metabolic rate change with precocial species.

In this study we measure the metabolic rate of brood parasite embryos and their hosts over the course of their development. We predicted that brood parasite embryos will exhibit a higher metabolic rate over their incubation period compared to non-parasitic species. Additionally, differences are expected between parasites with different levels of virulence (Kilner, 2005; Medina and Langmore, 2016). Highly virulent brood parasites are considered those which actively kill all the host young, whereas lower virulence species have a weaker effect, reducing the quantity or quality of the host young that fledge through food competition (Croston and Hauber, 2010). Given the strenuous physical nature of killing host young, high virulence parasites are predicted to have a higher metabolic rate than low virulence parasites, if embryo metabolism is adapted to hatchling behavioural requirements. Surprisingly little is known about the physiology of newly hatched brood parasites that permits them to achieve what are, evidently, difficult and demanding tasks. Coupling early hatching of the brood parasite with the intense physicality of their early life, raises the question of whether, and how, the embryo development of these species differs from non-parasitic birds.

Methods

Metabolic rate was measured as CO_2 production (VCO_2 ml/min) and was determined using a portable flow-through (pull set up) respirometry system ('FoxBox', Sable systems, USA) connected to a laptop computer, as used by other studies (Mortola et al., 2010; Ton and Martin, 2017; Goodchild et al., 2020). The focal egg was placed into a 50ml respirometry chamber through which air was pulled at a flow rate of between 200 and 400ml/min by the inbuilt pump of the FoxBox system. The airflow settings varied with incubation stage and size of the eggs and this rate was corrected for in calculations of CO_2. The excurrent air from the chamber subsequently passed through the respirometer where accurate flow rate and CO_2 were measured at constant pressure, at a rate of one measurement per second. Prior to entering the respirometry chamber, the incurrent air flow was dried using self-indicating Drierite ($CaSO_4$, W.A.Hammond CO LTD) and scrubbed of CO_2 using Soda Lime ($CaHNaO_2$). The empty chamber was initially 'flushed out' for 2-5 minutes, after which a starting baseline measurement of 5-10 minutes was recorded with no egg present in the chamber. After this baseline the chamber was opened, and an egg was placed inside. We then waited a minimum of 2 minutes for the air flow to expel the air that entered when the chamber was opened, which was visible as a drop then stabilising of the CO_2 levels on the connected laptop. The egg was then left in the chamber for 6-10 minutes after which the chamber was opened, the egg removed, and a further baseline of 5 minutes was recorded.

Sable systems Expedata software (v.1.2.02 Sable Systems International) was used for analysis and extraction of metabolic rates. The reading was baseline corrected for drift using start and end baselines, and the CO_2 output was converted to VCO_2 by multiplying CO_2 by the recorded flowrate. As incurrent air was scrubbed of CO_2 prior to entering the chamber it was not necessary to account for ambient CO_2. The basal metabolic rate of the egg was taken as the average of the most stable 4 minutes of VCO_2 measurements during the period where the egg was in the chamber. Due to the instability of O_2 measurements at low concentrations, analysis focused on VCO_2 measurements as a measure of metabolic rate, as has been done by other studies (O'Dea et al., 2004; Goodchild et al., 2020). VCO_2 was divided by egg weight to get VCO_2 per gram. All eggs were weighed at first measurement when freshly laid. In some cases, these eggs were not weighed at subsequent visits and in those cases their mass at these

stages was estimated based on the species-specific rate of mass loss demonstrated by other eggs.

Eggs were not removed from their nest until immediately before being placed into the respirometry chamber, to ensure temperatures during measurements were kept as close to incubation temperature as possible, and to prevent cooling of the eggs. Heat was not applied to the eggs during respiration measurements, as wild eggs are often unattended for periods of 10-20 minutes or longer during incubation recesses (Conway and Martin, 2000; Martin et al., 2018). Therefore, the short periods of removal from the nest for $\dot{V}CO_2$ measurements are unlikely to alter metabolic activity significantly or harm the eggs any more than these natural incubation breaks. Chamber temperature was monitored with an iButton^{tm} (Measurement Systems, Berkshire, UK) for the majority of recordings and was found to be within the specified thermal range for incubation breaks of these species.

Species and sample sizes

Detailed information on species breeding behaviours and field sites can be found in **Appendix item 1**.

Metabolic rate was recorded for the eggs of 12 bird species, five of which were brood parasites (Table 6.1). Metabolic rate was recorded repeatedly from the same eggs over the course of incubation, however, due to natural factors such as predation or egg death, some eggs do not have all recordings. As such, repeat measures per egg varied between 1 and 6 depending on egg survival and incubation length and this was accounted for in statistical analysis.

The eggs of several brood parasite species and their hosts (see Table 6.1.) were measured *in-situ* at multiple time points over the course of their development. Measurements were taken at any time between 8am and 4pm during the day. Nests of hosts were located and monitored to detect the presence of brood parasite eggs. The details of finding and monitoring nests differed between locations and species and is described in detail below. However, for all species, the nests were visited every 2-3 days for repeated respirometry measurements on selected eggs. All parasite eggs present were selected for measurement and additionally 1 or

2 host eggs were randomly selected and marked with pencil to allow reidentification. The measurements were taken close to the nest to minimise disturbance and the length of time the eggs were out of the nests. Eggs were not kept out of the nest for longer than 10 minutes per visit. Due to natural factors such as nest predation, abandonment or egg rejection, some selected eggs did not survive till hatching and are missing later incubation values. The feasibility of accurately recording start of incubation varied between species, so it was necessary to determine embryo age via candling and visual observation. To account for this, and to standardise across species with varying lengths of incubation, an embryo development stage was assigned. This method is described in **Chapter 3**, also see **Appendix item 2** for illustration and description of stages.

Table 6.1 List of species and numbers of eggs recorded for this study. Virulence definition based on descriptions in (Kilner, 2005). Mean incubation lengths per species extracted from the Handbook of birds of the world (Del Hoyo, J.; Elliot, S.A. & Sargatal, 1992).

Species	**n of eggs**	**parasite status**	**mean incubation length**	**location**
Acrocephalus arundinaceus	38	non-parasite	13	Czech Republic
Acrocephalus scirpaceus	17	non-parasite	12	Czech Republic
Cisticola juncidis	22	non-parasite	10	Zambia
Cuculus canorus	53	high virulence	12	Czech Republic
Estrilda astrild	21	non-parasite	11.5	Zambia
Indicator indicator	6	high virulence	16	Zambia
Indicator minor	12	high virulence	12	Zambia
Lybius torquatus	8	non-parasite	13	Zambia
Merops pusillus	8	non-parasite	19	Zambia
Molothrus ater	23	low virulence	11	Illinois,USA

Protonotaria citrea	23	non-parasite	13	Illinois,USA
Vidua macroura	15	low virulence	10	Zambia

Statistical methods

All data analysis was performed in R statistical software ((R Core Team 2020) using the integrated development environment 'R Studio' (R Studio Team 2015). Mean VCO_2 over the most stable 4 minutes of recording was taken as the metabolic rate of the embryo at that time point as was recorded as ml/min. In order to standardise across species with different incubation lengths, an embryo staging system (from 1 to 5) was employed as has been used in other studies (Spottiswoode and Colebrook-Robjent, 2007, **Chapter 3**). Supplementary Table S6.1 lists corresponding incubation days to incubation stage for each species.

Phylogenetically controlled mixed models were used for the primary analysis to control for non-independence of species. For this analysis, a phylogenetic tree of our focal species was constructed and downloaded from the online tree of life, using the R package 'rotl' (Michonneau et al., 2016) (Figure 6.1). The phylogenetic signal of VCO_2 was calculated as the percentage of variance explained by phylogeny as a proportion of total variance in the trait, and is presented as H^2. This value is directly comparable to Pagel's Λ (Hadfield and Nakagawa, 2010). The package 'MCMCglmm' was used to fit a phylogenetic mixed model (PMM), as this package can account for phylogeny and allows multiple measures per species to account for intraspecific variation (Hadfield and Nakagawa, 2010). Mean VCO_2 was the response variable in this model, with parasite status and embryo stage (1-5, continuous) as predictor variables. Parasite status was a categorical predictor with three levels (high virulence, low virulence, non-parasite). The designation of high and low virulence was based on parasite nestling behaviour, where species that actively kill the host offspring were deemed 'high virulence' and those that share the nest with or outcompete the host nestlings were 'low virulence'. This definition was based on description in (Kilner, 2005).
A secondary analysis was applied to the data where embryo stage was included as a categorical factor (1-5, categorical) to compare stage point differences between parasite status groups. All other components of the PMM were kept the same as above.

