

RESEARCH ARTICLE

Nesting behaviour influences species-specific gas exchange across avian eggshells

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ABSTRACT

Carefully controlled gas exchange across the eggshell is essential for the development of the avian embryo. Water vapour conductance (G_{H_2O}) across the shell, typically measured as mass loss during incubation, has been demonstrated to optimally ensure the healthy development of the embryo while avoiding desiccation. Accordingly, eggs exposed to sub-optimal gas exchange have reduced hatching success. We tested the association between eggshell G_{H_2O} and putative life-history correlates of adult birds, ecological nest parameters and physical characteristics of the egg itself to investigate how variation in G_{H_2O} has evolved to maintain optimal water loss across a diverse set of nest environments. We measured gas exchange through eggshell fragments in 151 British breeding bird species and fitted phylogenetically controlled, general linear models to test the relationship between G_{H_2O} and potential predictor parameters of each species. Of our 17 life-history traits, only two were retained in the final model: wet-incubating parent and nest type. Eggs of species where the parent habitually returned to the nest with wet plumage had significantly higher G_{H_2O} than those of parents that returned to the nest with dry plumage. Eggs of species nesting in ground burrows, cliffs and arboreal cups had significantly higher G_{H_2O} than those of species nesting on the ground in open nests or cups, in tree cavities and in shallow arboreal nests. Phylogenetic signal (measured as Pagel's λ) was intermediate in magnitude, suggesting that differences observed in the G_{H_2O} are dependent upon a combination of shared ancestry and species-specific life history and ecological traits. Although these data are correlational by nature, they are consistent with the hypothesis that parents constrained to return to the nest with wet plumage will increase the humidity of the nest environment, and the eggs of these species have evolved a higher G_{H_2O} to overcome this constraint and still achieve optimal water loss during incubation. We also suggest that eggs laid in cup nests and burrows may require a higher G_{H_2O} to overcome the increased humidity as a result from the confined nest microclimate lacking air movements through the nest. Taken together, these comparative data imply that species-specific levels of gas exchange across avian eggshells are variable and evolve in

response to ecological and physical variation resulting from parental and nesting behaviours.

KEY WORDS: Avian eggshells, Life history, Museum specimens, Nest environment, Permeability

INTRODUCTION

The striking diversity in shape, size and pigmentation of avian eggs (Hauber, 2014) provides an ideal model system for studying the causes and consequences of evolutionary diversity and adaptive function. The avian eggshell is a complex, multifunctional bioceramic (Fernandez et al., 1997). It actively shapes the developmental milieu of the embryo by protecting it from mechanical damage, facilitating gas exchange and providing calcium for bone growth (Ar et al., 1974; Maurer et al., 2011). Gas exchange across the shell relies on the diffusive properties of the eggshell and the environmental conditions in which the egg is placed, and is vital for the development of the embryo within the egg (Ar and Rahn, 1978; Ar and Rahn, 1980; Vleck et al., 1983; Rahn and Paganelli, 1990). Gas exchange contributes to the rate of water loss, estimated across the eggshell as water vapour conductance (G_{H_2O} ; $\text{mg day}^{-1} \text{ Torr}^{-1}$), which must be mediated in such a way that desiccation does not endanger the embryo, while sufficient water is lost for embryo growth and air cell formation (Ar and Rahn, 1980; Barrott, 1937; Romijn and Roos, 1938).

As birds breed in almost all terrestrial environments, including habitats with extreme levels of humidity, altitude and temperature (Lomholt, 1976; Sotherland et al., 1980; Davis et al., 1984; Davis and Ackerman, 1985; Arad et al., 1988; Carey et al., 1989; Carey et al., 1990; Walsberg and Schmidt, 1992; Carey, 1994), the structure of the eggshell is likely to play an important role in allowing bird species to successfully expand into and inhabit a wide variety of habitats. To fully understand the diversity of avian eggshell structure requires an analysis of the evolutionary basis of the structural adaptations for eggshells' gas exchange in different environments and nesting conditions, and across species with varying life histories (e.g. Portugal et al., 2014). Because all nutrients for embryonic development are deposited by the avian mother into the egg prior to laying, suitable levels of gas exchange and parental modulation of incubation temperatures constitute the only physical control of the requirements for embryonic development in birds (Ar et al., 1974; Hoyt et al., 1979; Paganelli, 1980; Visschedijk, 1980; Booth and Seymour, 1987). Here we examine how broad-scale evolutionary and ecological variation, species-specific breeding behaviour and phylogenetic relatedness can explain variation in gas transfer across the avian eggshell.

