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Title:
Common guillemot (*Uria aalge*) eggs are not self-cleaning

Running title:
Guillemot eggs are not self-cleaning

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Summary statement:

Despite reports in the media, there is no published evidence that common guillemot eggs are self-cleaning. Here, we test this idea and show how eggs really cope with debris.

Abstract

Birds are arguably the most evolutionarily successful extant vertebrate taxon, in part because of their ability to reproduce in virtually all terrestrial habitats. Common guillemots, *Uria aalge,* incubate their single egg in an unusual and harsh environment; on exposed cliff ledges, without a nest, and in close proximity to conspecifics. As a consequence, the surface of guillemot eggshells is frequently contaminated with faeces, dirt, water and other detritus, which may impede gas exchange or facilitate microbial infection of the developing embryo. Despite this, guillemot chicks survive incubation and hatch from eggs heavily covered with debris. To establish how guillemot eggs cope with external debris, we tested three hypotheses: (1) contamination by debris does not reduce gas exchange efficacy of the eggshell to a degree that may impede normal embryo development; (2) the guillemot eggshell surface is self-cleaning; and, (3) shell accessory material (SAM) prevents debris from blocking pores, allowing relatively unrestricted gas diffusion across the eggshell. We show that (1) natural debris reduces the conductance of gases across the guillemot eggshell by blocking gas exchange pores. Despite this problem, we find (2) no evidence that guillemot eggshells are self-cleaning, but instead show that (3) the presence of SAM on the eggshell surface largely prevents pore blockages from occurring. Our results demonstrate that SAM is a crucial feature of the eggshell surface in a species whose eggs are frequently in contact with debris, acting to minimise pore blockages and thus ensure a sufficient rate of gas diffusion for embryo development.

Introduction

Birds breed in virtually all terrestrial habitats, from deserts to polar regions, and even in wet environments (Deeming, 2002). This flexibility in breeding ecology (specifically, in habitat use) can be attributed to the fact that birds lay hard-shelled, desiccation-resistant eggs in a nest (or other incubation site) that is generally attended by one or both parents (Deeming, 2002). A consequence of laying eggs into a nest, which is then attended by a parent, is that the microclimate eggs are incubated in, and the conditions the avian embryo experiences during development, are largely independent of the wider environment (Ar, 1991; Deeming and Mainwaring, 2016; Rahn *et al.*, 1983; Rahn, 1991). In some species, however, bird eggs are exposed to extreme and potentially detrimental conditions due to the lack of a nest, limitations of incubation sites, or parental behaviours (Board, 1982).

The common guillemot, *Uria aalge,* breeds colonially on exposed and rocky cliff ledges which minimises predation of their eggs and chicks from terrestrial animals (Nettleship and Birkhead, 1985). To reduce the risk of losing eggs or chicks to aerial predators, guillemots also breed at very high densities (typically, 20 pairs per m^2) (Birkhead, 1977; Birkhead, 1993). One consequence of high density breeding is that colonies become 'unhygienic', with faecal material accumulating on the sea cliffs and breeding ledges. Contrary to previous suggestions (e.g. D'Alba *et al*., 2017), guillemot breeding sites are not usually dry, but are periodically wetted by rain leading to the formation of dirty puddles on the breeding ledges (Fig. S1; T. R. Birkhead pers. obs.). Since guillemots do not build a nest and instead incubate their single egg directly on bare rock ledges, their eggs are frequently exposed to a slurry of faeces, dirt, other detritus and water (henceforth, 'debris') during incubation (Birkhead, 2016; Birkhead *et al.*, 2017; Tschanz, 1990). Contamination of the eggshell by debris is almost inevitable as guillemots typically incubate their eggs between their legs (rarely with the egg entirely on top of their feet), and usually with the lower surface of the egg in direct contact with the substrate (Birkhead *et al.*, 2018; Manuwal *et al.*, 2001; Fig. S1).

Wet debris on the eggshell is likely to have a detrimental effect on embryonic survival since it may enter and block the gas exchange pores in the eggshell, reducing the gas exchange efficacy and also facilitate microbial invasion via the pore canals (Board, 1982). Both of these effects could compromise embryonic development through reduced water loss, carbon dioxide retention leading to hypercapnia (enhanced carbon dioxide in the embryo's blood), asphyxiation or infection, and ultimately result in embryo mortality (Ar and Deeming, 2009; Board and Fuller, 1993). Despite these potential risks, guillemot eggs covered with debris are known to hatch successfully (T. R. Birkhead pers. obs), suggesting that either (a) the debris that guillemot eggs are exposed to is relatively benign and does not compromise embryo survival, and/or (b) guillemot eggs possess adaptations to cope with the impact of debris.

Guillemot eggs could be unaffected by extensive debris cover if, due to intrinsic properties of the debris, it does not reduce the gas exchange efficacy of the shell. Coating either part of the blunt or pointed end of a chicken, *Gallus gallus domesticus*, egg with a man-made impermeable material (epoxy cement) has been shown to increase embryo mortality and levels of hatching failure (Tazawa, 1971). However, natural debris that adheres to the eggshell comes from a variety of sources and may include faecal material (which varies in its composition depending on the bird's diet e.g. guillemot's faeces contains small fish bones), dirt, sand, small stones, dust, feathers and vegetation. It is therefore likely to vary in gas permeability depending on its composition, and consequently may not have the same negative effects on embryo survival as impermeable cement.

Verbeek (1984) found that the water loss and hatching success of glaucous gull (*Larus glaucescens*) eggs were reduced when they were coated with gull faeces, but not when the eggs were coated with cormorant (*Phalacrocorax auritus, P. pelagicus*) faeces. This result is likely due to differences in the composition of faeces between species, and therefore the ability of gases to diffuse through. As a result, Verbeek (1984) suggested that birds that direct their faeces away from the nest site during incubation (like glaucous gulls) produce faeces that would inhibit gas exchange if it covered their egg(s); defecating away from the incubation site may therefore have evolved in response to the negative impact of faeces on embryo development. Birds whose faeces has little effect on eggshell conductance or hatching success may not be under the same selection to defecate away from their eggs or those of their neighbours in colonial breeding species. If Verbeek (1984) is correct, one might predict that guillemot faeces has little impact on gas exchange efficiency of the eggshell, since guillemots cannot not deliberately defecate away from their colony due to breeding at such high densities. In fact, although they propel their faeces away from themselves, the regularly propel their faeces onto their neighbours and their neighbours' eggs. In addition to faecal material, the debris on guillemot breeding ledges can include bones, stones, feathers, vegetation and soil, and thus may be porous and permeable to gases, allowing the relatively unrestricted diffusion of gases through it. However, if debris penetrates and blocks the gas exchange pores, it may still impede gas exchange by reducing the number of functional pores (open channels that allow the passage of gases through them) in the eggshell.

If guillemot eggs are affected by debris, one potential way they might cope is through 'self-cleaning' to remove contaminants, as suggested by Portugal *et al.*'s unpublished observations (https://phys.org/news/2013-07-unique-shell-guillemot-eggs-edge.html). Despite being widely covered by the media, including the BBC (http://www.bbc.co.uk/nature/23145291), The Guardian (https://www.theguardian.com/science/small-world/2013/jul/18/nanotech-roundup-cosmetic-fix-micro-batteries) and National Geographic (http://phenomena.nationalgeographic.com/2013/07/04/scientist-spills-water-discovers-self-cleaning-bird-egg/), this work remains unpublished (media reports were based on a conference presentation).

For a surface to be self-cleaning it must possess three properties; (i) high water repellency (known as super-hydrophobicity), with a stationary water contact angle of ~150°, (ii) low adhesion of extraneous debris to the eggshell surface and hence (iii) effortless removal of water and debris from the eggshell when water droplets make contact with its surface (Ensikat *et al.*, 2011; Genzer and Marmur, 2008; Yuan and Lee, 2013). According to Portugal *et al.*'s unpublished findings (https://phys.org/news/2013-07-unique-shell-guillemot-eggs-edge.html), the surface structure of guillemot eggshells makes them super-hydrophobic and consequently, self-cleaning. If true, debris should simply leave the surface of the shell every time the guillemot eggshell makes contact with water. The idea that guillemot eggs are self-cleaning seems biologically implausible since most guillemot eggshells remain contaminated with debris during the incubation period (Birkhead, 2016; Birkhead *et al.*, 2017), but the hypothesis has yet to be empirically tested.

