

Eggshell Permeability: A Standard Technique for Determining Interspecific Rates of Water Vapor Conductance

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ABSTRACT

Typically, eggshell water vapor conductance is measured on whole eggs, freshly collected at the commencement of a study. At times, however, it may not be possible to obtain whole fresh eggs but rather egg fragments or previously blown eggs. Here we evaluate and describe in detail a technique for modern laboratory analysis of eggshell conductance that uses fragments from fresh and museum eggs to determine eggshell water vapor conductance. We used fresh unincubated eggs of domesticated chickens (*Gallus gallus domesticus*), ducks (*Anas platyrhynchos domesticus*), and guinea fowl (*Numida meleagris*) to investigate the reliability, validity, and repeatability of the technique. To assess the suitability of museum samples, museum and freshly collected black-headed gull eggs (*Larus ridibundus*) were used. Fragments were cut out of the eggshell from the blunt end (B), equator (E), and pointy end (P). Eggshell fragments were glued to the top of a 0.25-mL micro test tube (Eppendorf) filled with 200 μ L of distilled water and placed in a desiccator at 25°C. Eppendorfs were weighed three times at 24-h intervals, and mass loss was assumed to be a result of water evaporation. We report the following results: (1) mass loss between weighing sessions was highly repeatable and consistent in all species; (2) the majority of intraspecific variability in eggshell water vapor conductance between different eggs of the same species was explained through the differences in water vapor conductance between the three eggshell parts of the same egg (B, E, and P); (3) the technique was sensitive enough to detect significant differences between the three domestic species; (4) there was no overall significant difference between water vapor conductance of museum and fresh black-headed gull eggs; (5) there

was no significant difference in water vapor conductance for egg fragments taken from the same egg both between different trials and within the same trial. We conclude, therefore, that this technique is an effective way of measuring interspecific water vapor conductance from eggshell fragments and that museum eggs are a suitable resource for such work.

Introduction

The gas and water transfer across the shell—which the avian embryo relies on for respiration, growth, and development—is dependent on and limited by the diffusive properties of gases across the eggshell and shell membrane (Ar and Rahn 1978; Vleck et al. 1983; Rahn and Paganelli 1990). The success of embryonic development is related to these functional properties of the eggshell, and the gas and water exchange between the embryo and its external environment occurs by diffusion through pores in the shell (Ar et al. 1974; Paganelli 1980; Visschedijk 1980; Booth and Seymour 1987). Total water loss through the shell during incubation must be within certain parameters for successful development, and in birds this is typically around 15% in naturally incubated eggs (Drent 1975; Ar and Rahn 1980). Too much water loss from the egg will result in dehydration and increased metabolism, resulting in rapid use of yolk resources (Barrott 1937); slower than average water loss will result in impaired growth (Romijn and Roos 1938; Ar and Rahn 1980). Therefore, measuring the rate of water loss—or rather its correlate, the water vapor conductance of the shell—is imperative for researchers wanting to understand the determinants behind water vapor conductance, such as pore density and eggshell thickness, along with ecological aspects and life-history traits of the incubating adult bird. In turn, a detailed understanding of eggshell water vapor conductance is required to model responses of eggs and incubation behavior to changes in environment, physiology, and climate and to monitor changes in eggshell physiology as a consequence of increased toxins in the human-perturbed environment (e.g., Finnlund et al. 1985; Nybø et al. 1995; Massaro and Davis 2005).

Typically, eggshell water vapor conductance is determined through the use of fresh whole eggs obtained at the commencement of a study (e.g., Ar et al. 1974; Morgan et al. 1978; Roudybush et al. 1980; Vleck et al. 1983; Grant et al. 1984; Finnlund et al. 1985; Nybø et al. 1995; Massaro and Davis 2005). These eggs are then placed in desiccators under constant conditions (temperature and humidity), and any loss in mass

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is assumed to be entirely due to the diffusion of water through the shell (Booth 1992). However, at times, it is not possible to obtain fresh whole eggs of the desired type, species, or developmental stage, and this is particularly pertinent for wild-living and endangered species. Therefore, it may be feasible to obtain only fragments of the eggshell or to use museum specimens that are not accessioned as specimens in the main collection. Typically, under these circumstances, water vapor conductance is calculated on the basis of the number of pores identified in the shell (e.g., Zimmermann and Hipfner 2007; Donaire and López-Martínez 2009). However, this technique is not always satisfactory, especially in the absence of a whole egg, because it takes no account of the interaction between pore number and pore length or diameter.