Quantifying patterns of interspecific variability in G_{H_2O} and the associated egg-mass loss across phylogenetically diverse taxa is essential to understand how flexibly birds have adapted to their diverse breeding environments. Typically, studies of eggshell G_{H_2O} have focused on closely related species and family groups of birds,

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Table 1. Putative predictions for a series of possible explanations for variation in water vapour conductance (G_{H_2O}) in the eggs of 151 British breeding birds

Hypothesis	Assumption
(i) Eggshell thickness	The water vapour travels for a shorter distance through the shell in thinner eggs.
(ii) Eggshell calcium content	Calcium-poor species should produce shells of lower density and thus facilitate rapid gas transfer.
(iii) Altitude	Enhanced diffusivity at low barometric pressure at higher altitudes increases water loss.
(iv) Nest structure ('open' ground versus 'closed' tree)	The air movement experienced by open nests facilitates eggshell gas transfer in comparison to cup nests, where eggs are more frequently on top of each other, and the cup shape may cause pockets of humidity.
(v) Nest placement (cavity versus open)	Cavity nesters have a higher humidity than the surrounding environment, and water vapour transfer is slowed down.
(vi) Clutch size	Evaporation from multiple eggs will create a nest atmosphere of greater humidity and reduced water vapour transfer.
(vii) Parental foraging style	The wet incubating parent returning to the nest will increase the nest's humidity, reducing water vapour transfer.

in an attempt to elucidate the ultimate and proximate causes of variation in the G_{H_2O} between related species (e.g. Vleck et al., 1983). Although the potential effects of nest environment and nest structure on G_{H_2O} have both been studied intensively, a focus on closely related taxa, even in comparative studies, means that potential confounds of shared phylogenetic affinities and life-history traits, which may play parallel or contrasting roles in determining the optimal G_{H_2O} , have not yet been identified (Cassey et al., 2010). Therefore, we measured surface-specific G_{H_2O} in the eggs of a broad taxonomic spectrum of 151 British breeding bird species spanning several orders, using a repeatable and standardised methodology. We tested ecological hypotheses on how modifications in the G_{H_2O} in the eggs of different lineages vary with respect to differences in the humidity and pressure of different nest environments. Based on extensive previous literature, we tested several physical and life-history variables that may explain variation in G_{H_2O} across species (detailed in Table 1). These can broadly be grouped into three categories: egg structure (predictions i and ii in Table 1), nest habitat and type (predictions iii–v) and life-history traits of the adult birds (predictions vi and vii).

RESULTS

Reliability of G_{H_2O} measurements

Mass loss between subsequent weighing sessions was highly repeatable for each eggshell fragment (Pearson's $r=0.99$, $n=1281$) and contributed to less than 5% of the total variability in G_{H_2O} between eggs. Nested ANOVA indicated that <50% of the variability in G_{H_2O} among individual eggs was explained through differences between the three eggshell regions: the blunt end (B), equator (E) and pointed end (P). However, the analysis of these three eggshell regions independently showed that individual egg (Egg ID) contributed 53.8, 59.6 and 67.0% of the total variability in G_{H_2O} for B, E and P, respectively.

To investigate further the contribution of individual variation in eggshell collection and preparation to the variation in G_{H_2O} , we analysed a subset of eggs that were donated to the Natural History Museum, Tring, as a single source collection compiled by a single collector. This subsequent analysis of the single largest collection of eggs from one donor (62 species and 42% of the collection in total) demonstrated that when analysed collectively, region (B, E and P), i.e. within-egg variability, was responsible for the largest percentage of variability (39.7%) in G_{H_2O} between eggs. In contrast, when the regions were separated, Egg ID was only responsible for 16.5, 9.6 and 2.4% of the variability between eggshells for B, E and P, respectively, with 77% of the variability in G_{H_2O} being explained by phylogenetic effects owing to species differences, within the same

avian families. From these results, we inferred that our methods are indeed sufficient to detect significant differences between species, and that an average species value' across all collections of G_{H_2O} is both obtainable and highly repeatable (Fig. 1).