Results

Increasing metabolic rate with (continuous) incubation stage

Metabolic rate (VCO_2) increased with continuous increasing incubation stage in all species in a non-linear fashion (MCMCglmm; linear term posterior mean = 0.09, 95% CI = 0.08, 0.10, quadratic term posterior mean = 0.03, 95% CI = 0.02, 0.04, cubic term posterior mean = 0.007, 95% CI = 0.0002, 0.04). A comparison of curves, with phylogenetic control incorporated, demonstrated that the metabolic rate of high-virulence brood parasites (Table 6.1) was significantly different in all levels of the polynomial fit, both from non-parasites (linear term posterior mean = 0.02, 95% CI [0.008, 0.04], quadratic term posterior mean = -0.02, 95% CI [-0.03, -0.007], cubic term posterior mean = -0.01, 95% CI [-0.03, -0.003], Figure 6.2) and from low-virulence brood parasites (linear term posterior mean = 0.02, 95% CI [-0.04, 0.004], quadratic term posterior mean = 0.01, 95% CI [-0.07, 0.03], cubic term posterior mean = 0.01, 95% CI [-0.005, 0.02], Figure 6.2). There was no difference in the metabolic rate between low-virulence and non-parasitic species (linear term= 0.005, 95% CI [-0.01, 0.03], quadratic term= -0.007, 95% CI [-0.02, 0.009], cubic term = -0.002, 95% CI [-0.02, 0.01], Figure 6.2). Overall, the phylogenetic signal (Pagel's Λ) of metabolic rate was high across these species (posterior Λ = 0.95, 95% CI [0.90, 0.99]).

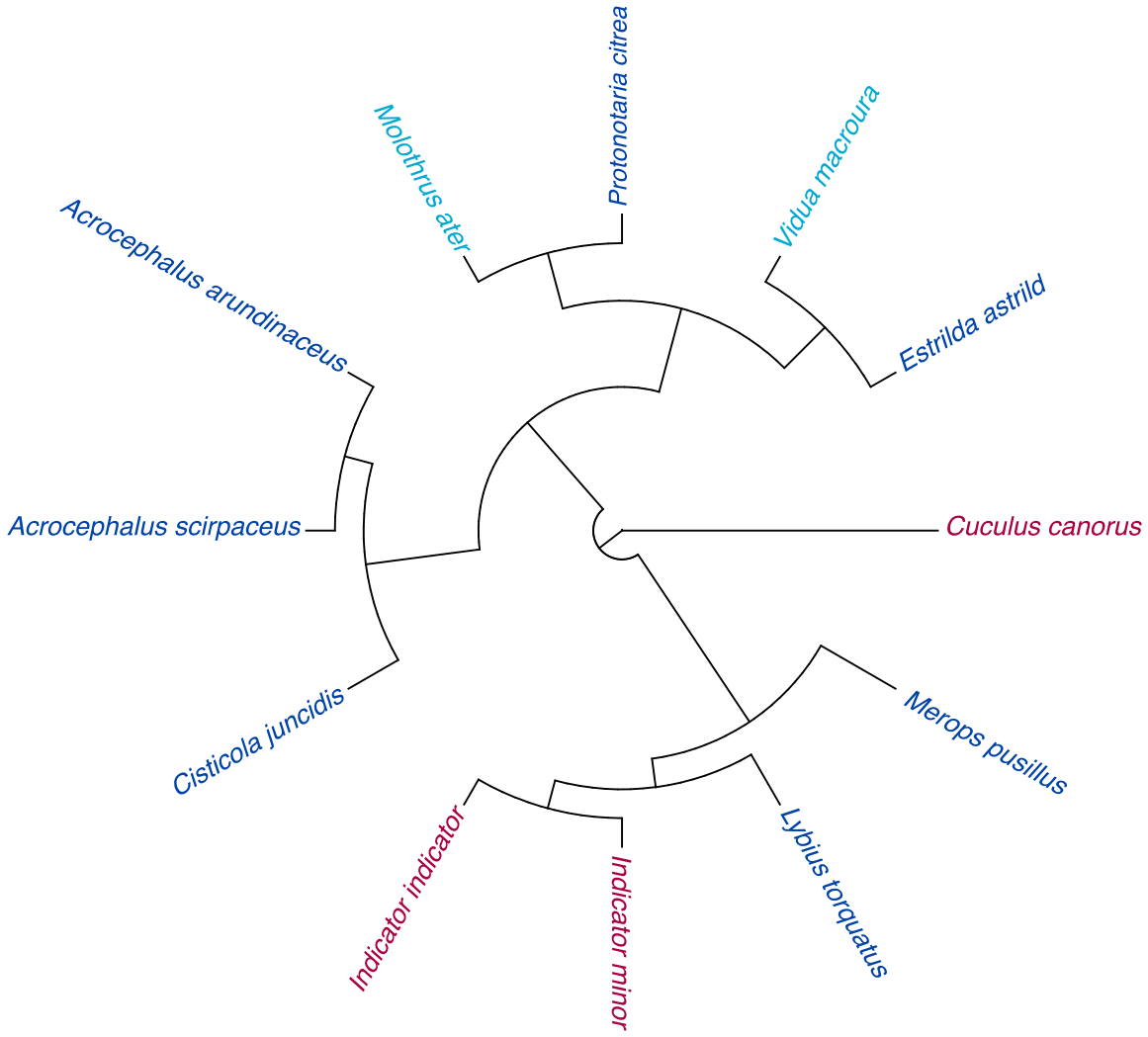

Figure 6.1 Phylogenetic tree of parasite and non-parasitic species included in this study. High virulence parasitic species are in red, low virulence in light blue and non-parasites in dark blue. Tree generated from the online tree of life using the R package 'rotl' (Michonneau et al., 2016). Maximum branch length set to 1.