Booth and Seymour (1987) briefly detailed a novel way of measuring eggshell water vapor conductance on shell fragments, during a study investigating megapode (Megapodiidae) incubation behavior and energetics. The approach involved gluing eggshell fragments to the top of glass vials filled with water before placing them in a desiccator and following the standard protocol detailed below. Therefore, it seems possible to be able to determine water vapor conductance from an eggshell fragment. However, the reliability, repeatability, and validity of this technique for measuring eggshell water vapor conductance has not been formally investigated or described sufficiently to be used in a modern laboratory setup (Booth and Seymour 1987; Balkan et al. 2006). In this article, we aim to present, in detail, the methodological and analytical aspects associated with this technique. In particular, we aim to clarify how repeatable the water vapor conductance is (1) between subsequent weighing sessions (2) for different fragments from the same egg, (3) between different trials for the same eggs and sections of the egg, and (4) within trials for the same eggs and sections of the egg. We also aim to test whether museum eggshell specimens are suitable for the study of eggshell water vapor conductance, particularly with this technique, by comparing the water vapor conductance of fresh and museum eggs of undetermined age of the same bird species.

Material and Methods

Egg Preparation

Unincubated eggs of the following species were obtained fresh from local retail outlets and specialized breeders: chickens (*Gallus gallus domesticus*, $n = 20$), guinea fowl (*Numida meleagris*, $n = 19$), and ducks (*Anas platyrhynchos domesticus*, $n = 18$). For the comparison of museum ($n = 9$) and fresh ($n = 15$) egg water vapor conductance, eggs of black-headed gulls (*Larus ridibundus*) were obtained, under license from Natural England (20092237), from a colony deserted as a result of nesting site flooding (June 2009). Museum eggs of black-headed gulls were obtained via an ongoing collaboration with the Natural History Museum, Tring.

Eggs were opened, emptied, and washed thoroughly using distilled water before being allowed to dry at room temperature. The eggshell fragments (approximately 225 mm²) were then

cut using a diamond-tipped dentist drill (Milnes Bros., Surrey). Booth and Seymour (1987) previously demonstrated that in malleefowl (*Leipoa ocellata*), certain regions of the eggshell were more porous, with the pointy end (P) being the most permeable region compared with the blunt end (B) and the equator (E). Therefore, to compare different regions and to investigate repeatability between sections, fragments from the P, B, and E were used for each egg.

Eggshell Thickness

Eggshell thickness measurements were taken using a modified Mitutoyo (Kawaski) micrometer (series 227-203, Absolute Digimatic) at its 1.5-N constant pressure setting. Both anvils of the micrometer were capped with an aluminium pin with a diameter of 1.35 mm, whose rounded tips (radius 0.35 mm) met when the micrometer was set to 0. The measurements were taken by resting the inside of the shell fragment on the stationary pin and tightening the opposite anvil until the hold signal appeared and the thickness reading was logged. Thickness was measured for one of the egg halves to an accuracy of 1 μm for B, E, and P three times each, resulting in a total of nine measurements per egg.

Water Vapor Conductance

The protocol for measuring eggshell water vapor conductance is based loosely on that of Booth and Seymour (1987), in that we created a conductance sampling unit using a tube and an eggshell fragment (Fig. 1). The lids of 0.25-mL micro test tubes, hereon referred to as Eppendorf tubes (surface area of 24.4 mm²), were removed before the tubes were filled with 200 μL of distilled water. Using water instead of fresh egg contents has been shown to have no effect on water vapor conductance (Taigen et al. 1978). Loctite glue (Loctite, Düsseldorf) 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 (see Fig. 1). The eggshell fragment was then gently pushed down to ensure contact with the glue and left for 4 h to dry (on the basis of manufacturers' recommendations). The Eppendorf tubes were then placed into 0.25-mL polymerase chain reaction trays (Fisher Scientific, Loughborough) for ease of handling and storage in the desiccator. Once the glue had dried, the eggshell fragments were checked to ensure the fragment was adhered securely, before superglue (RS Components, Corby) 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 Eppendorfs were placed into desiccators (Camlab, Over) and left for 24 h before the commencement of weighing. This 24-h period was discounted, since water vapor conductance tends to be much higher during the first 24 h as a result of the eggshell itself drying out (Booth 1992).