If the guillemot eggshell is not self-cleaning then the shell accessory material (SAM) on the surface of the eggshell could limit the impact of debris by preventing pore blockages (Board, 1982). Here, we use Board and Scott's (1980) more general terminology: 'shell accessory material' (henceforth, SAM), rather than 'cuticle' (implying organic material) or 'cover' (implying inorganic material) as SAM is semantically more appropriate (Board *et al.*, 1977). SAM is the outermost substance that sits on the exterior surface of the eggshell and can provide a variety of benefits including waterproofing (Board and Halls, 1973a,b; Sparks and Board, 1984), microbial defence (D'Alba *et al*., 2014; Gole *et al.*, 2014a,b; Ishikawa *et al.*, 2010; Wellman-Labadie *et al.,* 2008), desiccation resistance (Deeming, 1987; Thompson and Goldie, 1990), aesthetic properties – including gloss (Igic *et al.*, 2015), UV reflectance (Fecheyr-Lippens *et al*., 2015), colouration and patterning (Lang and Wells, 1987; Samiullah and Roberts, 2014) and, as a consequence, protection from harmful wavelengths of light (Lahti and Ardia, 2016; Maurer *et al.*, 2014). SAM may also provide increased shell strength (Portugal *et al.*, 2017; Tyler, 1969). This wide range of properties may be attributable to the composite nature of SAM, as well as its varied thickness and composition in different species (Mikhailov, 1997). Despite the variability that exists in SAM, D'Alba *et al.*, (2017) showed that SAM may possess some universal functions including modulating UV reflectance and providing a barrier against microbes across seven bird species studied. However, it is not clear whether SAM can also provide a barrier to debris, specifically, whether or not SAM can prevent debris from entering pores and blocking them.

Board and Perrott (1982) provided circumstantial, observational evidence that SAM may prevent pore blockages by debris in guinea fowl (*Numidia meleagris)* eggs incubated by domestic chickens. However, no manipulations of eggshell structure were performed to explicitly test the hypothesis that SAM prevents pore blockages. The adaptive role of SAM in the common guillemot's egg is not clear (but see D'Alba *et al.*, 2017 for some suggestions). It is therefore unknown if SAM mitigates the negative costs of debris on the guillemot eggshell by, for example, preventing pores from becoming blocked.

The aim of the present study was to establish how common guillemot embryos survive incubation in eggs with large amounts of debris on their shell surface, by testing the following three hypotheses:

(1) the properties of natural debris are such that contamination of the eggshell does not reduce the gas exchange efficacy of the shell;

(2) the guillemot eggshell is self-cleaning; and

(3) shell accessory material prevents pore blockages by debris, which in turn ensures sufficient gas exchange is permitted across the eggshell for embryonic development.

Materials and methods

Eggshell and debris sampling

Fresh eggs were collected in 2013-16 under licence from Skomer Island, Wales, UK. All eggs were drained of their contents before being washed in distilled water and allowed to air dry at room temperature before storage. A hand-held rotary saw (DREMEL Multi, DREMEL, USA) was used to cut fragments (~1 cm^2) from the eggshells for use in the experiments detailed below. Where possible, fragments were cut from areas of the eggshell that appeared to be clean and the fragments were then rinsed in distilled water and allowed to air dry. No soap or chemicals were used in the cleaning process as they can damage the surface of the shell and SAM (D. Jackson, pers. obs.). Natural debris was opportunistically collected directly into sterile eppendorfs from guillemot breeding ledges in 2014-17. Debris was stored dry or semi-dry and rehydrated prior to use in experiments. All debris was used within one year of collection, typically sooner within 1-2 months.

Effect of debris on eggshell gas conductance

Fragments from the blunt end (see Birkhead *et al.*, 2017 for sampling location) of each egg were carefully fixed to individual custom glass vials with an aperture diameter of approximately 0.3 - 0.5cm using super glue (Loctite, USA), so that the inside of the eggshell membrane was fixed to the glass vial, and left to dry for 24 hours. The seal between the eggshell and the glass vial was checked before any excess shell around the edge of the glass vial was removed with a hand-held rotary saw. Finally, a further layer of super glue was applied to the circumference of the eggshell fragment and glass vial and left to dry. Each fragment underwent two treatments, a "clean trial" followed by a "dirty trial". Before clean trials, eggshell fragments were carefully cleaned on the outer surface using a fine paintbrush to remove any dust and debris. For dirty trials, rehydrated natural debris (1g of natural debris mixed with 300μl of distiller water) was applied to the outer eggshell surface of fragments using a paintbrush until they were evenly coated and no eggshell surface was visible.

A Bruker Alpha FTIR Spectrometer fitted with an Alpha-T module cell at a resolution of 0.8cm^{-1} was used to record the spectra of gases within the glass vials. Sample scan and background scan times were set to 32 scans, the result spectrum was set to 'Absorbance', and the resulting spectrum was saved from the 360-7000cm^{-1} range. All spectra were baseline corrected using an independent background scan of laboratory air that was recorded before each series of measurements. To record the spectra readings, a glass vial with an eggshell fragment fixed to the top, was placed on to the extended finger of a gas cell (calcium fluoride windows, a 7cm path length and one gas-tight 'Youngs' valve) and sealed using a petroleum-based jelly. To create the carbon dioxide rich environment inside the gas cell, small pieces of dry ice were initially placed into the cell before the attachment of the glass vial. To avoid a build-up of pressure while the dry ice sublimed, the gas-tight tap was opened slightly and the gas cell attached to a gas bubbler. Once the dry ice had completely sublimed and no further bubbles were observed inside the gas bubbler, the gas-tight tap was closed, and the gas bubbler removed. Immediately after this, the gas cell was positioned onto the Alpha-T cell sample holder on the Bruker Alpha FTIR and an absorbance spectrum was recorded and saved. Another spectrum was recorded and saved one hour later to determine how much carbon dioxide had diffused through the shell within this time frame.

To quantify the rate constant of eggshell carbon dioxide gas diffusion for each fragment (henceforth, carbon dioxide conductance), integral measurements were taken between the absorption bands that correspond to carbon dioxide (3842.5 and 3763.15cm^{-1}) from the initial spectra and the spectra after one hour for each individual sample (see https://webbook.nist.gov/chemistry/). Integral values were standardised so that the initial value was 100. The carbon dioxide conductance was calculated by subtracting the standardised integral after an hour from the standardised initial integral.

The method described above was chosen over other methods to measure eggshell conductance of eggshell fragments (e.g. Portugal *et al.*, 2010) for two main reasons. Firstly, it directly measures the amount of carbon dioxide gas lost through the eggshell rather than predicting gas loss from measured mass loss. This potentially provides more precise measurements as the precision of weighing scales can be more limiting than the FTIR Spectrometer (J. E. Thompson pers. obs.), as well as providing more accurate data because gas loss is directly measured rather than predicted from mass loss. Secondly, and crucially, this method allowed us to repeat each trial on the same fragments when they were clean and dirty without damaging the fragment or the vessel the sample was attached onto, which would not be possible using Portugal *et al.'s,* (2010) approach. Even though we are measuring the change in carbon dioxide loss, water vapour, oxygen and carbon dioxide conductance are all linked (Rahn and Paganelli, 1990; Ar and Deeming, 2009) so all gases would likely be affected in a similar way and, therefore any restrictions on carbon dioxide conductance can theoretically be more broadly applied to any gas crossing the shell.

After the gas conductance of dirty fragments was measured, we cut the eggshell fragment off the glass vial and used X-ray micro computed tomography (microCT) to assess the extent to which eggshell pores were blocked by debris. Because the eggshell fragment needed to be cut off the glass vial for micro-CT scanning, we could not scan the eggshell fragments in between clean and dirty treatments, only once the gas conductance experiment was over and the eggshell fragment was dirty. Eggshell fragments were scanned in a Bruker Skyscan 1172 set to 100kV electron acceleration energy and 90uA current, with the sample 45.7mm from the X-ray source with a 1.0mm aluminium filter; and the camera 218mm away from the source. Camera resolution was set at 1048 x 2000 pixels, and a pixel size of 4.87μm. We used the same settings for each scan, collecting a total of 513 projection images over a 180° rotation using a rotation step size of 0.4° and a detector exposure of 885ms integrated over three averaged images resulting in a total scan time of 38 minutes. One eggshell fragment was scanned during each session. Projection images were reconstructed in NRECON software (version 1.6.10.2) after which image analysis was performed in CT analyser (CTAN, version 1.14.41), CTVOX (version 3.0) and CTVol (version 2.2.3.0; all the above software was provided by Bruker micro-CT, Kontich, Belgium). Reconstruction parameters used were: dynamic image range; minimum attenuation coefficient = 0.0025, maximum = 0.05, level 2 asymmetrical boxcar smoothing, ring artefact correction = 12, beam hardening correction of 20% and auto misalignment compensation. Resultant images were saved as 8-bit bitmaps.