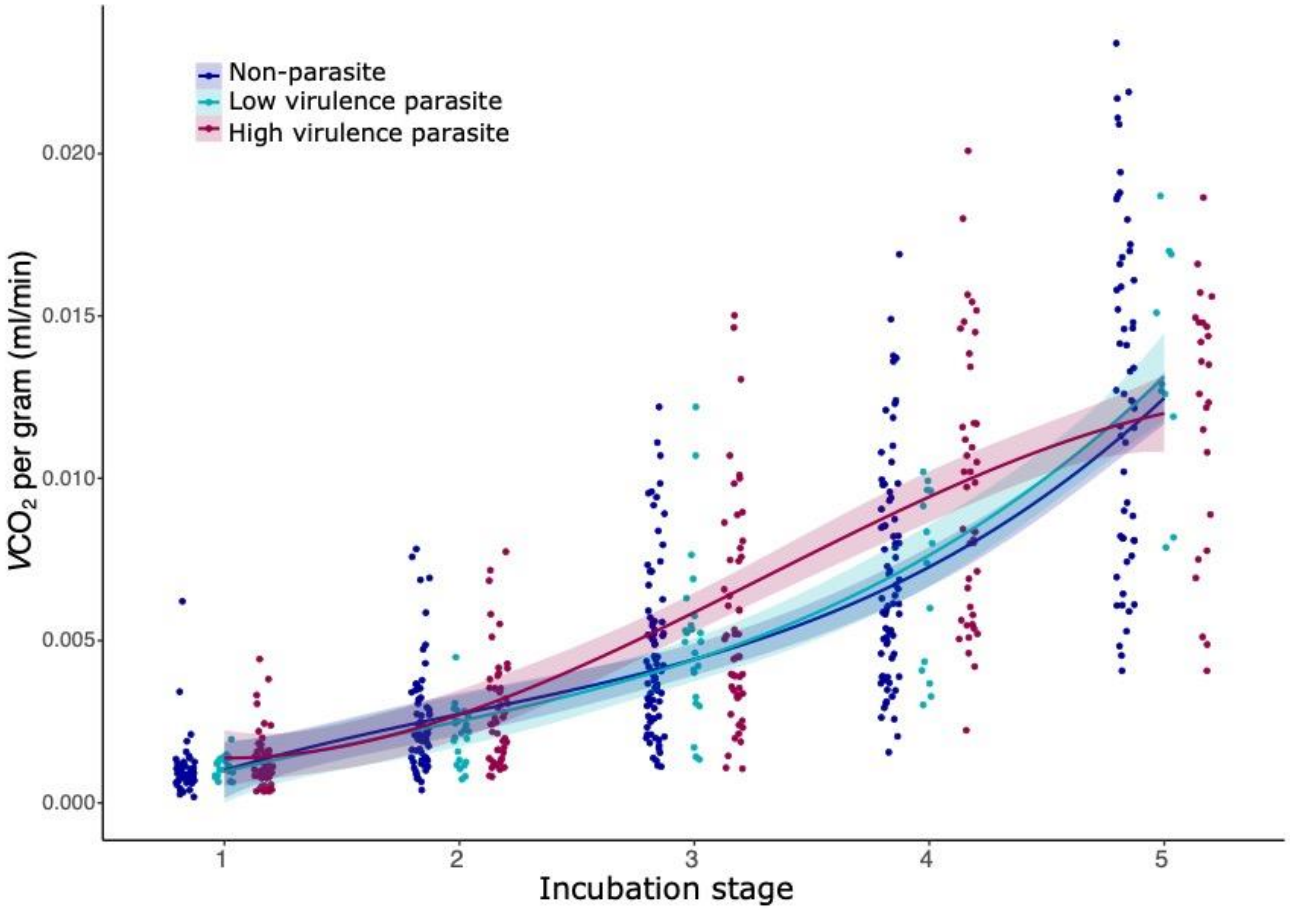

Figure 6.2 Metabolic rate corrected for egg mass (CO_2 per gram of egg, ml/min) of parasitic and non-parasitic species. Parasitic species are split between high virulence and low virulence based on Kilner (2005), see Table 6.1. High virulence brood parasites differ significantly from both other groups.

Comparison of metabolic rate between (descrete) incubation stages

When incubation stage was taken as a categorical predictor as opposed to a continuous factor, high-virulence brood parasites had a greater metabolic rate than non-parasites at incubation stage 3 and stage 4 (stage 3 posterior mean = 0.003, 95% CI [0.0010, 0.004]; stage 4 posterior mean = 0.004, 95%CI [0.003, 0.005], Figure 6.3). Additionally high-virulence parasites had a greater metabolic rate than low-virulence parasites in stage 4 only (posterior mean: -0.003, 95%CI [-0.006, -0.001], Figure 6.3). There was no difference between low-virulence parasites and non-parasites at this stage, nor was there a significant difference between any of the groups at any other incubation stages.

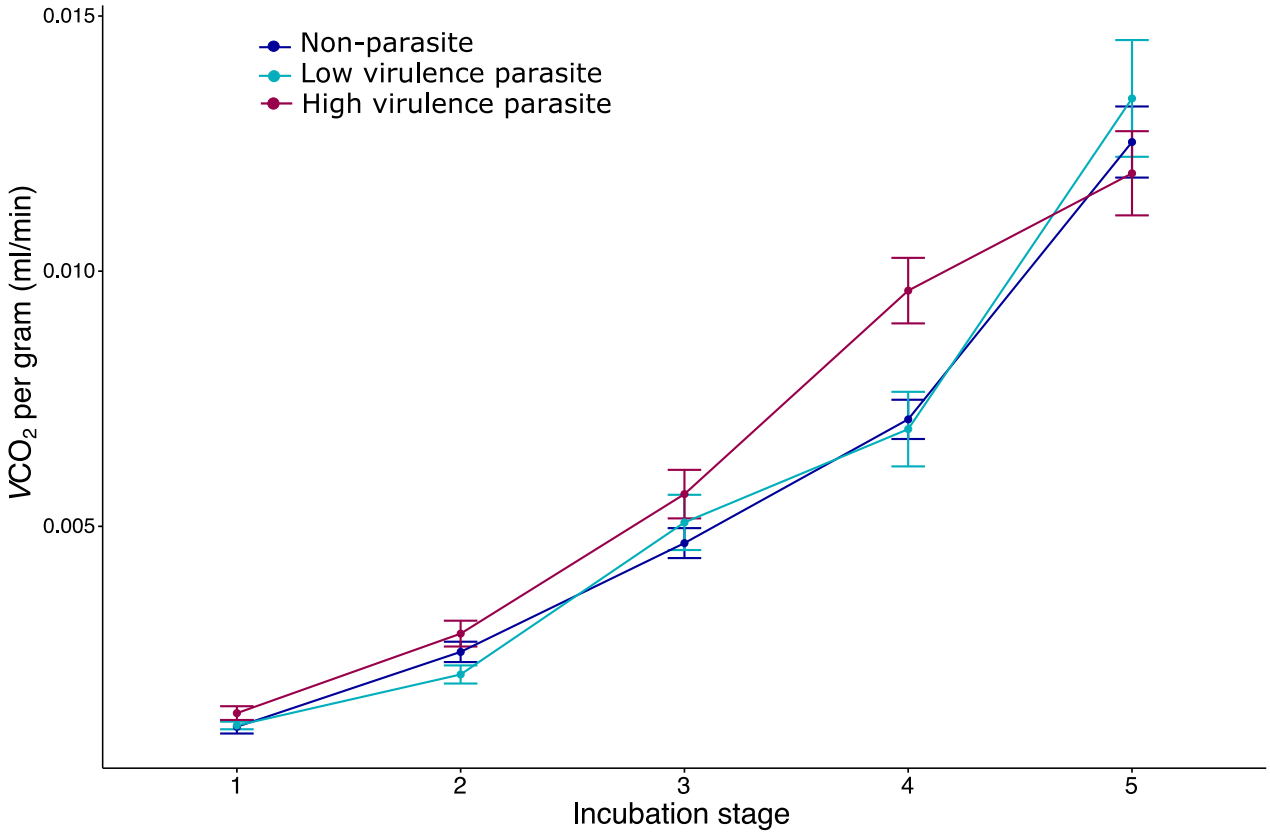

Figure 6.3 Metabolic rate at discrete incubation stages (see Methods) of parasitic and non-parasitic species. Metabolic rate is recorded as CO_2 production and corrected for mass (CO_2 per gram, ml/min). Parasitic species are split between high virulence and low virulence based on Kilner (2005). High virulence brood parasites differ significantly from both other groups at stage 4, and additionally, differ from non-parasitic species at stage 3.

Discussion

The pattern of increase in metabolic rate of high virulence brood parasite embryos was significantly different to that of low virulence parasites or non-parasitic species. The rate of production of CO_2, a proximate measure of metabolism, by high virulence embryos increased with increasing steepness during the early stages of incubation, before slowing down prior to hatching. This resulted in these embryos having significantly higher metabolic rate at stage 4 (approximately 80% into their incubation) compared to other species considered. However, by the stage 5 (end of incubation) the embryo metabolic rate of low virulence parasite and non-parasites increased sharply and there was no difference between the groups. In comparison, the pattern of metabolism appeared to follow similar progression in low virulence parasites and non-parasites, with no significant difference between these two groups at any stage of their incubation. The metabolic pattern of high virulence embryos indicates a greater energy consumption during development compared to other species, which would imply that either their eggs contain more energy stores (Hargitai et al., 2010; Török et al., 2004; but see Igic et al. 2015) or that less yolk is reserved post-hatching (Vleck and Vleck, 1996).

These results suggest that the embryos of high virulence parasites use their energy reserves differently from their hosts and from other brood parasites. For this study, we define high virulence in brood parasites as those whose hatchlings take action to kill the young of the host (Kilner, 2005). While the definition of virulence refers to the impact that the parasite is having on its host, what is more relevant for the parasites embryo's metabolism is the physical demands involved in killing the hosts young. High virulence brood parasite chicks destroy the hosts progeny by either pushing them out of the nest or biting and shaking them to death (Gloag et al., 2012). The energetic cost of these behaviours is high (Grim et al., 2009a; Hargitai et al., 2012), but this has not been quantitatively compared to the costs entailed by low virulence species who compete with the host young.