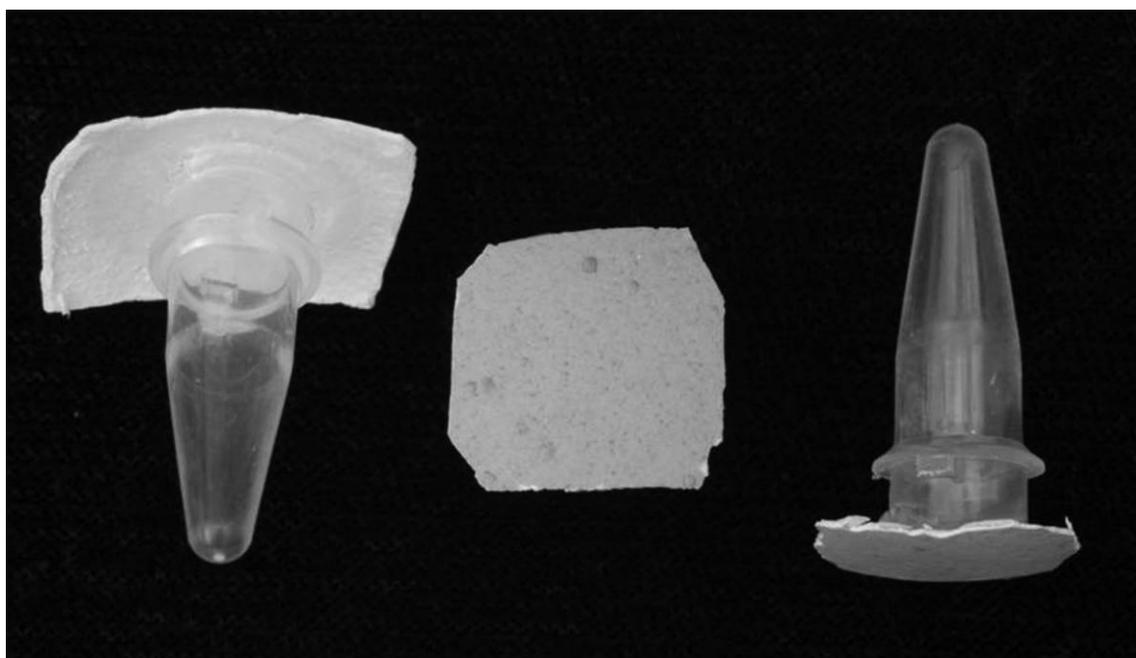


Figure 1. Photograph of chicken eggshell fragments glued onto an Eppendorf tube (see “Material and Methods”). A color version of this figure is available in the online edition of *Physiological and Biochemical Zoology*.

Two types of desiccators of different sizes were used: 305 mm × 305 mm × 305 mm (volume 28.4 L) and 178 mm × 305 mm × 305 mm (volume 16.5 L). The amount of self-indicating silica desiccant (Sigma, Gillingham) was scaled appropriately (500 g in the 16.5-L desiccator) to allow a comparison of water vapor conductance between the two desiccators (Booth 1992). The desiccators in turn were placed into a constant-temperature thermocabinet (260 L; Camlab) at $25^{\circ} \pm 1^{\circ}\text{C}$. Temperature, humidity, and pressure were monitored continuously via a logtag analyzer and an average logged every 1 min (Loggershop, Bournemouth). After 24 h, the Eppendorfs were weighed (g) to four decimal places (Sartorius, Göttingen) before being placed back into the desiccator. The Eppendorf and eggshell fragments were weighed at the same time of day on three successive days to give two values of 24-h water vapor conductance. After each weighing, the color of silica in the desiccator was checked and the silica sacs were turned upside down, following the guidelines set out by Booth (1992) for measuring whole fresh egg water vapor conductance.

To assess the constancy of eggshell water vapor conductance between different trials, replicate sections from the B, E, and P samples (thus six pieces per shell in total) were taken from the chicken shells and used in the same experimental setup but 1 wk later. Although it was not possible to undertake a repeated-measures design because of the destructive nature of the protocol, the two B, E, and P sections were taken from adjacent sections of the shell. To gauge the constancy between the B, E, and P sections within the trial, two adjacent sections of each were taken from the fresh and museum black-headed gull eggs and tested at the same time in the same desiccator.

Calculating Water Vapor Conductance

The water vapor conductance of a shell can be calculated following the Fick’s diffusion equation (Ar et al. 1974):

$$G_{\text{H}_2\text{O}} = \frac{\dot{M}_{\text{H}_2\text{O}}}{\Delta P_{\text{H}_2\text{O}}}, \quad (1)$$

where $G_{\text{H}_2\text{O}}$ is the water vapor conductance ($\text{mg d}^{-1} \text{ torr}^{-1}$), $\dot{M}_{\text{H}_2\text{O}}$ is the rate of mass loss (mg d^{-1}), and $\Delta P_{\text{H}_2\text{O}}$ is the water vapor pressure difference across the shell (torr).