Two 3D models – one for the shell and another for the debris – were created for each shell fragment by segmenting the images in CTAN. Shell models were created by initially resizing the data-set by a factor 2 with averaging in 3D on, before using automatic (otsu method) thresholding to segment the images, followed by low level despeckling of white and black pixels in 2D space (<10 pixels). The 3D .ctm model was then created using an adaptive rendering algorithm with smoothing on, a locality value of 1 and a tolerance of 0.05. Debris models were created by initially resizing the data-set by a factor 2 with averaging in 3D off, before manually thresholding for debris to segment the images, followed by low level despeckling of white (< 2 pixels) and black (<10 pixels) pixels in 2D space (<10 pixels). Again, the 3D .ctm model was then created using an adaptive rendering algorithm with smoothing on, a locality value of 1 and a tolerance of 0.05. Both models were loaded into CTVol, aligned, and pore channels were visually inspected to see if they were blocked by debris (Fig. S2). Due to the image processing protocols followed, we could detect air spaces (and blockages) no smaller than 10μm, so our method may have overestimated the number of blocked pores since any pores with small air spaces within the debris blockage would have been undetectable due to the resolution limit. This measure is therefore a proxy of the level of pore blockages within an eggshell fragment, rather than an absolute value. This methodology may introduce a bias if different types of debris are studied, but in each of our experiments debris was used from a single sample collected from the field, removing this issue. Only blockages inside the pore channel were counted, and not blockages at the surface of the pores, because the thresholding parameters used to identify debris could not distinguish between debris and the shell membranes, and potentially SAM on the shell surface.

The number of blocked pores was divided by the total number of pores to provide an estimate of the proportion of blocked pores per fragment. The thickness of debris on the surface of the shell (above each pore), and the length of each pore channel was measured in CTAN using the line measurement tool and averaged for each eggshell fragment. The thickness of the trueshell (the calcium carbonate layers of the eggshell, excluding the organic membranes) was also measured at 10 locations using the line measurement tool and averaged for each fragment (see Birkhead *et al.*, 2017).

Self-cleaning eggs

Using a method similar to Vorobyev and Guo (2015), we tested the most important property of self-cleaning surfaces; whether water droplets and debris readily leave the guillemot eggshell surface together. Ten freshly collected guillemot eggshells, and five museum samples were used in this study. Fragments were taken from the equator of each eggshell (see Birkhead *et al.*, 2017), and two fragments per eggshell were studied per treatment. An eggshell fragment was attached to a stand tilted at 8° and dust from a household vacuum cleaner (as used in Vorobyev and Guo, (2015)), was applied to the shell's surface. Over a series of fifteen to twenty droplets, 400µl of water was dripped on to the fragment and the shell was examined by eye. If the eggshell fragment contained a puddle of water carrying floating or stationary dust then the surface was deemed to not be self-cleaning, as water and debris still remained on the surface (see Introduction for definition of self-cleaning). If the surface did not contain any floating dust particles or any water, then the surface was classified as self-cleaning (Vorobyev and Guo, 2015). To validate this simple self-cleaning test, we repeated this trial using the following known self-cleaning materials; the fresh, young leaves of cauliflower (*Brassica oleracea var. botrytis*), broccoli (*Brassica oleracea var. italica*) and collard (spring) greens (*Brassica oleracea var. viridis*). After the dust trial on *Brassica* leaves, very little or no water remained on the surface of the leaves as it bounced off the samples removing debris with it (Movie 1), therefore validating the use of this simple self-cleaning test to determine if guillemot eggshells are self-cleaning. Self-cleaning tests were repeated using wet debris (a vial containing 2.5ml of semi-dry natural debris was diluted with 100µl of distilled water) and debris that had been allowed to dry onto the shell to assess if guillemot eggshell is self-cleaning against natural debris it would encounter during incubation.

After the self-cleaning experiment was conducted, eggshell fragments were washed in excess water and allowed to dry, to mimic a heavy rain shower and followed by natural drying. Eggshell fragments were then qualitatively assessed (yes, or no) – by eye, using a macro lens on a digital camera, and by microscope – to establish whether any debris remained on the shell surface.

Shell accessory material and pore blockages

To test the role of shell accessory material in preventing pore blockages by debris, we chemically manipulated eggshell fragments to remove shell accessory materials from the eggshell. Two pieces of shell (c. 1cm^2) were cut from the equator of five fresh eggs (see Birkhead *et al.*, 2017 for sampling location). One fragment acted as a control, and was washed in distilled water only, whereas the other fragment was first treated with thick household bleach (containing sodium hydroxide and hypochlorite: Original variety (unscented), Euroshopper, Booker, UK) to remove organic shell accessory material (see Fig. S3), and then also washed in distilled water. Both sodium hydroxide and sodium hypochlorite - key components of bleach – have been used to remove organic shell accessory material from the surface of the shell in previous studies (Deeming, 1987; Tullett *et al.*, 1976). Following the cleaning treatments, debris was carefully added to the surface of each shell fragment by squeezing a paintbrush loaded with wet debris (1g of natural debris mixed with 300μl of water) with forceps. The debris was allowed to air dry for at least 24 hours.

Eggshell fragments were scanned in a Bruker Skyscan 1172 using similar settings as detailed above, except that in this case a pixel size of 4μm was used, thus the sample was 48.7mm from the X-ray source with a 1.0mm aluminium filter, and the camera was 283mm away from the source. We collected 499 projection images each with an exposure time of 1475ms, leading to a scan time of 49min. These settings provided higher resolution data compared to those used above. A lower pixel size had to be used to scan the fragments used in the gas conductance trials to ensure that all of the eggshell exposed over the hole in the glass vial was scanned, whereas this was not a limitation here.

Two 3D models were created per shell fragment (one for the shell and another for the debris) in CTAN by thresholding for each material (automatically for the shell using otsu and manually for debris). Model creation parameters were the same as those discussed earlier except that shell models were created by initially resizing the data set by a factor 2 with averaging in 3D off. To account for differences in pore numbers between pairs of fragments, only the first fifteen pores that could be visualised by re-slicing the z-stack of reconstructed images were selected to assess pore blockages. The models were then loaded into CTVol, and pore channels were visually inspected to see if they were blocked by debris model (Fig. S2). As explained above, this measure provides a proxy rather than the absolute number of blocked pores. However, since we were able to use a higher scanning (and model) resolution in this experiment, detection of pore blockages and air spaces in between debris should have a limit of approximately 8μm.

Statistical analysis

All statistical analyses were performed in R (3.3.1 — R Development Core Team 2012). We used a paired t-test to test whether the presence of debris on the eggshell influenced carbon dioxide conductance. We used Pearson's product moment correlations to establish whether a correlation existed between the clean eggshell carbon dioxide (CO_2) conductance and (a) the number of pores in an eggshell fragment or (b) the length of those pores (measured both directly and by using the proxy of shell thickness). Pearson's product moment correlations were also used to establish whether a correlation existed between the relative change in CO_2 loss between clean and dirty fragments and the proportion of pores blocked in an eggshell fragment, or the thickness of the debris on the surface of the shell. Finally, paired t-tests were performed to assess whether SAM on the surface of guillemot eggshells limits the number of pores that are blocked by wet debris when it is applied to the outer surface of the shell.

Results

Effect of debris on eggshell gas conductance

The rate of gas exchange for clean eggshell fragments was positively correlated with the number of pores present in an eggshell fragment ($r = 0.733$, $p = 0.016$, $n = 10$), but not with either the mean length of pores ($r = 0.045$, $p = 0.902$, $n = 10$), nor the mean trueshell thickness ($r = -0.185$, $p = 0.610$, $n = 10$). After debris was applied to the eggshell, carbon dioxide conductance significantly decreased ($t = 3.02$, $df = 9$, $p = 0.014$; Fig. 1). The relative reduction in carbon dioxide conductance of the eggshell after the application of debris was negatively correlated with the proportion of pores in the eggshell that were blocked ($r = -0.821$, $p = 0.004$, $n = 10$), with fragments possessing a greater proportion of blocked pores showing a greater reduction in carbon dioxide conductance compared to when the fragments were clean (Fig. 2). The reduction in carbon dioxide conductance was not related to the average thickness of the debris on the eggshell above each pore ($r = -0.060$, $p = 0.870$, $n = 10$).

Self-cleaning eggs

None of the common guillemot eggshell fragments studied here demonstrated any self-cleaning ability against dust. All fragments were covered in a puddle of water containing dust at the end of the trial, which is characteristic of materials that are not super-hydrophobic and not self-cleaning (Movie 2; Vorobyev and Guo, 2015). None of the guillemot eggshell fragments demonstrated any self-cleaning ability against either wet or dry natural debris (Fig. 3; Movie 3). It was possible to remove some debris - but not all - by washing the eggshell with water, but a large volume of water had to be applied and debris removal appeared to depend on water volume and/or pressure. This is not necessarily biologically relevant with respect to the circumstances in which guillemots breed because even when it is raining, it is unlikely that a large volume of pressurised clean water will make contact with the eggshell surface all at once. Instead, it is more likely that dirty water and wet debris from the cliff ledges will come into contact with the egg. Even after excessive washing, fragments were not completely clean, with small amounts of debris and staining remaining (Fig. 3 & 4).