However, the differences we have observed in the ontogenetic pattern of metabolic rate between high and low virulence parasites suggest there are developmental differences which are likely linked to early-life demands. Unfortunately, little is known about the musculoskeletal requirements of chick killing and eviction, however observations of these behaviours indicate obvious strain for the newly hatched chick. Recordings of nestling greater honeyguides

(*Indicator indicator*) killing little bee-eater (*Merops pusillus*) chicks show the parasite young breathing heavily and requiring frequent rests (Spottiswoode and Koorevaar, 2012). Likewise, common cuckoo nestlings often take several days to completely evict the hosts clutch from the nest and suffered a reduced growth rate during this period (Grim et al., 2009a). This suggests a high level of aerobic capacity is likely to be necessary to achieve total removal of the host young without incurring long term costs (Anderson et al., 2009; Lindström, 1999). The establishment of efficient energy metabolism during embryo development could be fundamental for these birds.

The pattern of metabolic rate development in high virulence embryos showed similarities to the patterns observed in embryos of precocial species. The metabolic rate of embryos of altricial species generally increases continuously and at an accelerating rate through-out incubation, whereas in precocial species metabolic rate increases very steeply for the first 75-80% of incubation before the rate of increases slows down to a plateau prior to hatching (Vleck et al., 1979; Vleck and Vleck, 1980). A short plateau stage is likely present in altricial species; however, it is rarely detected as it may last only hours (Prinzinger and Dietz, 1995). High virulence parasite embryos showed a similar pattern to this, with a steep increase in their metabolic rate early in incubation and a slowing down of this rate of increase close to hatching. This is interesting as all species of high virulence avian brood parasites appear to be altricial at hatching. Both low virulence parasites and non-parasite species exhibited an accelerating increase in metabolic rate that is typical of altricial species, allowing them to 'catch up' with high virulence species by the end of incubation.

It is unclear how this parallel in metabolic rate pattern between precocial birds and altricial high virulence parasites is related to their embryo development. The reason for a plateau in precocial species is still not fully understood (Prinzinger and Dietz, 1995; Dietz et al., 1998), which makes it difficult to interpret the implications of this for parasitic species. It has been theorized that the plateau in precocial birds is a result of more efficient synthesis of tissue during later incubation (Dietz et al., 1998). As the embryo grows and develops, the costs of maintaining the established tissue increases along with continued growth costs, which fits with the continuous increase seen in altricial species. If precocial species can reduce the growth component of their energy costs through more efficient tissue synthesis, this might show as a plateau in their metabolic rate increase (Dietz et al., 1998). If this is the case, high virulence brood parasites might share this growth mechanism with precocial species. A reduction in

embryo activity in precocial species was similarly suggested to explain the plateau stage. This explanation is untested however, and would not explain the metabolic plateau in brood parasites, which are known to increase their embryo movement rates continuously throughout incubation (**Chapter 3**).

Although most brood parasite chicks are altricial in many visible ways, including a lack of down and closed eyes, it is possible that other uninvestigated aspect of their physiology may be more mature at hatching as a result of their increased metabolic rate. This could enable them to perform difficult virulence behaviours. Another factor in the interpretation of these results is eggshell conductance. Brood parasites have lower eggshell conductance than other species, potentially limiting high rates of gas exchange (Portugal et al., 2014; McClelland et al., 2019). Oxygen supply is a limiting factor in embryonic development of avian embryos, with any prolonged decrease in oxygen availability delaying or stunting development (Metcalfe et al., 1981). It has been suggested that eggshell conductance rarely limited metabolic rate of bird embryos (Vleck et al., 1979; Pearson et al., 2002), however, other studies have suggested that the characteristic prominent plateau in the metabolic rate of precocial embryos, which we also see in high virulence brood parasites, is a result of gas exchange approaching the limit of what is available through passive eggshell conductance (Rahn et al., 1974; Tzschentke and Rumpf, 2011; Mueller et al., 2015). Potentially, low eggshell conductance could influence the upper limit of gas exchange for brood parasite embryo development in this way.

Gas exchange is also regulated by the relative O_2 saturation and volume of blood passing through the capillaries of the chorioallantoic membrane (CAM) under the shell. The embryo's O_2 pulse, which is the amount of O_2 consumed per heartbeat, is strongly dependant on the heart stroke volume. In poultry, embryos with lower eggshell conductance or restricted oxygen availability have heavier hearts, indicating this might compensate for lower oxygen diffusion rates by increasing stroke volume (Dzialowski et al., 2002; Christensen et al., 2006). Likewise, common cuckoo hatchlings have both a low eggshell conductance and unusually large hearts at hatching (Cao et al., 2018). This suggests a more complicated relationship between eggshell conductance and embryo metabolism in brood parasites, whereby gas exchange could be enhanced by greater cardiac convection despite low conductance.

The present study included measurements from multiple families of birds and as such accounted for the effect of phylogenetic relationships statistically. In doing so we found that

there was a strong phylogenetic signal evident in the pattern of embryo metabolic rate among these species. The non-parasitic species we measured for comparison in this study were primarily hosts species and, in most cases, phylogenetically distanced from their parasite. As such, it would be useful for future research to measure this trait in non-parasitic close relatives of high virulence species to determine more conclusively whether the metabolic patterns observed are unique to parasitic species within these families. The discovery of a different pattern of metabolic rate development among high virulence parasites incites many new questions about how the physiology of these species differ from other birds, both as embryos and new hatchlings. Future studies should look to understanding the physical and energetic demands of virulence behaviour. This could entail comparative study of the muscle and skeletal systems of brood parasite hatchlings. Beyond its importance for the evolution of brood parasitism, determining why the metabolic rate of high virulence parasite embryos differ from other species would be beneficial to our wider understanding of avian developmental trajectories and avian ontogeny.

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Chapter 6 Supplementary Material

Supplementary table S6.1. Number of days after start of incubation that correspond to incubation stage for each species studied. 'Unk' means this information is unknown. "-" represents no data collected for these stages. "Inferred" means embryo stage was estimated based on proportion of incubation period completed as a proportion of mean incubation period for that species, and informed by published resources on development patterns in altricial species (Hemmings et al. 2016). "Field observation" means embryo stage was determined by candling in the field (see Appendix item 2).

Species	**Stage 1**	**Stage 2**	**Stage 3**	**Stage 4**	**Stage 5**	**Method**
Great reed warblers	3,4	5,6	7,8	9,10,11	12,13,14	field observation
Zitting cisticolas	1,2	3,4	5,6	7,8	9,10	field observation
Common cuckoos	2,3	4,5	6,7,	8,9,10	11,12	field observation
Common waxbills	2,3	4,5	6,7	8,9	10,11	field observation
Greater honeyguides	unk	unk	unk	unk	unk	field observation
Lesser honeyguides	unk	unk	unk	unk	unk	field observation
Little bee-eaters	unk	unk	unk	unk	unk	field observation
Black-collared barbets	unk	unk	unk	unk	unk	field observation
Brown-headed cowbirds	2,3	4,	5, 6	7,8	9,10	inferred
Prothonotary warblers	3,4	5,6	7,8	9,10	11,12	inferred
Pin-tailed whydahs	-	3,4	5,6	7,8	9,10	field observation
Domestic pigeons	3,4,5	6,7,8,9	10,11,12,13	14,15,16,17	18,19,20	field observation

CHAPTER 7. General Discussion

While behaviour evolution of brood parasites and their hosts has been studied extensively, little research had focused on the eggs of these species beyond egg appearance and mimicry. This is surprising because the eggs of brood parasites must be adapted to develop with the incubation, nest environment and care of an entirely different species. Furthermore, this reproductive strategy relies on the success of costly behaviours early in life (e.g., fast growth and either competing with or killing host young) for which the inchoate chick must hatch fully prepared. As such, this thesis aimed to bridge this gap in our knowledge about the physiological adaptations of a fundamental life-stage of these parasitic birds.

This thesis sought to identify egg and embryo adaptations to brood parasitism by measuring and comparing multiple aspects of the egg structures and the embryos development across a wide range of independent lineages of brood parasites. By comparing traits between phylogenetically independent brood parasites and contrasting this to the same traits in their non-parasitic relatives, we were able to account for the evolutionary histories of these birds and so better link the occurrence of these traits to parasitic reproduction.