Differences in G_{H_2O} across eggshells

There was a significant and consistent difference in the G_{H_2O} between the three eggshell regions ($F_{2,1281}=20.9$, $P<0.001$). For the majority of species (>80%), the B region, coinciding with the eventual location of the air cell, had a significantly lower G_{H_2O} compared with both E and P regions ($F_{2,1281}=25.76$, $P<0.001$). Mean values of G_{H_2O} for E and P were highly correlated ($r=0.65$, $n=144$, $P<0.001$), and not significantly different from each other (0.2559 ± 0.08 and 0.2532 ± 0.09 $\text{mg day}^{-1} \text{Torr}^{-1}$ for E and P, respectively, $B=0.2245\pm 0.07$ $\text{mg day}^{-1} \text{Torr}^{-1}$). Consequently, we considered the B and E regions only in subsequent analyses (all data are available in supplementary Tables S1 and S2).

Phylogenetic correlation

For all eggshell regions (i.e. whole eggshell or B and E regions separately), Pagel's λ values were intermediate between 0 and 1 and

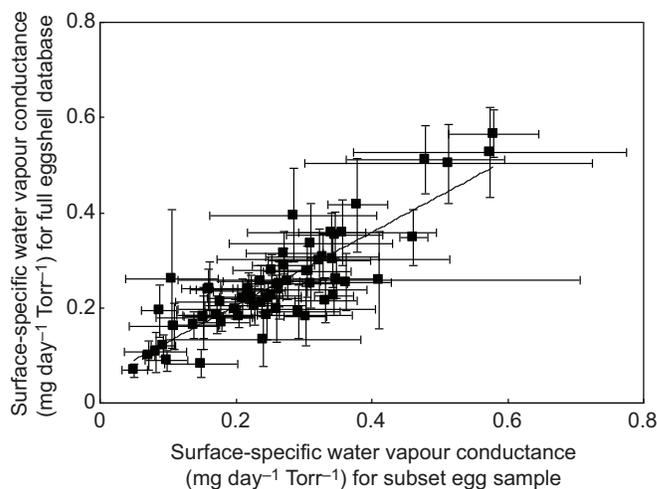


Fig. 1. Mean (\pm s.e.m.) surface-specific water vapour conductance (G_{H_2O}) for 62 British breeding bird species, sourced from a single museum donor, compared with the G_{H_2O} for the same species measured in eggs from multiple different donors. Analysis showed the values of G_{H_2O} to be highly repeatable for a species, and that egg donor origin was not a significant factor in the determination of average G_{H_2O} for a species. Values of G_{H_2O} for the three segments are combined (blunt end, equator and pointed end).

Table 2. Estimates of phylogenetic signal (Pagel's λ) for water vapour conductance (G_{H_2O}) for the blunt and equator regions of the eggshell, and the whole eggshell

Region	Maximum likelihood Pagel's λ	95% CI	Maximum likelihood	LR versus 0	LR versus 1
Blunt	0.65	0.60–0.70	136.07	28.98	72.92
Equator	0.65	0.63–0.67	134.81	31.18	47.43
Mean species	0.75	0.73–0.78	164.18	44.66	79.48

The 95% CI interval refers to the 95% sampling intervals across phylogenies. For all likelihood ratio (LR) statistics, the proportion of trees where λ significantly differed from either 0 or 1 was 100%.

significantly different from both (Table 2), suggesting that some phylogenetic signal is retained in G_{H_2O} . A Pagel's λ value of 0 indicates that values of the G_{H_2O} trait are independent of phylogeny, while a Pagel's λ value of 1 indicates that values of G_{H_2O} are evolving according to Brownian motion on the given phylogeny (e.g. Cassey et al., 2010). Intermediate values of Pagel's λ imply that traits have evolved according to a process in which the effect of phylogeny is weaker than in the Brownian model. These patterns were detected regardless of whether life-history traits were included in the phylogenetic model (Table 2).

Of our 17 life-history and habitat variables, only two were retained in the final model of G_{H_2O} variation across species. Using mean G_{H_2O} values for the whole eggshell of each species, the model that best explained the variation in G_{H_2O} ($r^2=0.17$, $F_{5,132}=5.38$, $P<0.001$) retained only wet incubating parent ($F_{1,132}=6.08$, $P<0.001$; Fig. 2) and nest type ($F_{5,132}=4.99$, $P<0.001$; Fig. 3) as significant predictor variables. The relative contribution of the non-significant variables included in the initial full model, which were removed, are presented in supplementary material Table S3.