Shell accessory material and pore blockages

The removal of SAM from eggshell fragments resulted in a significant increase in the proportion of pores that were blocked after the experimental application of natural debris to the shell surface, compared to control fragments where SAM was still present ($t = 4.74$, $df = 4$, $p = 0.009$; Fig. 5).

Discussion

Our results show that debris contaminating the surface of guillemot eggshells during incubation reduces the gas exchange efficacy of the eggshell, and the eggshell is not self-cleaning to help resolve this problem. Instead, the full impact of debris on the gas exchange efficacy of eggshell is minimised by shell accessory material (SAM). SAM protects pores, reducing the number that are blocked by debris, which in turn minimises the reduction in eggshell gas conductance caused by debris on the eggshell.

The drivers of eggshell gas conductance

Our data suggest that pore number is the primary driver of gas conductance in guillemot eggshell fragments. This is contrary to the predictions of Zimmerman and Hipfner (2007) who suggest that shell thickness (i.e. pore length) and pore size are the key drivers of porosity and therefore gas conductance in common guillemot eggs. The fact that pore length (shell thickness) does not drive eggshell gas conductance is consistent with ideas initially presented by Ar and Rahn (1985) and Rahn and Paganelli (1990), as well as in the discussions of Portugal *et al.*, (2010) and Maurer *et al.*, (2012), which allude to the fact that shell thickness is not a determinant of water vapour conductance. In the present study, we were unable to use micro-CT to scan clean fragments that were used in our gas conductance trials (see Methods for further details), so we cannot explicitly link pore size to eggshell conductance. However, evidence from other studies suggests that the role of pore size is likely to be minor compared to that of pore number or density (Ar and Rahn, 1985, Rahn and Paganelli, 1990; Rokitka and Rahn 1987, Simkiss 1986; see Table 1).

If pore number is the main driver of gas conductance across the eggshell, then predictions made using the calculations based on the traditional theoretical formulae presented in Ar *et al.*, (1974) and Ar and Rahn (1985), based on Fick's law of diffusion, may be incorrect as they erroneously include terms for pore length (shell thickness) and pore area. Previous research has suggested that calculated versus measured conductance values are not consistent; in fact, measured values can be three times lower than calculated values (Tøien *et al.*, 1988). Including pore size and pore length (shell thickness) could be one reason for this discrepancy, alongside a lack of consideration of the effects of (1) SAM (Thompson and Goldie, 1990; Tøien *et al.*, 1988), (2) convective and diffusive resistance (Tøien *et al.*, 1988), and (3) internal heat changes due to the metabolic rate of the developing embryo. In addition, historical methods used to study shell thickness and porosity were imprecise, unreliable and inaccurate. For example, pore size was likely overestimated in previous studies because the minimum cross-sectional dimensions (e.g. area or radius) could not always be measured as they are within the pore channel, and therefore measures from the inner surface of the shell were used instead under the presumption that these dimensions were the limiting dimensions (see Birkhead *et al.*, 2017). Furthermore, shell thickness measures are not always the same as pore length (see supplementary material). Further investigation into the drivers of eggshell gas conductance is needed, particularly with the advent of more precise and accurate methods for measuring eggshell parameters and gas conductance. Gaining a better understanding of what drives eggshell conductance is particularly important because predicted gas conductance values are used in a variety of ways, including for inferring the nesting conditions of extinct birds and dinosaurs (e.g. Deeming, 2006; Deeming and Reynolds,

2016) and drawing comparative conclusions about species' developmental biology (e.g. Jaeckle *et al.*, 2012).

The role of shell accessory materials in protecting pores

Our finding that eggshell gas conductance is driven by pore number is important because it means that any blockages within pores impose a serious restriction on gas exchange through reducing the number of functional pores (i.e. unblocked, complete pores that gases can diffuse through) available for gas exchange. Our results show that internal pore blockages by debris have a direct effect on the gas exchange efficacy of the eggshell, as was previously suggested by Board (1982) and Board and Perrott (1982). In a previous study, we suggested that the pyriform shape of common guillemot eggs, and the distribution of pores across the eggshell, may help to minimise the effects of eggshell contamination on the developing embryo (Birkhead *et al.*, 2017). The orientation of the guillemot's pyriform egg during incubation is such that the blunt end of the egg (where porosity is highest) generally does not come into contact with the substrate, so most debris is concentrated on the pointed end of the egg where porosity is low. This potentially minimises the overall number of pores that become blocked and maximises the number of functional pores available for gas exchange. However, debris on the elongated, pointed end of the egg could still lead to a large reduction in overall eggshell gas exchange, and, despite the egg's shape, debris is still sometimes seen on the blunt end. We show here that SAM prevents pores becoming blocked by debris, a finding consistent with Board and Perrott's (1982) observations that nesting debris penetrates pores and may reduce the total area of eggshell available for gases to diffuse through. SAM could therefore minimise the negative effects of debris covering the eggshell surface by minimising the number of pores that become blocked.

How SAM prevents pore blockages is not clear. One possibility is that the SAM acts as a physical barrier to the penetration of debris, as seemed to be the case for helmeted guinea fowl eggs (Board and Perrott, 1982). Alternatively, SAM may provide water resistance to the eggshell, which prevents aqueous debris from entering eggshell pores (Board, 1981). Either way, if SAM is removed or damaged, the pores become vulnerable to blockages. Natural cracking of SAM can occur due to dehydration, and cracks could leave pores vulnerable, which may explain why some of the untreated eggshell fragments we studied to assess the impact of debris on eggshell conductance had a large proportion of blocked pores (see Fig. S4). Some eggshells also had poor quality SAM or a patchy SAM coverage meaning pores were uncovered and left vulnerable (Fig. S3), and in addition, our limited imaging and blockage detection resolution may have lead us to consistently overestimate the proportion of blocked pores (see methods). Although this would not invalidate our overall findings, it could explain the unexpectedly high proportion of blocked pores found in untreated eggshells when debris was added onto the surface of the shell. Whether SAM plays the same role on the eggs of other species that are directly exposed to debris (e.g. the blue footed booby, *Sula nebouxii*, (Mayani-Paras *et al.*, 2015)), remains to be tested.

Guillemot eggs are not self-cleaning

Despite suggestions of previous researchers, we found no evidence that the guillemot eggshell surface is self-cleaning. Common guillemot eggshells lack the three important properties which would make them self-cleaning:

(1) They are not super-hydrophobic. Reported water contact angles are lower than 150°. For example, Portugal *et al.* reported values of approximately 120° (Portugal, S. as reported by Yong, 2013 in http://phenomena.nationalgeographic.com/2013/07/04/scientist-spills-water- discovers-selfcleaning-bird-egg/) while D'Alba *et al.*, (2017) reported values of just over 90°. The latter is potentially lower due to eggshell treatment with ethanol in that study.

(2) Debris strongly adheres to the guillemot eggshell surface (see Fig. 3 in Birkhead *et al.*, 2017). Our self-cleaning trials corroborate observations that debris cannot easily be washed off most guillemot eggshells. Instead scrubbing or wiping with excess amounts of clean water is required to remove debris, and this is still often unsuccessful, implying that debris has high adhesion with the shell (J. E. Thompson and D. Jackson, pers. obs.). Furthermore, it is worth noting that even apparently clean sections of naturally incubated eggs usually contain staining or particles of debris when viewed at high magnification, illustrating that debris does indeed adhere to the eggshell surface (Fig. 4).

(3) Consequently, natural debris on the guillemot eggshell surface does not readily leave when water makes contact with it and the eggshell (Fig. 3; Movie 3).

The fact that guillemot eggshells do not possess self-cleaning properties becomes intuitive when we consider how debris interacts with the eggshell surface. A single application of wet debris can not only cover the eggshell surface, but also cause pore blockages that reduce the ability of gases to pass through the shell. A self-cleaning surface on its own would thus be insufficient to maintain adequate gas exchange across the eggshell, unless there was also a unique mechanism to un-block pore channels. Given that SAM prevents pore blockages, and that the presence of debris does not appear to limit the ability of gases to diffuse across the eggshell, there would be little selection on guillemot eggshell structure for self-cleaning properties in the context of eggshell conductance.

Instead of evolving self-cleaning eggs, guillemots may avoid the problem of their eggs becoming excessively covered in debris during incubation via an altogether different mechanism: egg turning. Egg turning is the process where incubating parents turn their eggs around along the longitudinal axis, which is important for normal embryonic development and subsequent hatching (Deeming and Reynolds, 2016). Turning may physically remove debris via abrasion and limit an excessive build-up of material on the surface of the shell (Board and Scott, 1980; Board, 1982), which could affect embryo development by reducing gas conductance, increasing the risk of embryonic infection or interfering with contact incubation and thermoregulation. Anecdotal observations suggest incubation and egg turning limits the build-up of material on common guillemot eggs, as abandoned, un-incubated eggs soon become completely covered in debris (T. R. Birkhead pers. obs; see Fig S1 for an example). Furthermore, Verbeek (1984) suggested that abrasion of faecal material from the surface of glaucous gull eggs may have partially restored their hatching success, although this was not based on direct experimental evidence. However, guillemot eggs that are partially or largely covered with debris still tend to hatch (T. R. Birkhead pers. obs.), indicating that complete debris removal is not essential for normal embryo development in this species.