The traits I investigated for this thesis can be broadly grouped into eggshell adaptations (**Chapters 2, 4 and 5**) and embryo adaptions (**Chapters 3 and 6**). However, there is significant overlap between these categories; the eggshell, the egg contents and the embryo are highly interconnected and cannot be considered in isolation. **Chapter 4** differs from the other sections of this thesis in that is does not specifically relate to brood parasites but is instead filling an important gap in the evolution of eggshell physiology, and links to the results of the other eggshell structural chapters.

The principal findings emerging from this thesis are as follows:

1) Brood parasite eggshells have a lower water vapour conductance GH_2O compared to their hosts and for their phylogenetic position, and this was evident across phylogenetically distinct lineages (**Chapter 2**). There was no statistically significant difference between high virulence parasites and low virulence parasites, however there was a trend towards lower GH_2O in high virulence species.
2) Brood parasite embryos had a greater rate of movement throughout incubation compared to their hosts and non-parasite species (**Chapter 3**). Three out of 5 parasite species measured had higher movement rates than their hosts, and importantly this was not related to their level of virulence.
3) Both embryo movement (**Chapter 3**) and metabolic rate (**Chapter 6**) were higher in brood parasites compared to non-parasitic species, and increased more steeply during incubation. However, while movement increased in a linear manner, metabolic rate followed a non-linear (quadratic) increase throughout incubation.
4) Metabolic rate change over development in highly virulent brood parasites was significantly different from that of low virulence brood parasites as well as non-parasite (**Chapter 6**). The shape of the metabolic growth curve of high virulence brood parasites was visually similar to that of precocial bird species, in that it exhibited a clear 'plateau', as a slowing of the increase in metabolism towards hatching is observed.
5) Overall, the eggshell surface properties (wettability and surface roughness) of brood parasite eggs were not significantly different than non-parasites (**Chapter 5**). Nor were traits of brood parasite eggs more similar to that of their hosts than they were to any other species' eggs. However, pigmented regions of cuckoo finch eggshells were significantly rougher than background regions, whereas this was not the case for the host eggs they were mimicking.

Below I discuss possible relationships between physical traits that were uncovered in brood parasite eggs and their embryo behaviour and development, and suggest avenues for future research.

Eggshell structure and composition in brood parasites and beyond

This thesis investigated multiple aspects of brood parasite eggshell structure using museum eggs and fresh eggs collected, with permits, from our field sites. While the combination of museum and field-based collection broadened the range of species across which comparisons could be made, the sample size was still potentially low for many species as we were constrained by what was available within the destructive collection of the museums, and what could be collected from the field at any given time. Regardless, I found strong patterns in many of these eggshell traits across brood parasites. In **Chapter 2**, I found that brood parasites had a lower water vapour conductance than expected, both for their phylogenetic position and when compared to their respective hosts. This result matches what has been found previously in a single parasite species, common cuckoos, although it was contrary to what had been predicted by previous studies based on pore counts. This result suggested a convergence on eggshell structure driven by parasitism across different lineages (McClelland et al., 2019). However, when I compared the composition (calcium carbonate content) and the surface properties (hydrophobicity and surface roughness), in **Chapter 5** we did not find any significant difference between parasites and non-parasites. This indicated that either these are not traits that are selected for by a parasitic reproductive strategy, or that these traits are too strongly controlled by other selective forces to show adaptations to parasitism. However, it is likely that lower eggshell conductance in brood parasites correlates to their rates of embryo gas exchange, which I explored in **Chapter 6**.

Chapter 4 showed, among other results, a trend across bird families for eggshells with a lower proportion of calcium carbonate in species with thicker eggshells. We had expected the opposite pattern, where thicker eggs contained a higher calcium component; this result might reflect that calcium is an expensive mineral for birds to procure. Although brood parasites lay eggs that are thicker than average for their egg size, when we compared several parasite species to hosts, we did not find them to have either a lower or higher calcium carbonate content (**Chapter 5**). This might suggest that for their given shell thickness, brood parasites have a higher-than-expected calcium content. However, as methodology was marginally different (temperature of eggshell incineration) between how calcium carbonate content was assessed in **Chapter 4** and **Chapter 5**, we did not think it was appropriate to compare these datasets.

There is evidence, at least in captivity, that brown-headed cowbirds reduce their egg-laying rate when calcium is restricted (Holford and Roby, 1993). In the wild, however, parasites can compensate for any limit in diet-based calcium by consuming host eggs at laying. Overall, the relationship between brood parasites and calcium is potentially more complicated than could be assessed in this study.

The surface roughness of the eggshells of multiple brood parasite species, along with their hosts (and a selection of related non-host species for phylogenetic control), were measured using optical profilometry (**Chapter 5**). This method compared the nanoscale peaks and troughs of the eggshell and assigned a measure of roughness based on this. We did not see any significant patterns in the roughness of brood parasites compared to their hosts. However, the eggs used for this measurement came from multiple sources which were stored with varying degrees of care. It possible that the outermost cuticle of some eggs was worn away since initial laying, as the cuticle of eggs is very thin, potentially water soluble and most often organic (D'Alba et al., 2014, 2017). This could potentially affect the biological relevance of this measure, if the eggshell surfaces measured were not the outermost layer present in the nests, at least at initial laying. In **Chapter 5**, I speculate that a smoother eggshell surface could benefit brood parasites by increasing the difficulty of rejection by grasp ejection (i.e. grasping the intact egg in their beak). This idea could be further investigated by comparison between parasites whose hosts primarily eject suspicious eggs by grasping and those that reject eggs by puncturing them; I did not have the sample size or spread of species necessary to fully test this idea in this project.

Interestingly, I did find a difference in the surface roughness between the pigmented and unpigmented regions of cuckoo finch eggs, where the pigment spots were rougher than the background (**Chapter 5, Figure 5.6**). This difference was not seen in eggs of their hosts, tawny-flanked prinias, who's pattern these eggs were mimicking. This result suggests a difference in how or where the pigment is deposited in the eggshell, as a more superficial pigment deposition would influence the surface properties more so than pigment that is deposited deeper in the shell layers. Alternatively, there could be differences in the composition or quality of the pigment itself. In other host-parasite system, the chemical basis of eggshell pigment mimicry has been explored. In common cuckoos the pigments and pigment concentration of the eggshell are similar to their host races (Igic et al., 2012).

Conversely, in striped cuckoos a mimetic colouring is achieved through a different composition of pigments than their hosts (Dainson et al., 2018). This indicated that the mechanisms by which parasites achieve eggshell pattern mimicry might differ from their hosts. To my knowledge, the vertical location of pigment deposition has not been compared between any parasite and their host, but our results hint that a difference might exist between cuckoo finches and prinias.

Chapter 5 also investigated wettability (a.k.a. hydrophobicity) and did not find a difference in this measure between parasites and non-parasites. However, there was a high degree of variation within species, which may have obscured any potential differences. Despite high variation, the methodology for this measurement was well validated, as published by my colleagues (Attard et al. 2021, *in press*), since measurements taken on different points of the same egg gave comparable results. Again, potential wear or degradation of the cuticle could explain the high degree of interspecific variation seen within species. Alternatively, wettability is potentially influenced by organic material deposited on the eggshell surface, such as uropygial gland oils (Martín-Vivaldi et al., 2014), which could degrade on museum stored eggs, over time.

Overall, the results of this thesis highlight the complexity of factors that influence selection on eggshell structure, composition, and function. Much research is focused on the biomechanics of chicken and poultry eggshells because of their relevance to the food industry (Samiullah and Roberts, 2014; Wellman-Labadie et al., 2008). Yet, even for chicken eggs, there is still much uncertainty on how the shell is formed and its structural properties. Discoveries on domestic fowl eggs are often generalised to all birds, despite centuries of artificial selections, and the diversity in form and function of wild bird eggs (Gautron et al., 2021; Wilson, 2017). The results of **Chapter 4**, while providing a first step towards answering these questions, indicates that less is known than we thought about how species differ in their eggshell formation. Relating these result more specifically to brood parasite eggs, I propose that this group of birds are likely to show many other features of their eggshell mechanics that differ from their non-parasitic relatives.