Species with wet incubating parents had a G_{H_2O} that was, on average, $0.042 \text{ mg day}^{-1} \text{ Torr}^{-1}$ higher than those species where parents do not return to the nest wet. The rate of diversification in G_{H_2O} was approximately five times greater between species in which parents incubate with wet plumage than it was between species in

which parents incubate with dry plumage [as represented by the branch lengths in Fig. 2, relative rate estimate=4.72 (2.94–67.95% CI), likelihood ratio test=39.93, d.f.=1, $P<0.001$]. Eggs of species nesting in ground burrows, cliffs and arboreal cups had significantly higher G_{H_2O} than those nesting on the ground in open nests or cups, in tree cavities and in shallow arboreal nests (Fig. 3).

For G_{H_2O} of the B region only, nest type ($F_{5,132}=4.48$, $P<0.001$) and wet parent ($F_{1,132}=11.86$, $P<0.001$) were the only significant predictor variables retained in the model ($r^2=0.18$, $F_{5,132}=5.96$, $P<0.001$). For the E region only, nest type ($F_{5,132}=4.93$, $P<0.001$) and average eggshell thickness ($F_{1,132}=7.22$, $P<0.001$) were the only significant predictor variables retained ($r^2=0.17$, $F_{5,132}=5.38$, $P<0.001$), with an increase in thickness (estimate \pm s.e.m.= 0.16 ± 0.05) resulting in an increase in G_{H_2O} .

DISCUSSION

Differences observed in the rate of water vapour conductance (G_{H_2O}) across the avian eggshell covary with a combination of both shared ancestry (phylogenetic relatedness) and several life-history and ecological traits (Table 2). Species-specific behavioural and environmental parameters can play an important role in influencing G_{H_2O} (Deeming, 2002), and our study discovered that nest type and whether the incubating parent returns to the nest wet were the only statistically significant factors that predicted interspecific variability

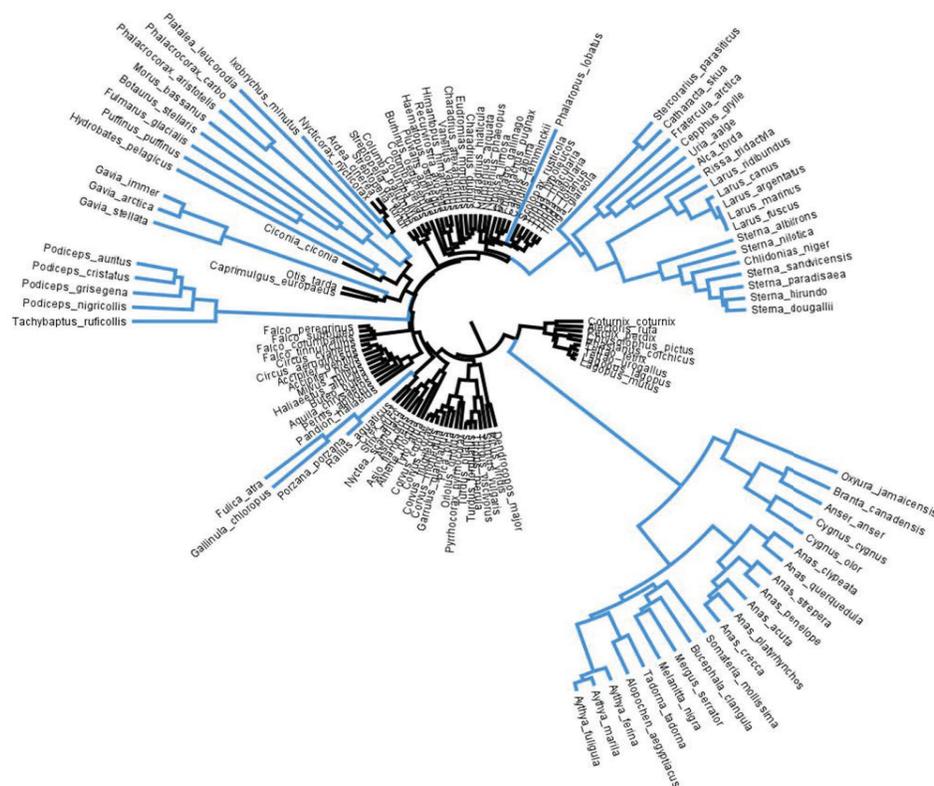


Fig. 2. Phylogenetic tree of the 151 species of British breeding birds. Those species which are classified 'wet parents' (habitually return to the nest wet) are coloured blue. The remaining species (coloured black) are 'dry parents'. The branch length are proportional to the rate of diversification in G_{H_2O} , which was ~5.5 times greater in the wet incubating parents group when compared with those species that return to the nest dry.