Conclusion

The findings of the present study suggest that the effect of debris contaminating the surface of common guillemot eggs is minimised by the presence of SAM, which reduces the number of pores that become blocked. This, in combination with the fact that the pyriform shape of the guillemot egg minimises the amount of debris that covers the highly porous blunt end of the egg (Birkhead et al. 2017), ensures that a high proportion of pores remain functional during incubation and guillemot eggs are able to maintain efficient gas exchange despite being covered in debris. The ability of SAM to minimise pore blockages by debris, rather than the egg's shape or pore distribution, is presumably crucial when eggs are heavily covered with debris. It seems likely that the presence of functional SAM, rather than solely the egg's shape, allows guillemot eggs to maintain gas exchange despite being covered in debris throughout the 32-day incubation period, allowing the embryo to develop normally.

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Competing interests

No competing interests declared.

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Data availability

Data are available in the supplementary material (Datasets 1 & 2).

Author contributions

TRB conceived the study, DJ, JET, NH and TRB conceived and planned the experiments. DJ and JET carried out the experiments. DJ and JET analysed the data. DJ took the lead in writing the manuscript with support and input from TRB, NH and JET.

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Figure legends

Figure 1. The effect of debris on carbon dioxide loss. The rate of carbon dioxide loss significantly decreased after the application of natural debris onto the eggshell (paired t-test: $t = 3.02$, $df = 9$, $p = 0.0144$, $n=10$). Boxes are the interquartile range, black line within the box is the median, the whiskers show the highest and lowest values and the circles are the individual data points.

Figure 2. The effect of the percentage of pores blocked by debris on the percent change in carbon dioxide conductance through guillemot eggshell covered with debris compared to when the eggshell was clean. The relative reduction in carbon dioxide conductance of the eggshell after the application of debris was negatively correlated with the proportion of pores in the eggshell that were blocked (Pearson's product moment correlation: $r = -0.821$, $p = 0.004$, $n = 10$). Change in carbon dioxide conductance was calculated as: ((dirty gas conductance - clean gas conductance) / clean gas conductance) x 100. The red line is the line of best fit.

Figure 3. Example of a self-cleaning trial involving dried on debris. The large patch in the centre of the eggshell fragment is the debris – the two smaller dark patches either side are pigment on the eggshell surface. (A) An eggshell fragment with debris on the surface, (B) the same fragment after the first drop of water has fallen onto the shell surface, (C) at the end of the trial water and debris remained on the eggshell surface illustrating that the sample is not self-cleaning. (D) After the trial, excess clean water was used to wash off the debris. Even after this cleaning, debris remained on the eggshell surface as stains or remnants.

Figure 4. Natural debris on common guillemot shells (debris is light brown; darker brown/black patches in these images are eggshell pigment). (A) and (B) are images from a stereoscopic microscope showing the remnants of debris remaining on a guillemot fragment after washing with excess water. Scale bar for (A) 1000μm and (B) 100μm. (C) and (D) are images from a stereoscopic microscope showing natural debris on common guillemot eggshell. Scale bar for (C) is 1000μm and (D) 100μm. (C) An un-manipulated

piece of guillemot eggshell showing natural debris staining, but also a patch that, to the naked eye, looks clean. The rectangle marks the "clean" area shown in (D). (D) A high magnification image of a piece of "clean" eggshell showing that even here, there are small particles of debris on the shell surface, a few of which are marked with arrows.

Figure 5. The effect of shell accessory removal on the percentage of pores blocked by natural debris. The proportion of pores blocked by debris significantly increased after the removal of shell accessory material using bleach (paired t-test: $t = 4.74$, $df = 4$, $p = 0.00904$, $n=5$). Boxes are the interquartile range, black line within the box is the median, the whiskers show the highest and lowest values, and the circles are the individual data points.

Table 1. The linear regression relationships between measured or calculated eggshell parameters and observed gas conductance in the eggs of 21 species of Anatidae. The total number of pores per egg ($R^2 = 0.624$) and the total pore circumference ($R^2 = 0.633$) explain more variation in observed gas conductance than does calculated gas conductance using the traditional calculation ($R^2 = 0.371$), highlighting an issue with the assumption that pore area and shell thickness are determinants of gas conductance. The fact that total pore area per egg ($R^2 = 0.485$) explains less variation than the total number of pores per egg, and pore area is not significantly associated with observed gas conductance, suggests that pore area does not drive eggshell gas conductance.

Parameter	**Calculation**	**Adjusted R^2**	**Regression equation**	**P value**	**Source**
Total pore circumference[1] (μm)	2 x π x pore radius x pores per egg	0.633	y = 0.0153x + 5.35	< 0.0001	Re-calculated from Hoyt *et al*.'s, (1979) data using Simkiss's (1986) formula
Calculated gas conductance[2] (mg Day^{-1} $Torr^{-1}$)	(2.24 x pore area x pores per egg) / shell thickness	0.371	y = 0.575x + 9.41	0.00202	Calculated by Hoyt *et al.,* (1979)
Total pore area ($μm^2$)	Measured pore area x pores per egg	0.485	y = 0.0079x + 9.63	0.000271	Calculated from data in Hoyt *et al*., (1979)
Pores per egg[3]	Calculated from surface area and measured pore density	0.624	y = 0.00157x + 2.52	< 0.0001	Data from Hoyt *et al*., (1979)
Shell thickness (mm)	Measured	0.267	y = 56.7x - 3.32	0.00968	Data from Hoyt *et al*., (1979)
Pore area ($μm^2$)	Average measured area of a pore	0.00479	y = 0.0143x + 14.5	0.308	Data from Hoyt *et al*., (1979)

[1] based on Stefan's law of diffusion

[2] constant*total pore area*pore length^{-1} based on Fick's law of diffusion

[3] it is worth noting that Ar and Rahn (1985)'s regression analysis of pore number against eggshell gas conductance on 134 different species' eggs had an R^2 value of 0.89.

Movie captions:

Movie 1: Validation of self-cleaning trial using a fresh cauliflower (*Brassica oleracea var. botrytis*) leaf.

Movie 2: Dust self-cleaning trial on common guillemot (*Uria aalge*) eggshell.

Movie 3: Wet natural debris self-cleaning trial on common guillemot (*Uria aalge*) eggshell followed by a dry natural debris self-cleaning trial.