Embryo behaviour and energetics

The embryonic period of an animal's development is important for dictating future phenotype and fitness (Gorman and Nager, 2004; Metcalfe and Monaghan, 2001). Avian embryos make excellent systems for studying general vertebrate embryology as they develop externally to the mother and so can be easily observed and manipulated (Douarin and Dieterlen-Lièvre, 2013; Mueller et al., 2015). This has led to much research on chicken eggs over the last century (Mueller et al., 2015; Romanoff and Romanoff, 1949), however, as with eggshell mechanics, a lot of what has been discovered in domestic species is assumed to apply to all birds. Embryo development and energetics have been investigated in only a handful of wild species, so little is known about variation across birds (Prinzinger et al., 1995; Vleck and Hoyt, 2009). This hinders attempt to fit my results on embryo energetics and behaviour of brood parasites into the context of 'normal' bird development. However, a benefit of brood parasites as a study system is the conveniently available host species with which to compare. As such, many of the results of **Chapter 3** and **Chapter 6** make use of the host species as examples of non-parasite embryos. This method has the potential to introduce some error, as frequent host species may have evolved modifications to their embryo development as a result of co-evolving with their parasite. To counter this, we had planned to include more species which were neither parasite or hosts in this research, and in particular to include close relatives of parasite species, such as non-parasitic cuckoos. Unfortunately, due to the Covid-19 pandemic I was unable to collect these data. It would also be valuable for future research to build on these results with comparison within avian families between parasites and non-parasites. This would help to separate embryo adaptations that are common to bird families, such as *Cuculidae*, and adaptions that have specifically arisen as a response to intra- or inter-specific parasitic behaviour.

For **Chapter 3** and **6** particularly, I was fortunate enough to have collaborators across several continents allowing me to visit their established fieldsites to collect my data. For **Chapter 3,** I was also able to send the 'EggBuddy' devices to some collaborators letting them record the embryo movement on my behalf when travel was impossible or too expensive, and transfer the videos back for embryo movement to be counted by myself or by collaborating M.Sc. students (M.R and M.C, see author contributions in **Chapter 3**). As a result, **Chapter 3** on embryo movement included non-parasitic cuckoo species (African black coucals and white-browed coucals) which were suitable phylogenetic controls for the common cuckoo.

Unfortunately for **Chapter 6** on embryo respiration, I was unable to collect data on non-parasite *Cuculidae* species. Although the strong similarities in patterns of metabolic rate between common cuckoos and the other high virulence brood parasite species measured (honeyguide species) is strong evidence that this metabolic ontogeny is related to parasitism, a comparison to non-parasitic relatives would have helped to solidify this.

Chapter 3 found that embryo movement rates increased more steeply in brood parasite embryos than in non-parasite species. Embryo movement has been shown in other species (primarily domestic birds) to increase the number of primary muscle fibres in muscle complexes (Hammond et al., 2007; Heywood et al., 2005; Pitsillides, 2006), which in turn controls post-natal muscle growth potential. We could not directly compare the muscle development of the individual hatchlings of the eggs we were comparing, as that would have entailed invasive procedures for which we did not have permits. However, newly hatched common cuckoos have previously been shown to have a greater muscle fibre density in their hatching muscles compared to their hosts (Honza et al., 2015). This suggests that embryo movement is a plausible explanation for this muscle development. Whether other muscles of the common cuckoo, or other brood parasite hatchlings are similarly enhanced has not been investigated. An invasive study, involving monitoring embryo movement and then sacrifice and dissection of freshly hatched parasite chicks, would be required to ascertain a direct link between embryo movement and muscles in these species. This would also allow one to pinpoint any specialised qualities to the muscles involved with in eviction and chick-killing. Such an experiment was outside the scope of this project.

A better understanding of brood parasite nestling physiology, especially immediately after hatching, would greatly improve our understanding of how highly virulent species achieve the task of killing host young. Very few papers have looked at the condition of freshly hatched brood parasites, apart from the paper discussed above on the hatching muscle, (Honza et al., 2015), and Cao et al. (2018) who measured hatchling heart size. Consequently, we do not know whether obligate brood parasites possess any adaptations to their muscles or skeleton compared to typical altricial chicks. Birds on the altricial end of the development spectrum hatch in what is considered an under-developed state, even described as 'embryo-like' (Starck and Ricklefs, 1998). This means that they hatch with eyes sealed shut, no down or feathers, unable to thermoregulate, and with limited motor abilities (Starck, 1993; Starck and Ricklefs, 1998). Additionally, the skeleton of altricial young is less ossified at hatching

compared to precocial species, especially the long bones associated with hatchling locomotion (Blom and Lilja, 2004). This raises the question of whether brood parasites hatch in a typical altricial state, given the strength exhibited shortly after hatching. To my knowledge, the level of ossification of hatching brood parasites has not been assessed. In particular, future research could determine the ossification of the leg bones of cuckoo nestlings prior to eviction, which would be expected to require rigidity for this activity. Mechanical stress on unossified bones can cause permanent deformity in vertebrates (Caine et al., 2021; Cobcroft et al., 2001), as such if the long bones of the brood parasite chick are unossified, the mechanical strain of eviction could be detrimental.

It has been shown in many species of precocial birds that the level of maturity at hatching can differ between body parts (Aourir et al., 2016; Dial and Carrier, 2012), such as in mallards (*Anas platyrhynchos*) where hindlimbs are highly functional for locomotion at hatching whereas forelimb (wing) growth is delayed (Dial and Carrier, 2012). Speculatively, it is possible that evictor species of brood parasites could evolve a 'prioritisation' of limb development to acquire the ability to evict eggs. This could further link to the result of greater embryo movement in brood parasites (**Chapter 3**), as increased embryo motility in chicken embryos has been associated with longer leg bones (Hammond et al., 2007).

I found significant differences in the metabolic rate of high virulence (chick-killing) brood parasites and low virulence (nestmate tolerant) species in **Chapter 6**. The metabolic rate of embryos was measured as the production of CO_2. I had originally planned to also record O_2 consumption, however the measurement of O_2 was very sensitive to temperature and atmospheric pressure changes, which made it unsuitable for recording in the field.
The metabolic rate of all embryos increases over development as the tissue mass (and hence maintenance costs) increase, along with the growth costs. However, in highly virulent species the sharp increase in metabolic rate slowed down during the last 20% of incubation. This is similar to the patterns that are seen in precocial species (Vleck et al., 1979; Vleck and Vleck, 1980). The 'plateau' stage of metabolism ontogeny is associated with precocial development, however more recently several studies have identified a plateau in altricial species also (Hatzofe and Ar, 2003; Prinzinger et al., 1997, 1995). However, plateaus in altricial species, when they occur, happen earlier in development and for a shorter duration (Prinzinger and Dietz, 1995). Given the methodology of **Chapter 6**, where eggs were recorded every few days, it is possible that a short plateau stage was missed in our non-parasite and low virulence

parasite embryos. Regardless, a clear difference remains between these groups in shape of the curve and as a result the higher rate at stage 4 (80% of incubation) in high virulence brood parasites.

The reason for the plateau stage in precocial bird has not been definitively explained. As a result it is hard to draw conclusions on what the presence of a plateau in brood parasites indicates for their development. Dietz et al. (1998) suggested that the plateau in precocial species could be the result of a reduction in embryo movement later in incubation. However, our results in **Chapter 3** rule this out as an explanation for the plateau in virulent brood parasites. Another question that has not been addressed is how much energy embryos expend on movement. While I recorded both embryo movement rates and metabolic rates in many of the same eggs in this study, it was impossible to record both simultaneously with my equipment and set-up. While both embryo movement and metabolic rate increased over development in all species (**Chapter 3** and **Chapter 6**), they did not increase in the same manner. Whilst it would be difficult to apply to wild species, it would be insightful for future research to measure the metabolic cost of movement with modified incubators that could continuously measure both embryo movement (using infrared light disruption) and CO_2 production during incubation. This would also give a finer scale picture of how both factors change over incubation.