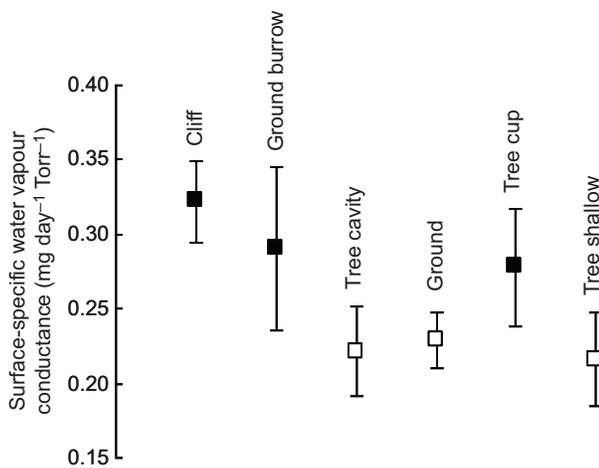


Fig. 3. Mean (\pm s.e.m.) water vapour conductance (G_{H_2O}) for six nest types and nest locations, measured in 151 species of British breeding birds. Nest types/locations indicated with filled squares had significantly higher G_{H_2O} values than those indicated with open squares.

in G_{H_2O} of bird eggs in a large sample of representative British species.

Whereas G_{H_2O} is vital for successful incubation, nest site choice for the adult bird will likely involve a trade-off between a suitable nest microclimate for optimum egg-water loss and minimising nest predation risks, the latter being the most important predictor of nesting success across birds (Ricklefs, 1969). The full effects on embryonic development, hatching success, post-hatching growth and overall fitness of sub-optimal egg-water loss are not understood. However, it is clear that eggs and clutches incubated under conditions that lead to sub-optimal water loss have reduced hatching success (e.g. Buhr, 1995; Yildirim and Yetisir, 2004). Yet, the long-term effects of water loss on fledgling success, when the eggs hatch, remain unknown, especially because water loss is likely to covary with fluctuations in incubation temperature, which in turn has crucial fitness impacts on post-hatching development (Pérez et al., 2008; DuRant et al., 2010).

Importantly, in our results clutch size and developmental mode were not significant main predictor variables of gas transfer across our relatively limited geographic sampling of British bird eggs (see Carey, 1994; Deeming, 2002). We originally hypothesised (see Table 1) that species with large clutch sizes would have higher G_{H_2O} than those species with relatively smaller clutches, in response to the increased humidity that a greater number of eggs present in the nest might produce. However, behaviours of the incubating adult bird, such as egg turning using the feet (Morgan et al., 2003; Skutch, 1976), beak (Haftorn, 1994; Handrich, 1989) and tremble-thrusting (Vleck, 1981), may effectively mix the air and reduce the chance of pockets of humidity developing over time, so that the eggs of species with large clutches do not require an adaptation to their G_{H_2O} .

Our study helps clarify to what extent the incubating parent influences the G_{H_2O} in the developing egg. We have shown that both the behaviour of the incubating adult (returning to the nest with wet or dry plumage) and its choice of nest site can affect the rate of gas exchange across the eggshell. Previously it was found that the adult bird can contribute to the control of the nest microclimate, and as much as 30–45% of moisture in the nest's atmosphere is provided by the incubating parent (e.g. Andersen and Steen, 1986). This percentage would be even greater if the eggs and nest are in a confined area such as a burrow, cavity or cupped nest. Our results

are consistent with previous studies that revealed significantly higher G_{H_2O} in species that use underground burrow nests and cup nests (Fig. 3) (Rahn et al., 1977; Ackerman and Platter-Reiger, 1979; Carey, 1980; Rahn and Hammel, 1982). However, the result reported here, namely that the G_{H_2O} of eggs found in ground-nesting species and cavities was lower than these aforementioned groups, is unexpected (see Deeming, 2002). In the considerably smaller phylogenetic sample of bird species (seven members of the order Pelecaniformes, and three members of the order Charadriiformes) used by Vleck et al. (Vleck et al., 1983), ground-nesting birds typically laid eggs with a high rate of G_{H_2O} , potentially to overcome the lack of wind and air movements, which can result in a humid nest microenvironment. Similarly, eggs of cavity-nesting birds studied previously show comparable traits of increased G_{H_2O} .