Figures

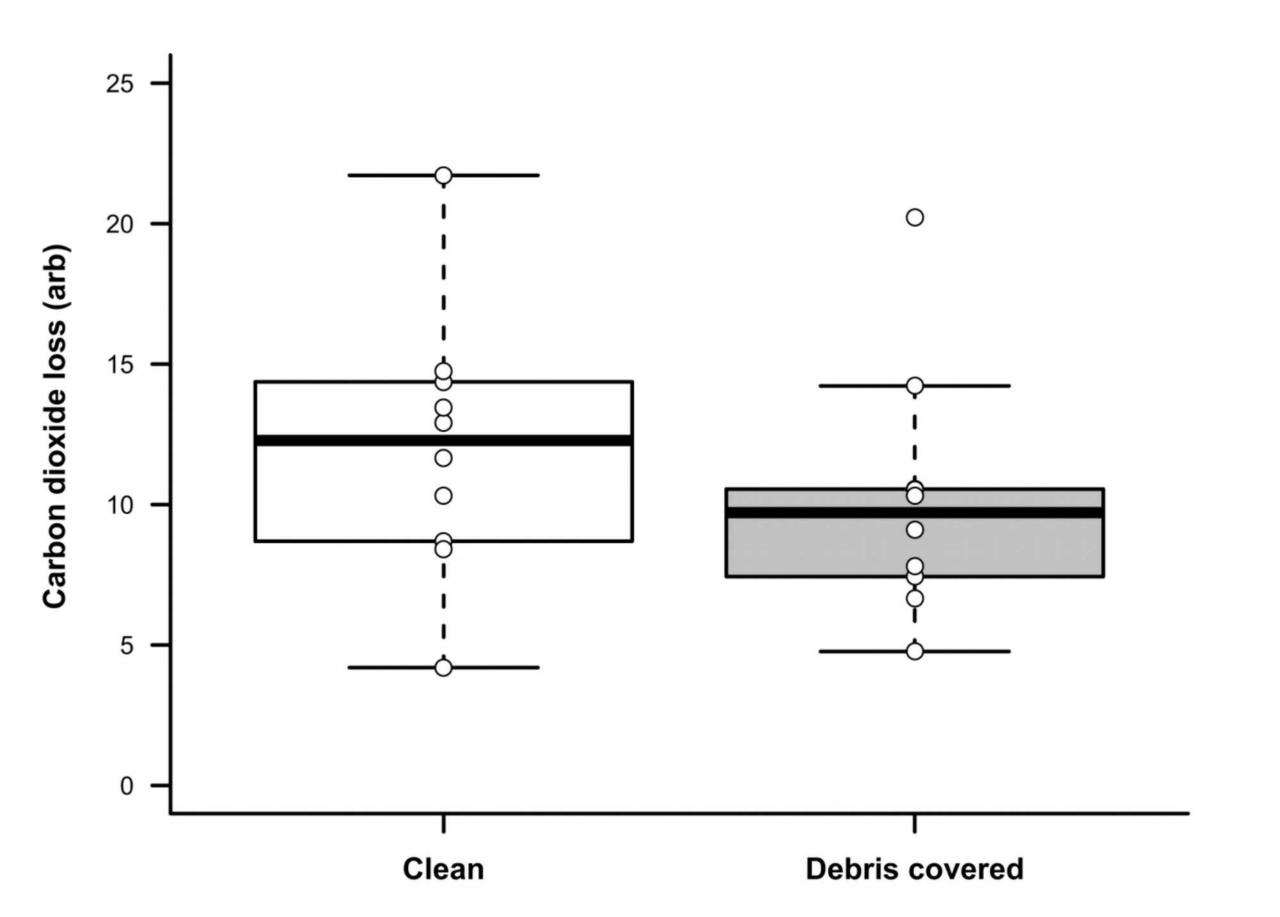

Figure 1. The effect of debris on carbon dioxide loss.

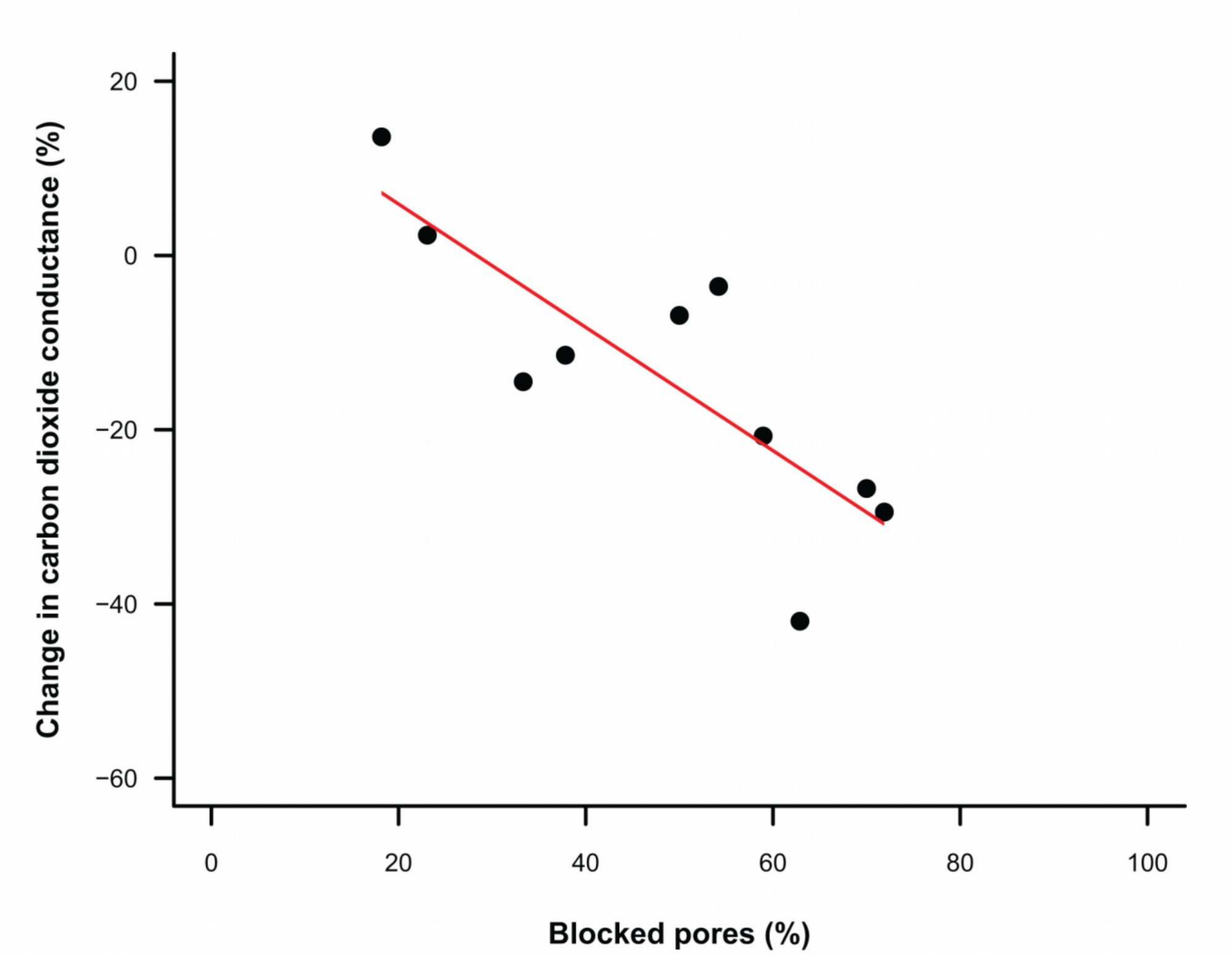

Figure 2. The effect of the percentage of pores blocked by debris on the percent change in carbon dioxide conductance through guillemot eggshell covered with debris compared to when the eggshell was clean.

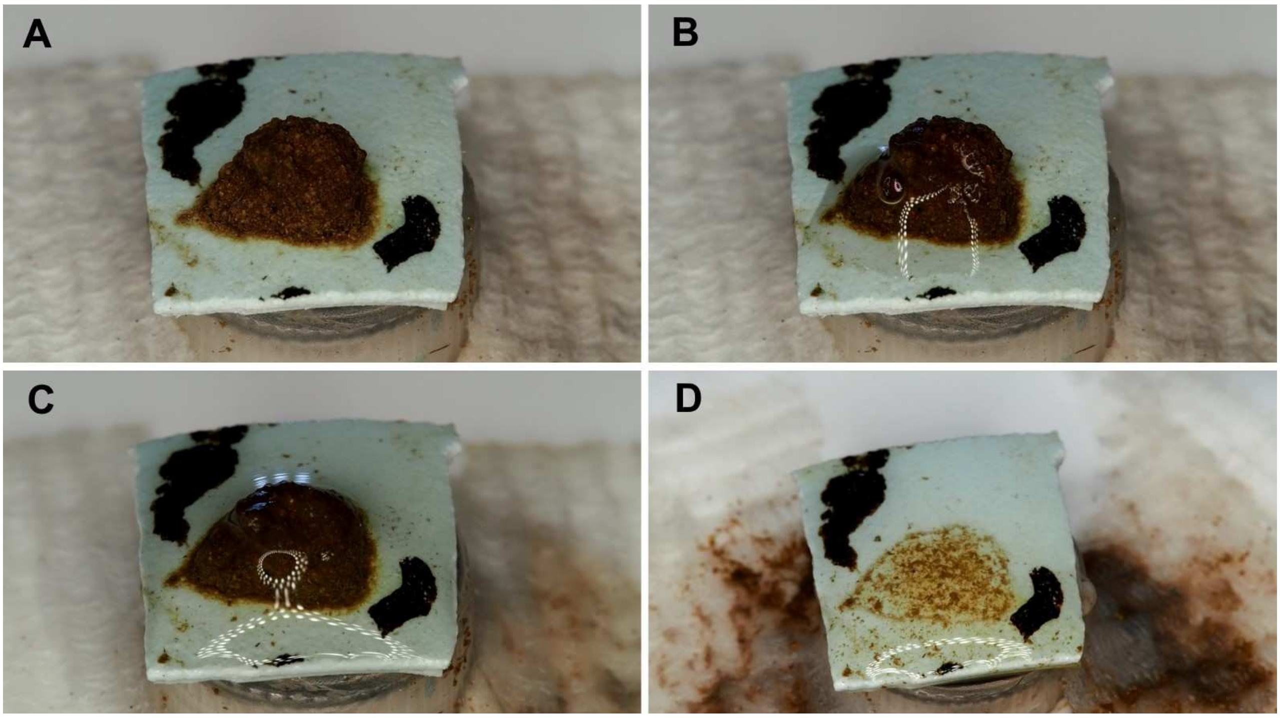

Figure 3. Example of a self-cleaning trial involving dried on debris.