If embryo movement is adaptive for muscle or skeletal development as suggested, it would be expected to entail an energetic cost. The cost of movement, and the higher metabolic rate in high virulence brood parasites, must presumably be paid with energy reserves of the yolk. Whether or not the yolks of brood parasite eggs are more energy rich than other species is debated. Török et al. (2004) and Hargitai et al. (2010) showed that the yolks of common cuckoos are heavier than their hosts and larger than expected for the egg size. Yet, Igic et al. (2015) showed that the concentration of triacylglycerols, the primary energy-reserve lipid of egg yolk, was lower in common cuckoos compared to two of their hosts, including great reed warblers. However, the results of **Chapter 6** suggest that since the metabolic rate is higher or equal throughout incubation, the total energy consumed during development by common cuckoos (included in the high virulent brood parasites category) must be greater than that of great reed warblers (included in the non-parasite category). What energy reserves are being used for this metabolic activity cannot be answered by this study but deserves further investigation.

Additionally, it should be considered that the hatching process of brood parasites is known to be especially strenuous due to their thicker eggshells (Igic et al., 2017; Spaw and Rohwer, 1987), which requires a greater number of pecks to break out off (Honza et al., 2001; Yoon, 2013). This process also requires energy supplied by the egg, and so at least some of the yolk supplies must be retained for this. The liver may also play a role in this process. Chicken embryos form and store glycogen in their livers early in development, which they later convert and metabolise as glucose to fuel hatching (Freeman, 1969; Wittmann and Weiss, 1981). Previous work has shown that common cuckoos have enlarged livers (compared to hosts) at hatching (Cao et al., 2018), which could suggest greater glycogen storage. However, it is not clear what the cost of glycogen production is for the developing embryo.

Concluding remarks

Overall, this thesis provides a window into the remarkable biological construct that is the brood parasite egg. Brood parasitism is an extreme reproductive strategy that has evolved multiple times due to its benefit in shedding the cost of parentage. However, how it is physiologically achieved is often taken for granted. This thesis helps to bring insight into how the eggs and embryos of these species differ from their hosts to 'beat' their hosts. I believe that this work begins to scratch the surface of what there is to be discovered about the physiology of brood parasite embryos and the structural ingenuity of their eggs. Additionally, I hope that this thesis has highlighted the need to consider the phylogenetic range of parasites and how they have each evolved unique (or sometimes very similar) strategies to solving many of the same challenges. And beyond the field of brood parasite research, I hope that our results might inspire some new ideas for the study of avian and vertebrate evolution in general.

Covid-19 pandemic impact on thesis

The Covid-19 pandemic took grip just over halfway through my PhD project with resultant frequent lockdowns, lab closures and bans on travel and fieldwork. I was fortunate to have completed a lot of fieldwork and data collection already by that time. However, my final fieldtrip to Zambia was cut short and I had to cancel a fieldtrip planned to Panama to collect

data on Greater Ani's (*Crotophaga major*) (as a non-parasitic cuckoo) in collaboration with Dr Christina Riehl (Princeton University). Additionally, I had planned in 2020 to collect more metabolic rate data on non-parasitic passerines for a phylogenetic control. Due to the loss of these field seasons **Chapters 3** and **6** are missing several species that were originally to be included. Another aspect that I had intended to investigate in this project was the energetic cost of eviction in common cuckoos. During fieldwork on common cuckoos in the Czech Republic in 2019, I piloted a set up to measure this with some success. This involved placing a young cuckoo chick into an old nest inside a metabolic chamber and enticing it to evict and artificial egg. Unfortunately, my intention to run this experiment in the summer of 2020 could not go ahead.

In addition to disruption to fieldwork, the engineering lab where I was collecting eggshell biomechanics data at the Open University was closed to visitors for much of 2020 delaying **Chapter 5**. Thanks to excellent collaborators, who were able access the labs and to help collect this data for me, I eventually got most of the eggshells measured. However, the spread of species and sample size of this chapter was still reduced. The life history investigation of eggshell calcium carbonate content (**Chapter 4**) was originally intended to be combine with **Chapter 5** on eggshell mechanics. However, it became a larger research question as the pandemic meant I had a lot more time, and no ability to collect data for much of the year. Overall, I was lucky to have a lot of assistance to mitigate the impact of the pandemic and my project was less negatively impacted than many of my cohort.

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Appendices

1. Appendices item 1.

Field site and focal species details (Chapter 3 and 6)

Zambia (dry season)

Fieldwork in Zambia took place in the Choma region (16°49'S, 26°59'E) at a field site established by Prof. Claire Spottiswoode (University of Cambridge). The field site was on a land owned by local farmers, and the habitat is a mixture of native mixed (miombo) wood-land and tobacco fields. Data were collected on greater honeyguides (*Indicator indicator*) and their hosts, black-collared barbets (*Lybius torquatus*), and lesser honeyguides (*Indicator minor)* and their hosts, little bee-eaters (*Merops pusillus*), at a field site on a farm in the Choma District of Zambia during the dry season (May to November) of 2016, 2017 and 2018. Further details of the field site can be read in (Spottiswoode, 2013). Black-collared barbet nests were located in tree cavities and accessed by openings that were cut into the cavity wall above the nest and covered by strips of bark between visits. The nests of little bee-eaters were located in underground tunnels dug into the side of aardvark (*Orycteropus afer*) burrows. These could be accessed by digging down to the nest from above.

Nests were visited and embryo movement recordings taken every 2–3 days during the incubation period. Nests were often located after the beginning of incubation, and incubation stage (described in Appendix item 2) was estimated by candling the egg and assessing embryo development (Lokemoen and Koford, 1996). Exact incubation day was unknown for these nests. Embryo movement measurements were taken from the parasite egg in each nest located, along with any live host eggs present. Greater honeyguide females often puncture the host eggs when they lay their own (Spottiswoode, 2013), and so most parasitized nests of little bee-eaters did not contain live host eggs; therefore, measurements were also taken from non-parasitized little bee-eater nests. We were unable to get

measurements from greater honeyguides during early incubation (prior to developmental stage 2).

Zambia (wet season)

Wet season data were collected at the same field site described above. Data were collected from pin-tailed whydahs (*Vidua macroura*) eggs, and the eggs of their hosts, the common waxbill (*Estrilda astrild*). Additionally data were collected on zitting cisticolas (*Cisticola juncidis*), which are common hosts of cuckoo finches (*Anomalospiza imberbis*), however low parasitism rates during the 2019 and 2020 breeding season meant insufficient data were collected on this parasite to include it in this study. Zitting cisticola data were included as a non-parasitic species for phylogenetic comparison.
These data were gathered at the same field site described above during the wet season (December to April) of 2019 and 2020. Both common waxbills and zitting cisticolas build cup nests close to the ground in grassy habitat which are easily accessed. Nests were located by local field assistants and were measured every two days. Egg stage was estimated by candling and incubation commencement and hatching date was known for most nests (see Table S6.1). No measurements were recorded for pin-tailed whydahs at stage 1 due to difficulty locating nests and low parasitism rates during these years.

Czech Republic

Fieldwork in the Czech Republic took place at a field site near Mutěnice, Czech Republic (48°54'N,16°59'E) in the south of the country. This site was established and maintained by Dr Marcel Honza (Czech academy of science). The habitat at this site was primarily artificial aquacultural ponds with dense reed beds around the banks. Data were collected from common cuckoos parasitizing great reed warblers (*Acrocephalus arundinaceus) and Eurasian reed warblers (Acrocephalus scirpaceus)*. The nests of great reed warblers were located in these narrow strips of reed beds surrounding the ponds. Parasitized nests were visited either every day or every two days during incubation and eggs were briefly removed and brought to the bank of the pond for measurement. The eggs were replaced with decoys while measurements were taken and returned to the nest within 10 minutes. Some abandon or rejected eggs were transferred to the lab and incubated until hatching. For details about incubation procedure see Honza et al. 2001. Embryo movement (**Chapter 3**) measurements

were taken from these eggs. Measurements from incubator-hatched eggs (n= 18 of 68) and wild-hatched eggs were not statistically different in EM rate ($t_{(41)} = 0.906$ $p = 0.366$), so these data were combined for analysis. The chicks which hatched from these eggs were returned to other nests at the field site.