This equation is adapted from equation (2) (Ar. et al. 1974) through a change of units from mass to volume:

$$\dot{V}_{\text{H}_2\text{O}} = K_{\text{H}_2\text{O}} \times A \times \Delta P_{\text{H}_2\text{O}}, \quad (2)$$

where $\dot{V}_{\text{H}_2\text{O}}$ is the diffusive rate of water loss (cm^3 standard temperature and pressure [STP] s^{-1}), $K_{\text{H}_2\text{O}}$ is the water vapor conductance constant of the shell ($\text{cm}^3 \text{ STP cm}^{-2} \text{ torr}^{-1} \text{ s}^{-1}$), and A is the surface area of the shell (cm^2).

By using the adapted equation (1), it is possible to express the volume of water vapor ($\dot{V}_{\text{H}_2\text{O}}$) as a mass unit of water ($\dot{M}_{\text{H}_2\text{O}}$) and to express the conductance of the shell, G , independently of the surface area. A variety of operational definitions have been termed for the water loss across the eggshell, or “porosity” (reviewed in Ar et al. 1974). In this study, we explicitly focus on water vapor conductance under standard conditions (*sensu* Ar et al. 1974) because this is suited best to interspecific comparisons. Only values obtained under such conditions are comparable at different barometric pressures.

Mass loss from the Eppendorf and eggshell fragments was expressed as water loss per 24 h and corrected to standard baro-

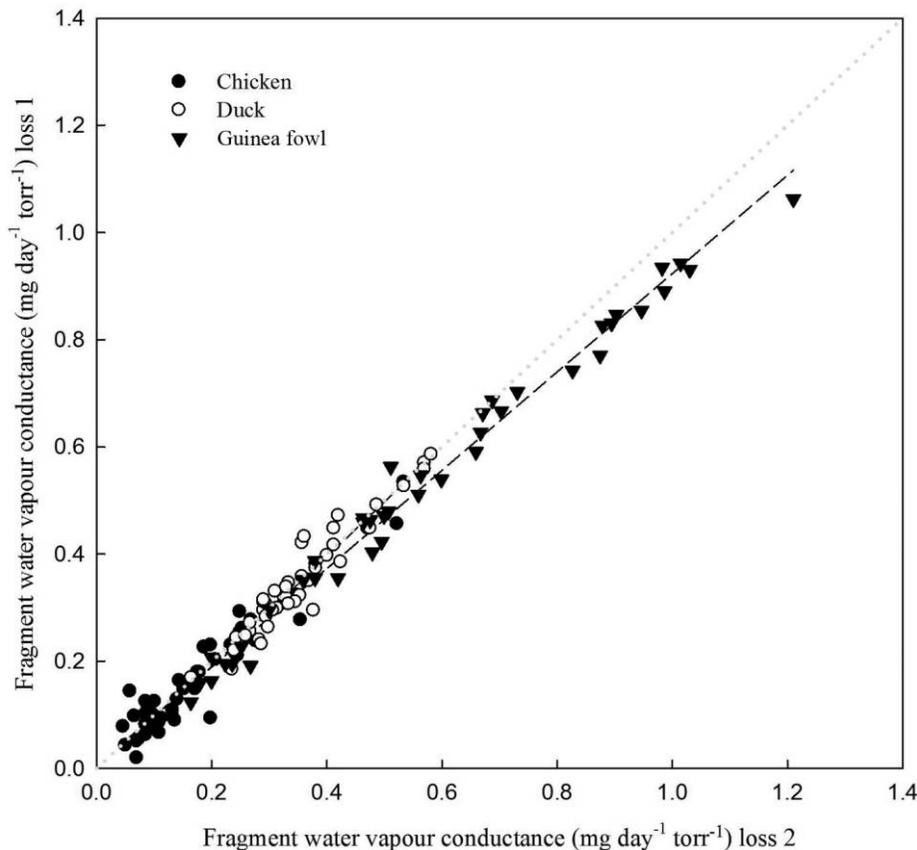


Figure 2. Bivariate scatterplot of eggshell fragment water vapor conductance, expressed as mass loss ($\text{mg d}^{-1} \text{ torr}^{-1}$), over two consecutive 24-h periods for chickens (*solid circles*), guinea fowl (*triangles*), and ducks (*open circles*). Lines of best fit are calculated from ordinary least squares regression. The correlation coefficients for all three species were high (0.95, 0.97, and 0.99 for chickens, guinea fowl, and ducks, respectively). The dotted gray line represents the line of equality.

metric pressure of 760 torr (Ar et al. 1974), since the rate of diffusion is inversely proportional to the total pressure. The corrected value was then divided by the water vapor pressure difference across the shell. Since with fresh desiccant the desiccator atmosphere is close to zero humidity, the water vapor pressure difference was assumed to be equal to the water vapor pressure of saturation at the egg temperature (23.77 torr at 25°C). 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 ($n = 11$).