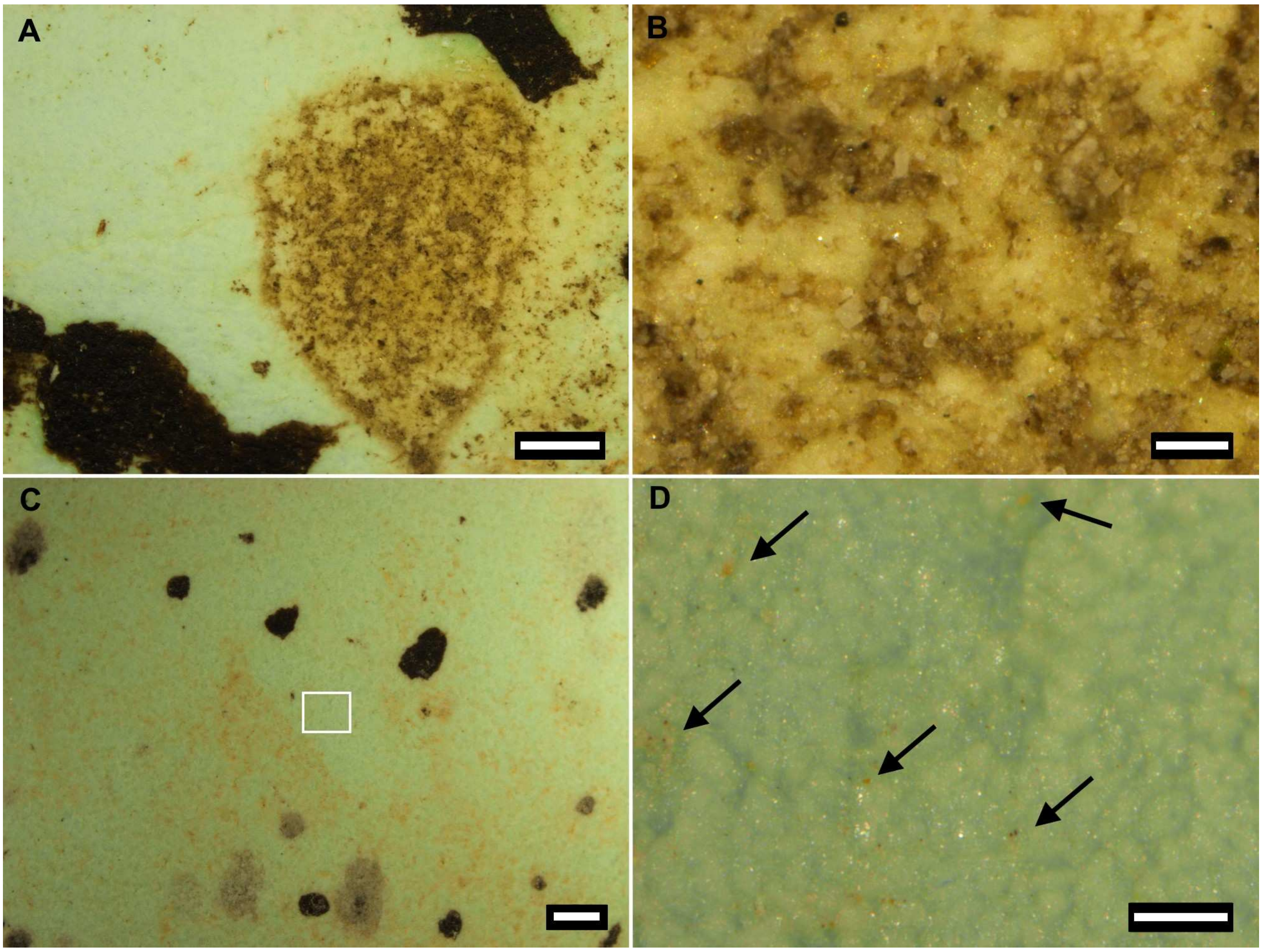

Figure 4. Natural debris on common guillemot shells (debris is light brown; darker brown/black patches in these images are eggshell pigment).

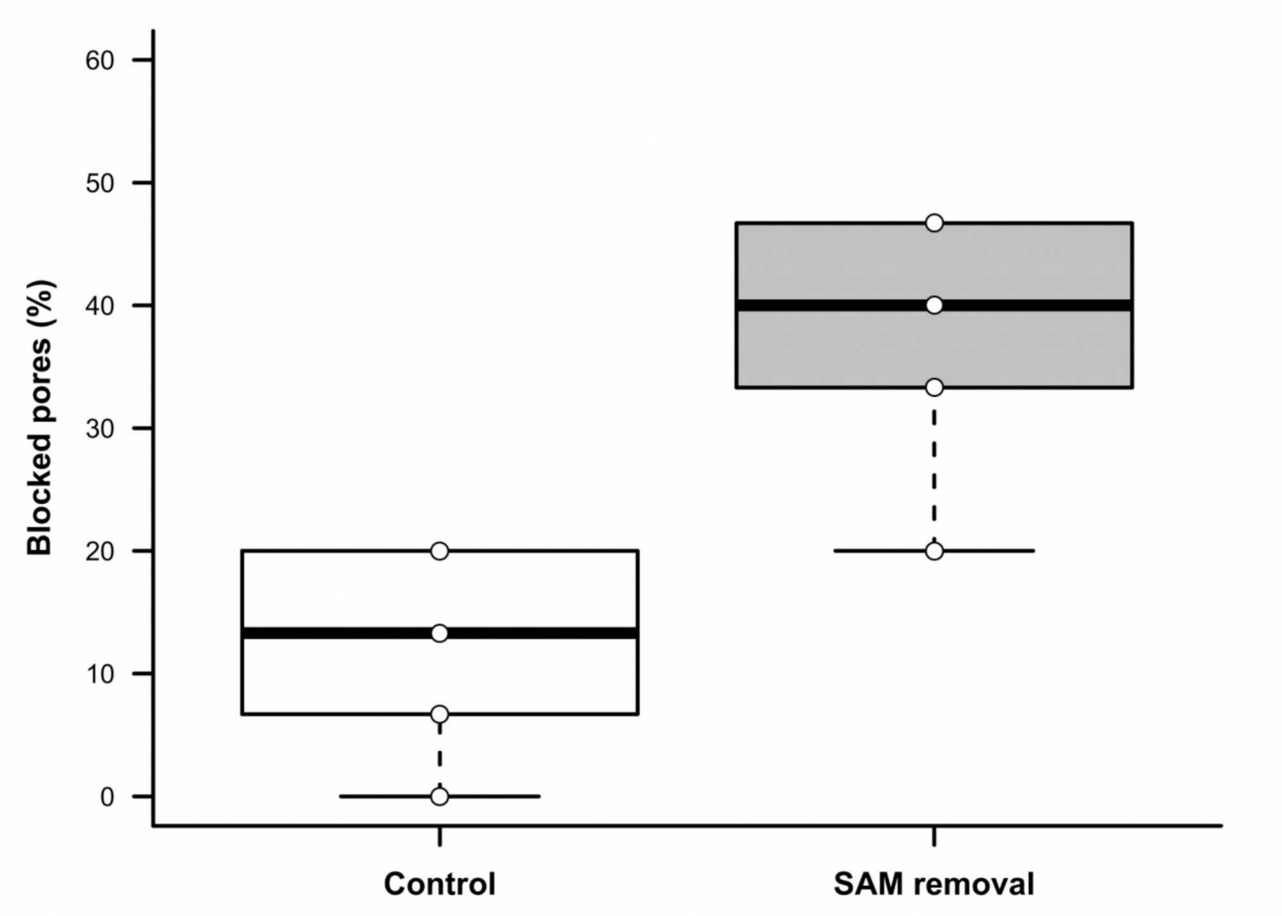

Figure 5. The effect of shell accessory removal on the percentage of pores blocked by natural debris.

Supplementary materials for:

Common guillemot (*Uria aalge*) eggs are not self-cleaning

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Figure S1. Images illustrating the conditions within a guillemot breeding colony. Note the puddles of water and debris on the ledges. All images were taken at sites on Skomer Island, Wales, UK by TRB. Additional images and videos of guillemots incubating their eggs can be seen on Wildscreen Arkive e.g. https://www.arkive.org/guillemot/uria-aalge/image-A24724.html and https://www.arkive.org/guillemot/uria-aalge/video-09c.html.