Illinois, USA

Fieldwork in the USA was undertaken in the Cache River watershed, southern Illinois (centred on 37°24'N,88°53'W) at a site maintained by the research group of Prof. Mark Hauber (University of Illinois Urbana-Champaign). This site was primarily swamp and flooded deciduous forests. Measurements were collected on brown-headed cowbirds in a nest box breeding population of prothonotary warblers (*Protonotaria citrea)* (for further details see Hoover and Hauber 2007) during the summer of 2018. Nest boxes were sited on public land and had high rates of parasitism during the study year (~ 80%). The egg monitor was set up on dry land close to the nest box and eggs were removed for less than 10 minutes for measurements. One host egg in each nest was measured in addition to any parasite eggs. For nests that contained two cowbird eggs, both parasitic eggs were measured. Eggs were measured every two days during incubation.

Tanzania

Measurements were collected on classically polyandrous African black coucals (Centropus grillii) and white-browed coucals (*C. superciliosus*) in the Usangu wetland in south-western Tanzania (8°41’S, 34°5’E) at a field site run by Dr Wolfgang Goymann. Coucals build dome-shaped nests in dense vegetation. These nests were located either by observing birds carrying nesting material or incubating birds back to the nest, or by following birds equipped with radio-transmitters (for further details see Goymann et al. 2015, 2016). The egg monitor was set up ca. 5–10m from the nest and eggs were removed for less than 10 minutes for measurements. Eggs were measured every four days during incubation.

Note: Only embryo movement data (**Chapter 3**) was recorded in these species. Metabolic rate (**Chapter 6**) was not recorded on any species at this fieldsite.

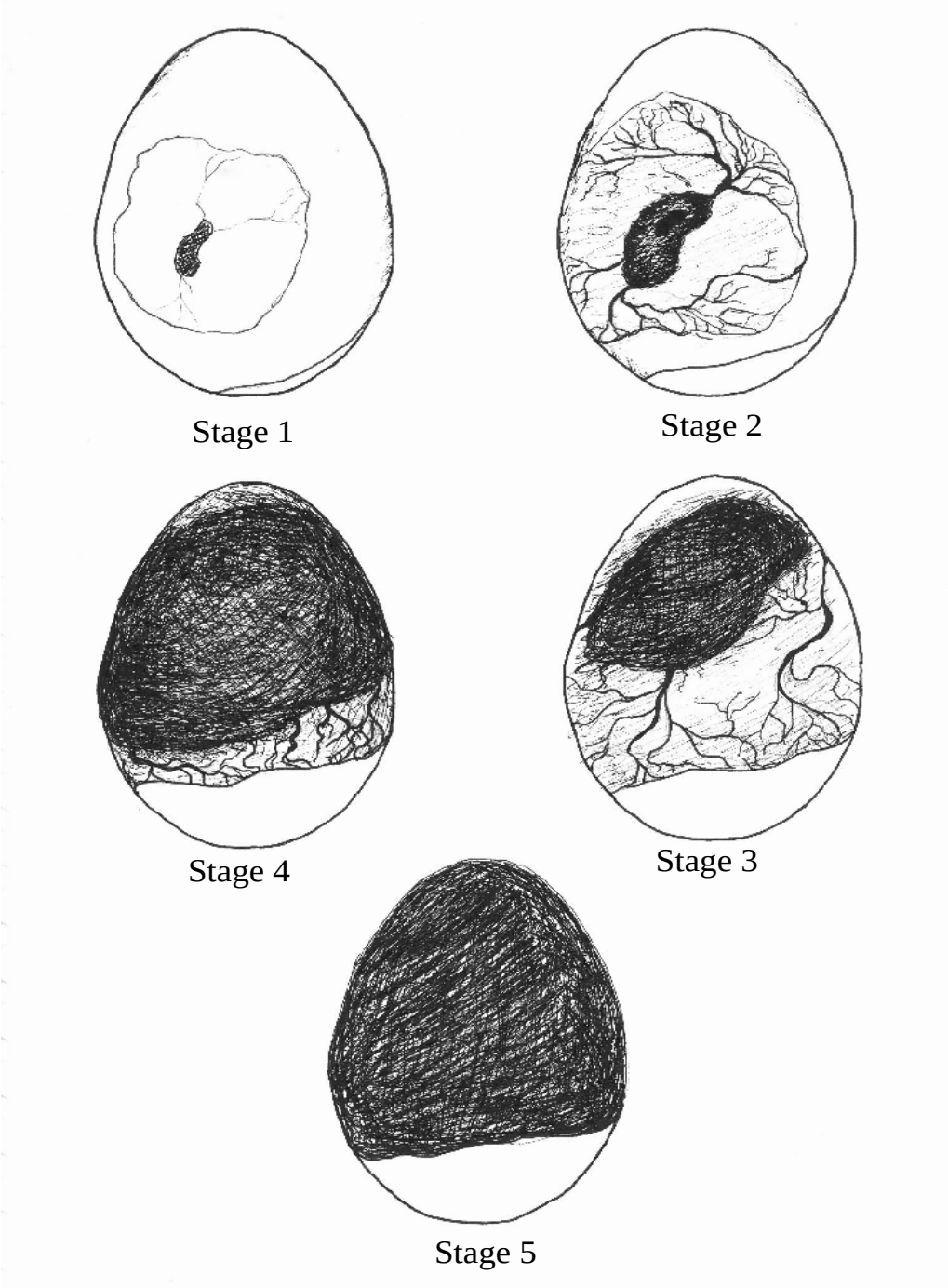
Domestic pigeons (UK)

Measurements were taken on the eggs of domestic homing pigeons (Columba livia) in the UK. Pigeons nested in purpose-built housing lofts (2.1m x 1.8m) on the campus of Royal Holloway University of London (51°25'N, 0°33'W), and recordings were taken at the lofts on alternate days between incubation days 3 and 20. Husbandry details available in (Portugal et al., 2017).

Note: Data on domestic pigeons were included in **Chapter 3,** for embryo movement but embryo metabolic rate was not measured in this species (**Chapter 6)**.

2. Appendices item 2.

Incubation stages assigned to eggs at sequential stages of embryonic development



Appendix Figure 1.

Incubation stages assigned to eggs at sequential stages of embryonic development. Stage 1: Albumen light orange. Small dark red embryo visible attached to the membrane on one side of the egg. A first circular blood vessel sometimes visible encircling the embryo. Air cell very small, barely visible. Stage 2: Embryo approximate double the size of stage 1 and more defined. Dark spot of the heart in centre of embryo visible, beating of the heart visible later in this stage. A 'spiderweb' network of veins visible extending from the embryo along the membrane and connecting with the circular blood vessel initially visible in stage 1. Air cell clearly discernible. Stage 3: Embryo much larger and takes up about 1/3 of the egg interior. Usually difficult to see clear details of the embryo as it has moved further in from the shell membranes and is obscured by blood vessels. Movement of the embryo is easy to see during candling during this stage. The albumen appears much redder as blood vessels extend through most of the egg. The air cell is approximately 50% larger than stage 2. Stage 4: Embryo takes up a large proportion of the egg interior, at least 50%, and appears as a dark mass. A band of clear and highly vascularised (red) albumen is visible between the dark mass of the air cell. Air-cell is the same size or slightly larger than in stage 3. Stage 5: The egg appears almost entirely dark except for the air cell as the embryo takes up all the available space. Blood vessels mostly obscured by the embryo. Air cell similar in size to stage 4. Embryo has not yet internally pipped (pierced the air cell).

Publications

Two peer reviewed publications have so far resulted from the research from this thesis and are attached here.

Chapter 2.

McClelland, S.C., Jamie, G.A., Waters, K., Caldas, L., Spottiswoode, C.N., and Portugal, S.J. (2019). Convergent evolution of reduced eggshell conductance in avian brood parasites. Philos. Trans. R. Soc. B Biol. Sci. *374*, 20180194.

Chapter 4.

McClelland, S.C., Cassey, P., Maurer, G., and Portugal, S.J. (2019). How much calcium to shell out? Life history strategy influences eggshell calcium content across avian families, J. R. Soc. Interface. *In press*

A third publication (based on **Chapter 3**) has recently been returned with minor revisions from review at Proceedings of the Royal Society B. However, this publication has not been included in this thesis.