Statistical Analysis

In each species, Pearson correlation coefficients were calculated for the mass loss between weighing sessions, across all fragments. Nested ANOVA was used to partition the percentage of variability in water vapor conductance that was directly attributable to egg section and individual egg. We analyzed whether the differences in water vapor conductance were associated with the species identity, eggshell thickness, and shell section using generalized linear mixed models (accounting, where necessary, for repeated measures from the same egg, and fragments within

an egg, as random effects). Paired *t*-tests were calculated for differences between replicate fragments of the same shell section between trials (a trial being an experimental run of Eppendorfs and eggshell fragments in a desiccator). All analyses were conducted in SAS (ver. 9.2; SAS Institute, Cary, NC).

Results

Within-Trial Comparisons

Mass loss between subsequent weighing sessions was highly repeatable for all species, as demonstrated by their respective correlations (Fig. 2; chickens: correlation coefficient = 0.95, $n = 110$; guinea fowl: 0.97, $n = 41$; ducks: 0.99, $n = 44$). In the guinea fowl eggs, >98% of the variability in eggshell water vapor conductance across different eggs (Table 1) was explained through differences among the three eggshell parts (B, E, and P). In the chicken and duck eggs, the percentage of the total variability attributed to differences among parts within individual eggs was between 78% and 86%, respectively (Table 1). For all three species, the percentage of the total variability in water vapor conductance between the two subsequent weighing sessions was very low (<5%).

Table 1: Nested ANOVA for the variability in water vapor conductance among individuals and among egg parts (blunt end, equator, and pointy end) in the eggs of chickens, guinea fowl, and ducks

Species and Level	df	Variance Component
Chicken:		
Individual egg	19	.00192 (17.17%)
Egg part	34	.00872 (78.07%)
Guinea fowl:		
Individual egg	18	-.0079 (0%)
Egg part	22	.0793 (98.02%)
Duck:		
Individual egg	17	.0009 (9.90%)
Egg part	27	.0083 (85.69%)

Note. The negative estimate for the variance component among individual eggs in the guinea fowl indicates that the variability at this level is less than predicted from the variability observed at the smaller levels (among egg parts and among consecutive 24-h periods). Following convention, the estimate was set to 0, the factor was removed from the model, and the estimates were recalculated for the remaining factors (Fletcher and Underwood 2002).

Species Differences

The technique detected consistent (statistically significant) differences between the three domestic species (Fig. 3; $F_{2,131} = 66.94$, $P < 0.001$), with guinea fowl having the highest rate of water vapor conductance (mean \pm SE) and chickens the least (1.72 ± 0.02 and 0.53 ± 0.01 mg d⁻¹ torr⁻¹, respectively, for the three sections combined for illustrative purposes) and ducks being intermediate (1.05 ± 0.01 mg d⁻¹ torr⁻¹). Within the

three species, there were significant differences in the water vapor conductance among the three individual shell sections (Fig. 3). The interaction between species and shell sections (B, E, P) was highly significant ($F_{4,131} = 3.64$, $P < 0.001$). The intraspecific differences between B, E, and P in water vapor conductance were not consistent among species, however. In ducks, the P segment was significantly less permeable than the B and E parts ($F_{2,41} = 5.45$, $P = 0.008$; Fig. 3). In guinea fowl ($F_{2,37} = 1.72$, $P = 0.192$) and chickens ($F_{2,49} = 0.10$, $P = 0.901$), the three eggshell segments showed no significant difference in water vapor conductance. Shell thickness had no significant effect on water vapor conductance for chickens (slope \pm SE = 0.058 ± 0.153 , $F_{1,49} = 0.14$, $P = 0.708$), guinea fowl (slope \pm SE = -0.234 ± 0.808 , $F_{1,49} = 0.08$, $P = 0.774$), or ducks (slope \pm SE = 0.583 ± 0.526 , $F_{1,49} = 1.23$, $P = 0.275$).