Measuring the G_{H_2O} across the eggshell under standard conditions reveals adaptations that facilitate optimal water loss during incubation in very different nest environments. To achieve optimal water loss, the G_{H_2O} is likely to be very similar between all species under typical nest conditions. Only under standard laboratory conditions will these differences in G_{H_2O} due to structural adaptations of the avian eggshell become apparent. Measuring the G_{H_2O} under standard conditions, however, may result in certain functional differences between the eggs of particular species being missed. For example, the eggs of eared grebes (*Podiceps nigricollis*) are covered in rotting vegetation when the incubating parents make recesses, and frequently can be partially submerged in water (Davis et al., 1984; Lomholt, 1984; Board, 1982; Sutherland et al., 1984). The extreme changes in humidity and temperature associated with this nest environment may mean that the G_{H_2O} of grebe eggs is more flexible and responsive to changes in the environment, and the eggs more resilient to desiccation and wetting, thus being able to cope with wider temperatures and humidity ranges. Therefore, it may be that under standard conditions, eggs of species that are better adapted and equipped to cope with changes in environmental factors (e.g. temperature and humidity) will not exhibit these differences in physical properties.

It would be worthwhile for future studies to test the G_{H_2O} in eggs of certain species under different temperatures and humidity ranges, or specifically replicating particular nesting microhabitats, to ascertain a thermal and external humidity tolerance zone of G_{H_2O} as they are related to species-specific environmental factors and behavioural patterns during incubation.

MATERIALS AND METHODS

Egg samples and preparation

Eggs of 151 British breeding bird species were obtained from the destructive collection of the Natural History Museum, Tring (UK) (Cassey et al., 2010; Cassey et al., 2012; Maurer et al., 2014; Portugal et al., 2010a; Portugal et al., 2010b; Russell et al., 2010). Previously, we demonstrated that G_{H_2O} of museum and fresh eggs of the same species does not differ significantly (Portugal et al., 2010a). Eggshell parameters [length (mm), breadth (mm), mass (g) and thickness (μ m)] were measured directly on the sampled eggs. Only species with an eggshell >30 mm in length and >0.075 mm thick were included, as smaller shells were unsuitable for reliable G_{H_2O} measurements (see Portugal et al., 2010a).

Three different regions of the eggshell were used for G_{H_2O} measurements: the blunt end (B), equator (E) and pointed end (P). These fragments of the eggshell (~225 mm²) were cut from the shell using a diamond-tipped dentist drill (Milnes Bros., Surrey, UK). Prior to G_{H_2O} being measured, eggshell thickness was recorded using a Mitutoyo Series 227–203 constant measurement force micrometer (as described in Maurer et al., 2010; Maurer et al., 2012).

Detailed information about eggshell preparation and measurements of the G_{H_2O} can be found elsewhere (Portugal et al., 2010a; see also Booth and Seymour, 1987; Maurer et al., 2011). Briefly, the eggshell fragments were

glued to the top of Eppendorf tubes (surface area of 24.4 mm²) that had been previously filled with 200 µl of distilled water. The Eppendorf units were placed into desiccators (Camlab, Over, Cambridgeshire, UK), which in turn were housed in a constant-temperature thermocabinet (Camlab) at 30±1°C. Temperature, humidity and pressure were monitored continuously via a logtag analyser and an average was logged every 1 min (Loggershop, Bournemouth, Dorset, UK). After 24 h, the Eppendorfs were weighed (g) to four decimal places (Sartorius, Göttingen, Germany) before being returned to the desiccators. The Eppendorf tubes and eggshell fragments were weighed at the same time of day on three successive days to give two values of 24 h G_{H_2O} . Any mass loss was assumed to be the result of water loss (*sensu* Booth and Seymour, 1987). Calculation of G_{H_2O} was conducted as previously described (Portugal et al., 2010a).

Life-history and ecological data of a representative avian phylogenetic sample from Britain

Species were classified following Sibley and Monroe (Sibley and Monroe, 1990). 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* (Cramp et al., 1977–1994). In addition, supplementary data were obtained from family- and species-specific monographs, and field guides to nests (sources available on request).

The following variables were recorded from the literature: adult body mass (g), clutch size (modal number of eggs), incubation length (days), shared incubation between two parents (no/yes), median breeding range (degrees latitude), nest type [cup/non-cup (open)], nest concealed (no/yes), ground nesting (no/yes), arboreal nesting (no/yes), cavity nesting (no/yes), cliff nesting (no/yes), diet (calcium rich/herbivore), development mode (precocial/altricial), migration (no/yes), whether the parental foraging style meant that adults returned habitually to the nest with wet plumage (no/yes wet incubating parent variable; see Results), and general breeding habitat (open/closed) [see Cassey et al. (Cassey et al., 2010) for full description]. All variables in our data set could be assigned for all species.

Body mass of adult birds was taken as a mean for both sexes, primarily from the *Handbook of Avian Body Masses* (Dunning, 2007). Breeding latitude was compiled from data tabulated by Orme et al. (Orme et al., 2005; Orme et al., 2006). Nest type was recorded as evidence of cup building versus open shallow nests, based on the description in the literature. The open category includes shallow nests (e.g. oystercatcher, *Haematopus ostralegus*), and complete absence of nest [e.g. Eurasian stone curlew (*Burhinus oedicnemus*), common guillemot (*Uria aalge*)]. Further detail was then added on whether the nest was placed concealed in vegetation or camouflaged. This information was based on the location of the nest, the nest structure and its components (nest material). A nest in open view (0) was not considered hidden [e.g. osprey (*Pandion haliaetus*), pied avocet (*Recurvirostra avosetta*), common coot (*Fulica atra*)]; by contrast, a nest that is concealed in a crevice, in a burrow or by foliage was considered hidden (Ar and Rahn, 1978). The migratory nature of a species was determined from the illustrative maps in the *Handbook of the Birds of the World* (Del Hoyo et al., 1992–2010), by establishing whether the entire population underwent a full move from one region to another, and as such, did not include locally dispersing species and partial migrants, which were also scored as non-migratory. General breeding habitat was assigned based on the descriptions of McNaught and Owens (McNaught and Owens, 2002), where habitats were broadly defined as closed (closed woodland, reed beds, rank grass) or open (arid regions, grasslands, heathland, wooded grasslands, open woodland, marsh).

The average degree of maculation of the eggshell was determined by three observers from specimens studied at the Natural History Museum, Tring, collection [see Cassey et al. (Cassey et al., 2010) for repeatability estimates]. For each species, the eggs were assessed for presence and coverage of maculation using a three-point scoring system and points were averaged between observers to obtain a maculation score. Maculation was recorded as '0' if the egg was immaculate, '1' for maculation present but with a clear, dominant background colour, and '2' for widespread maculation that covered the entirety of the egg (see also Brulez et al., 2014). The full species matrix of life-history traits and eggshell parameters can be found in supplementary material Tables S1 and S2.

Phylogenetic methods and analysis

We revised and updated a recent phylogenetic hypothesis of British birds (Thomas, 2008). The phylogeny was based on sequence data from 12 protein-coding mitochondrial genes and included 151 British breeding bird species. The published tree was extended by: (1) adding sequence data for 15 more species, (2) increasing the number of genes included where available and, (3) replacing the data on Thomas' (Thomas, 2008) three surrogate species with recently published data on the focal species (little bittern, *Ixobrychus minutus*; European bee-eater, *Merops apiaster*; and European golden plover, *Pluvialis apricaria*). Each gene was aligned by eye in SE-AL v. 2.0a11 (Rambaut, 2002). All sequence data were collected from GenBank (Benson et al., 2007) using Geneious v. 4.8.5 (Drummond et al., 2009), and sequence accessions and full alignments are available on request.