Between-Trial Comparisons

Between different trials, analysis of chicken egg fragments showed that the key source of variability in individual egg water vapor conductance was again between the B, E, and P segments rather than between trials of the same eggs and egg segments (Fig. 4), demonstrating that trial is not a significant contributor to variability in eggshell water vapor conductance. The water vapor conductance for each egg part of an individual egg was not significantly different for the three sections B (paired t -test, $t = 1.324$, $n = 15$, $P = 0.207$), E (paired t -test, $t = 1.706$, $n = 17$, $P = 0.107$), or P (paired t -test, $t = -0.787$, $n = 18$, $P = 0.436$); as a result, the overall water vapor conductance (the three sections combined) for each individual egg was not

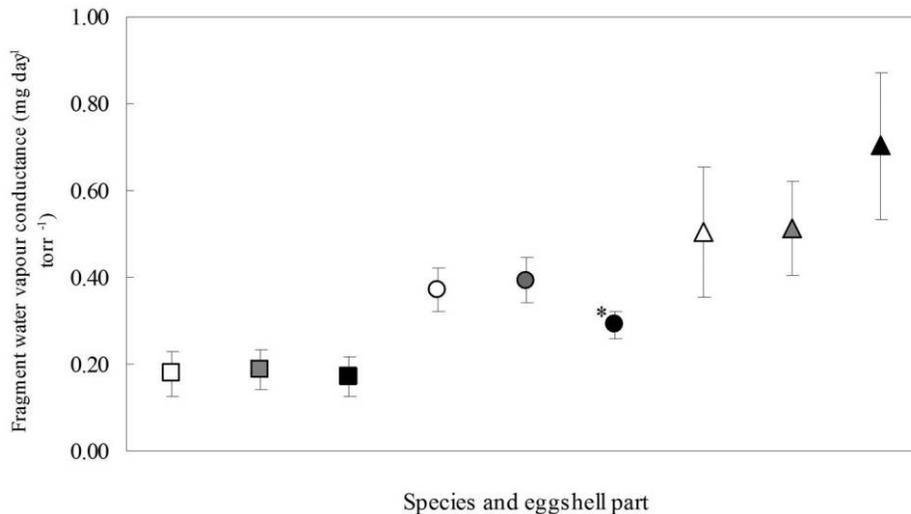


Figure 3. Rate of eggshell water vapor conductance (mg d⁻¹ torr⁻¹) for chickens (squares), ducks (circles), and guinea fowl (triangles). Water vapor conductance is presented for three different eggshell sections, blunt end (B; white symbols), equator (E; gray symbols), and pointy end (P; black symbols). The Eppendorf technique was sensitive enough to detect significant differences ($\alpha = 0.05$) between all three domestic species and a significant difference between P from the other eggshell parts in the duck (asterisk). Error bars are standard errors.

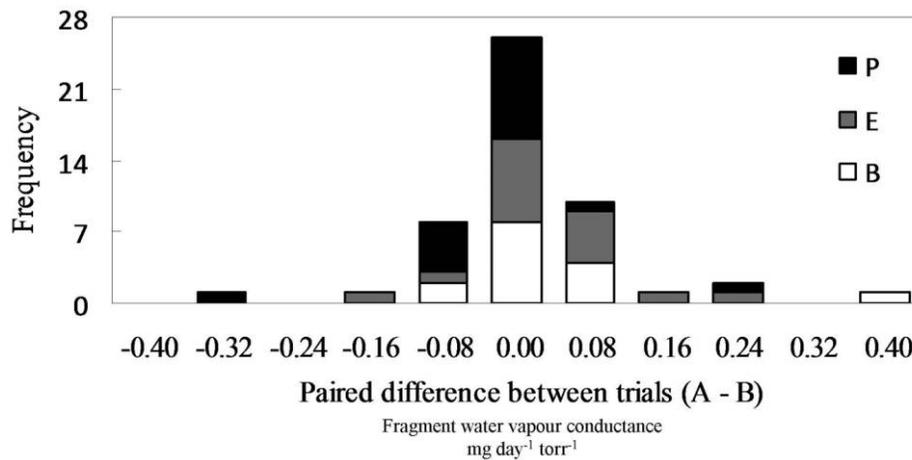


Figure 4. Repeatability of water vapor conductance in chicken eggs between two separate trials for three eggshell segments, blunt end (B; white), equator (E; gray), and pointy end (P; black). Each of the two segments was adjacent to each other on the whole eggshell. Paired *t*-tests showed no significant differences in water vapor conductance for each of the three segments (B, E, and P) between the two trials.

significantly different between the two separate trials (paired *t*-test, $t = 1.202$, $n = 50$, $P = 0.235$).

Comparison between Museum and Fresh Eggshell Specimens and Within-Trial Comparisons

There was no overall significant difference in water vapor conductance between the museum and freshly collected black-headed gull eggs (Fig. 5; $F_{1,97} = 0.79$, $P = 0.375$; 0.77 ± 0.06 and 0.72 ± 0.04 mg d⁻¹ torr⁻¹ for fresh and museum gull eggs, respectively, for the three sections combined for illustrative purposes). Similar to the duck eggs, there was a significant effect of eggshell part on water vapor conductance in the gulls eggs ($F_{2,97} = 5.61$, $P = 0.005$), with B having a significantly lower water vapor conductance (0.18 mg d⁻¹ torr⁻¹) in comparison to E and P (0.26 and 0.28 mg d⁻¹ torr⁻¹, respectively; $P < 0.01$ for both; Fig. 5). Subsequently, controlling for the random effects of different trials nested within eggshell part did not change the outcome of this ($F_{2,74} = 6.27$, $P = 0.003$). Within-trial analysis showed no significant difference in water vapor conductance of the B, E, and P replicates for each egg within the same trial ($F_{1,97} = 1.27$, $P = 0.262$).

Discussion

Typically, eggshell water vapor conductance is measured on whole fresh eggs. Here, we examined in greater detail, allowing independent replication, a technique first described in brief by Booth and Seymour (1987). Our approach enables egg fragments and museum specimens to be used for the determination of the water vapor conductance of eggshells of species that cannot be sampled as whole eggs for ethical or practical reasons. Importantly, and in deviation from Booth and Seymour (1987), our approach was to leave the inner shell membrane in place, since for whole-egg conductance measurements the membrane

is usually not removed. The technique demonstrated high levels of repeatability for 24-h water vapor conductance and high levels of repeatability both within and between trials (i.e., for the same egg fragment measured within the same trial and for the same egg area between different trials). However, we note that for two of the individual sections, the differences were greater than the mean of their two trials. This occurred in 4% of the samples but did not affect the overall outcome; however, removal of the two cases greatly increased the overall correlation between trials (two cases in, $r = 0.33$, $n = 50$, $P = 0.002$; two cases out, $r = 0.59$, $n = 38$, $P > 0.001$). This confirms the requirement to carefully check the distribution of the data before further analysis. The technique found significant differences between the chickens, guinea fowl, and ducks, suggesting that it is sensitive enough to detect subtle changes in water vapor conductance, even though the surface area of the shell and water volume of the Eppendorf are relatively small. The high level of variability between the three segments concurs with what has been previously found (e.g., Booth and Seymour 1987) and confirms how essential it is to measure the water vapor conductance of different shell segments if a whole or fresh egg is not available. The lack of significant difference within shell fragments from the same egg both between and within trials allows inter- and intraspecific comparisons to be made between trials as long as the standard conditions are maintained (constant temperature, zero humidity, and the same quantity of desiccant). The use of shell fragments also allows further analysis and investigations to be conducted on the shell fragment for which water vapor conductance was determined. Such analyses of the shell fragment could potentially include pigment extraction (Gorchein et al. 2009), ashing to determine calcium content (Reynolds 2001), analysis of protein composition (Mikšík et al. 2007), and microscopy of the shell structure (Nys et al. 2004). It is also possible to extrapolate the values of water

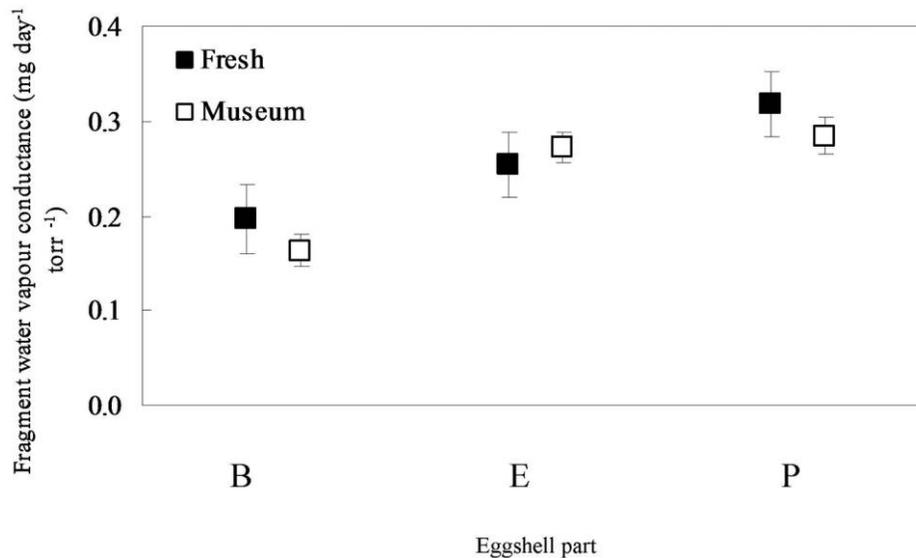


Figure 5. Comparison of water vapor conductance ($\text{mg d}^{-1} \text{ torr}^{-1}$) between freshly collected (*solid squares*) and museum specimen (*open squares*) black-headed gull eggs. Water vapor conductance is presented for three eggshell segments, the blunt end (B), equator (E), and point end (P). A nested ANOVA showed no overall significant difference in water vapor conductance between the museum and fresh eggs. Error bars are standard errors.