We used BEAST 1.5.4 for phylogenetic analyses using a codon-specific GTR+ Γ substitution model in which substitution rates, among-site rate variation and state frequencies at third codon positions were unlinked (GTR+CP₁₁₂+ Γ). We used a Yule prior on the branching process and an uncorrelated relaxed clock in which rate variation among branches was drawn from a log-normal distribution. We applied two topology constraints to the phylogeny by defining the monophyly of the widely accepted Neoaves and Galloanserae clades. Note that this is more liberal than the 11 constraints used by Thomas (Thomas, 2008) and allows us to better account for the uncertainty in topology in the deeper nodes of avian phylogeny. We conducted two runs, one each for 40 and 50 million generations, sampling trees every 10,000 generations. We assessed mixing within runs and convergence between runs using Tracer v. 1.5.0 (Drummond and Rambaut, 2007) based on visual inspection of traces and effective sample sizes of tree parameters (node ages of the two constrained nodes), posterior log-likelihoods and substitution model parameters. Both runs converged rapidly and we discarded 10% of generations from each run as burn-in. We combined the post-burnin samples of the two runs to yield a posterior distribution in which the majority of parameters had effective sample sizes of >500 (and all >100). For use in subsequent phylogenetic analyses (see below) we subsampled down to 1000 trees (drawn from the full posterior distribution of >8000 trees) and pruned each tree to the 49 species in the eggshell data set. We also extracted the maximum clade credibility tree from the full tree distribution for use as a single 'best' representative tree.

We estimated Pagel's λ (Pagel, 1997; Pagel, 1999; Shackleton et al., 2000), using the R-library *motmot* (available from <http://r-forge.r-project.org/>), as a measure of the strength of phylogenetic signal in the G_{H_2O} variables. Pagel's λ varies from 0 to 1, where 0 indicates no phylogenetic signal in the data and 1 is consistent with a Brownian motion model of trait evolution in which the phylogeny accurately reflects the covariances between species for a given trait (for details, see Shackleton et al., 2000). To assess the effects of phylogenetic error, we repeated the λ fitting procedure with a distribution of 1000 phylogenetic hypotheses (see above).

We tested hypotheses on the correlates of G_{H_2O} using the R-library CAIC (available from <http://r-forge.r-project.org/>) to fit phylogenetically controlled general linear models. Specifically, we used the function *pglmEstLambda* to fit Pagel's λ simultaneously with each regression model in order to appropriately correct for phylogenetic signal in the residuals. We first tested the correlation between G_{H_2O} variables without any other covariates and repeated this over 1000 phylogenies. We then fitted full regression models including all relevant explanatory variables with G_{H_2O} as response variables. From the full models we simplified the model by removal of each statistically non-significant explanatory variable in turn ($\alpha=0.05$). We also added each removed variable back into the final reduced model one by one to assess model robustness. The initial full model and model simplification were conducted on the maximum clade credibility tree only. To assess the robustness of parameter estimates and significance to phylogenetic uncertainty, we subsequently ran the simplified model across all 1000 phylogenetic hypotheses.

For a single variable [parental feeding mode (wet/dry)], we tested the *a posteriori* hypothesis that the rate of between species diversification in G_{H_2O} would be higher among species with wet incubating parents than species with dry incubating species because of constraints on G_{H_2O} imposed by dry incubation. To test this hypothesis, we used the relative phenotypic rates test proposed by Thomas et al. (Thomas et al., 2006) implemented in the R

library ‘motmot’. This test compares a model in which the rate of phenotypic diversification is constant lineages with a model in which rates differ between lineages with wet incubating parents and lineages with dry incubating parents. Ancestral states for incubation type were reconstructed using the ‘ace’ function in the R library ‘ape’ (Paradis et al., 2004). Note that results for the rates test were qualitatively unaffected by choice of ancestral state reconstruction methods.

Statistical analysis

Pearson correlation coefficients (Pearson’s r) were calculated for the mass loss between weighing sessions, across all fragments. Nested ANOVA (SAS v9.2 Proc NESTED) was conducted to partition the percentage of variability in G_{H_2O} that was directly attributable to egg section (within an egg), individual egg (within a species) and individual species (within a family). We analysed whether the differences in G_{H_2O} were associated with the species identity, eggshell thickness and shell section using generalised linear mixed models (SAS v9.2 Proc GLIMMIX; accounting for repeated measures from replicate fragments within an egg as a random effect). Preliminary tests confirmed that eggshell thickness and adult body mass were highly correlated with each other, with >78% of the variation explained. Therefore, in subsequent analyses, only eggshell thickness, and not adult body mass, was included. No other measures were correlated at levels >50%.

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Competing interests

The authors declare no competing financial interests.

Author contributions

S.J.P. and P.C. designed the study. S.J.P. and G.M. ran the experiments. S.J.P. and P.C. analysed the physiology data. G.T. undertook the phylogenetic analysis. M.E.H. and T.G. contributed samples and expertise. S.J.P. wrote the manuscript with input from all authors.

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Supplementary material

Supplementary material available online at <http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.103291/-DC1>

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