vapor conductance from the eggshell fragments to obtain a whole-egg value of conductance rate. This can be achieved through calculation of the total egg surface area (see Paganelli et al. 1974) and then dividing the value by the surface area of the Eppendorf tube (24.4 mm^2 in this instance). The resultant figure gives a measure of how many of the “Eppendorf areas” would fit into the total surface area of the whole eggshell, and a mean value of water vapor conductance rate for one fragment (obtained from the B, E, and P regions) can be multiplied by the total number of Eppendorf areas found in the shell to give an approximate value for whole-egg water vapor conductance (in $\text{mg d}^{-1} \text{ torr}^{-1}$). For example, in this study, the mean surface area of the chicken eggs was 51.2 cm^2 . This equates to 213.3 Eppendorf areas (0.24 cm^2 per Eppendorf tube) within the surface area of the chicken eggshell. The mean rate of water vapor conductance for the three segments (B, E, P) is $0.18 \text{ mg d}^{-1} \text{ torr}^{-1}$ (see Fig. 3), which equates to a whole-egg value of $38.3 \text{ mg d}^{-1} \text{ torr}^{-1}$. The chicken eggs in this study showed no significant differences in the rate of water vapor conductance between the three regions (B, E, and P), indicating that extrapolating these values to a whole egg through the use of a mean water vapor conductance for the three regions combined is relatively straightforward. If, however, there is a significant difference between the three regions in the rate of water vapor conductance, it may be possible to take samples from intermediate regions to see how conductance varies and use this data to make extrapolations to the whole egg.

Using equation (1) relaxes the requirement for surface area to be known for the calculation of eggshell water vapor conductance to be calculated. However, the use of a standard Eppendorf tube will mean that each eggshell fragment has the

same surface area for evaporation, making interspecific comparisons simpler, since no corrections to egg size are required for direct comparisons of water vapor conductance. The procedure of using an Eppendorf tube may have limitations as to which species and their eggs can be used for this technique, owing to the size of the aperture of the tube (24.4 mm^2) and the sectioning of the shell. On the basis of the diameter of the Eppendorf we used, we note that an egg $<30 \text{ mm}$ long and $<0.075 \text{ mm}$ thick (notably many species from the order Passeriformes) will be too small for use or too fragile to cut. In this study, Eppendorf tubes were used, but it would be possible to use any plastic tubing or glass vial, smaller examples of which may be suitable for smaller eggs. However, sufficient mass losses may be difficult to record from such a small water volume and surface area unless the period between weighing was increased beyond 24 h to, for example, 72 h. With small eggs, it also becomes difficult to apply the required pressure to form a sufficient glue seal between the eggshell surface and tube or vial without breaking the shell, since the eggs are thinner and more prone to damage.

The lack of a significant difference between the museum and fresh black-headed gull eggs provides evidence that it can be appropriate to use museum eggs for studies of this type, making it a useful technique when no fresh eggs are available. In this study, the sampling of museum eggs was made possible through a collaboration between the Centre for Ornithology, University of Birmingham, and the Natural History Museum (Tring), that provided a limited number of data-poor eggs available for destructive analysis. Two potential and linked problems of using museum eggs for this type of study are the lack of information regarding how it was prepared for inclusion in the museum

collection and the developmental stage of the embryo at collection. Both these problems can be minimized by careful inspection of the sample, however. A review of techniques used to remove egg contents and treat museum eggshells found that cleaning eggs with pepsin, trypsin, and wine vinegar were the most damaging to the structure of the eggshell (Scharleman 2002). We are unable to know exactly what treatment the museum eggs in the study received, particularly before admittance to the museum collection. Frequently, however, the size of the blow hole can be a useful tool to estimate the stage during incubation at which the egg was taken and blown. Those with a small blow hole will have usually been collected at the early stage of incubation when the contents of the egg are more liquid, and such aggressive treatments like acids and vinegar are not required to dissolve an embryo. Museum eggs of this type should be preferentially selected for water vapor conductance analysis where possible within the confines of the experimental design (i.e., taking into account incubation stage).

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