1098

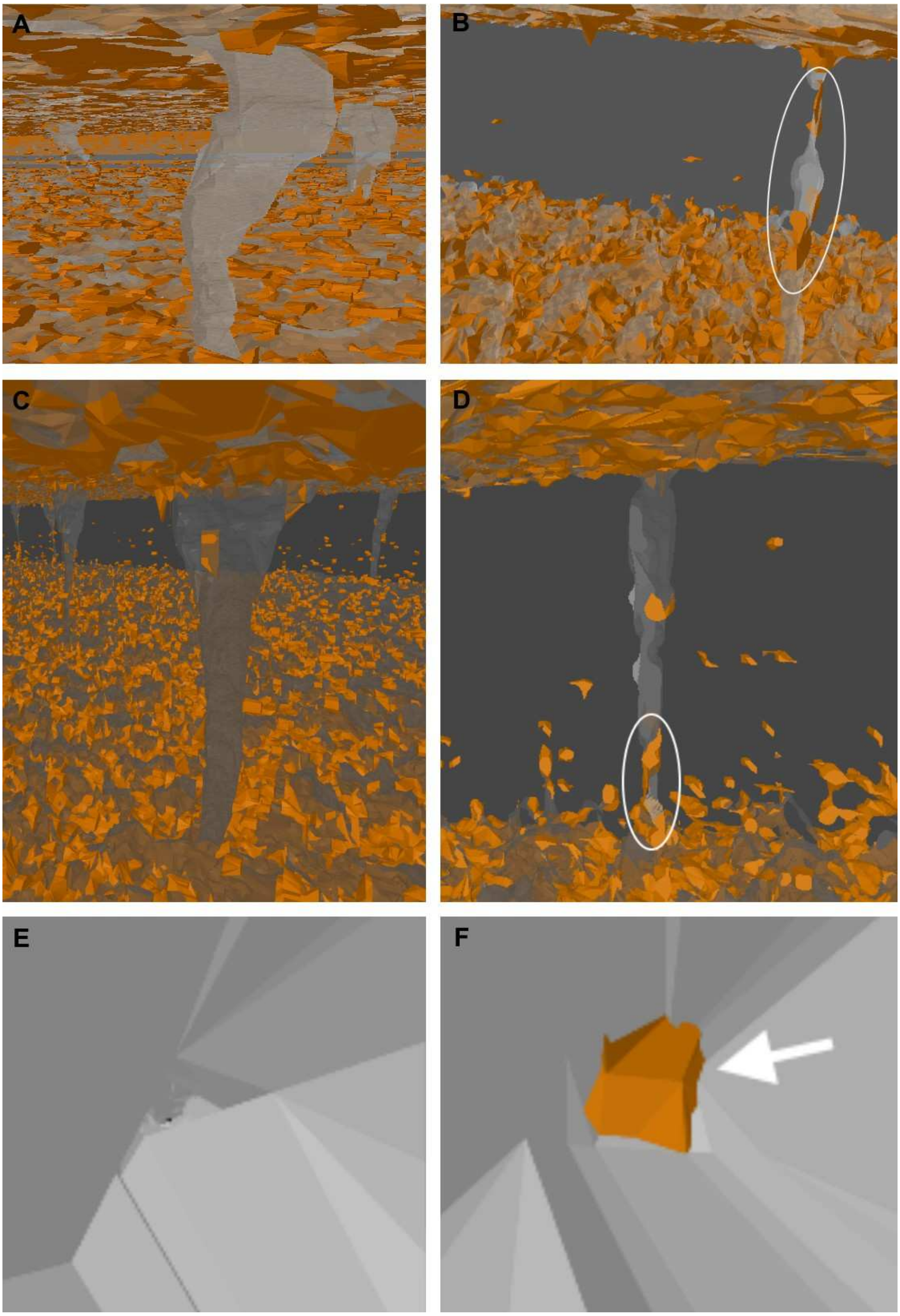

Figure S2. Examples of unblocked (A, C and E) and blocked (B, D and F) eggshell models, created from microCT data. The orange model represents the debris (and other organic matter like the shell membranes) and the translucent grey-white model represents the eggshell. The top two rows of images (A, B, C and D) show a cross section through the shell with the shell transparent and the pore channels (empty air space) visible in translucent grey. The top of the image is the exterior surface of the shell. The bottom two images (E and F) are the view looking down through a pore channel from near the exterior surface of the shell. The black dot in the middle of the E is the empty space on the other side of the pore channel (i.e. looking through the pore opening on the inner surface of the shell). The white circles and arrow highlight blockages within a pore channel caused by debris. All pores were checked for blockages both ways, but only pores that had a solid block i.e. no air spaces in the orange debris model (illustrated by the arrow) were considered blocked.

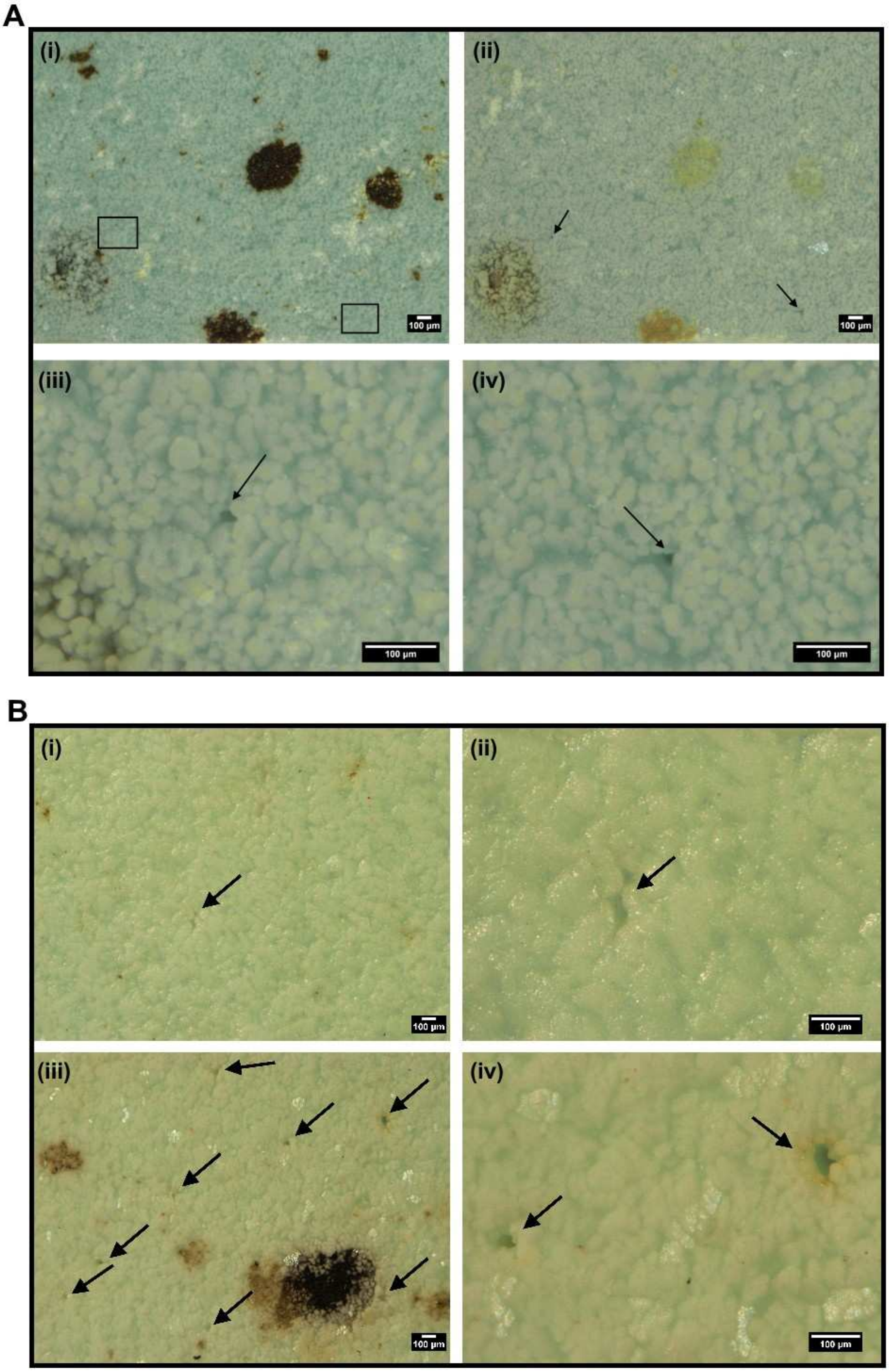

Figure S3. Removal of shell accessory material with bleach (A) and the natural variation in shell accessory material presence over pores between eggs (B).

A - (i) Untreated eggshell. Rectangles mark where two pores are that only become visible after treatment with bleach because they are covered in SAM. (ii) Eggshell treated with bleach. The SAM have been removed from the eggshell, and as a result, there is much more definition in the shell surface topography, pigment has been removed and pores (indicated with black arrows) are now visible because they are no longer covered in SAM. (iii) A higher magnification image of the open pore visible on the left hand side of top right image. (iv) A higher magnification image of the open pore visible on the right hand side of the top right image.

B - Images (i) and (ii) are from one of the eggs used in our study that showed a low proportion of blocked pores after debris application and (iii) and (iv) are from one of the eggs used that had the highest proportion of blocked pores after debris application. In images (i) and (ii), only one pore is clearly visible and it is covered in shell accessory materials (ii), whereas the pores in the other egg are not covered by shell accessory material (iii and iv), which may explain why this egg showed such a high proportion of blocked pores when debris was applied to the surface. All images were taken at a clean region of the equator of each egg and these imaging locations (i and iii) were haphazardly selected. Arrows indicate the location of visible pores.

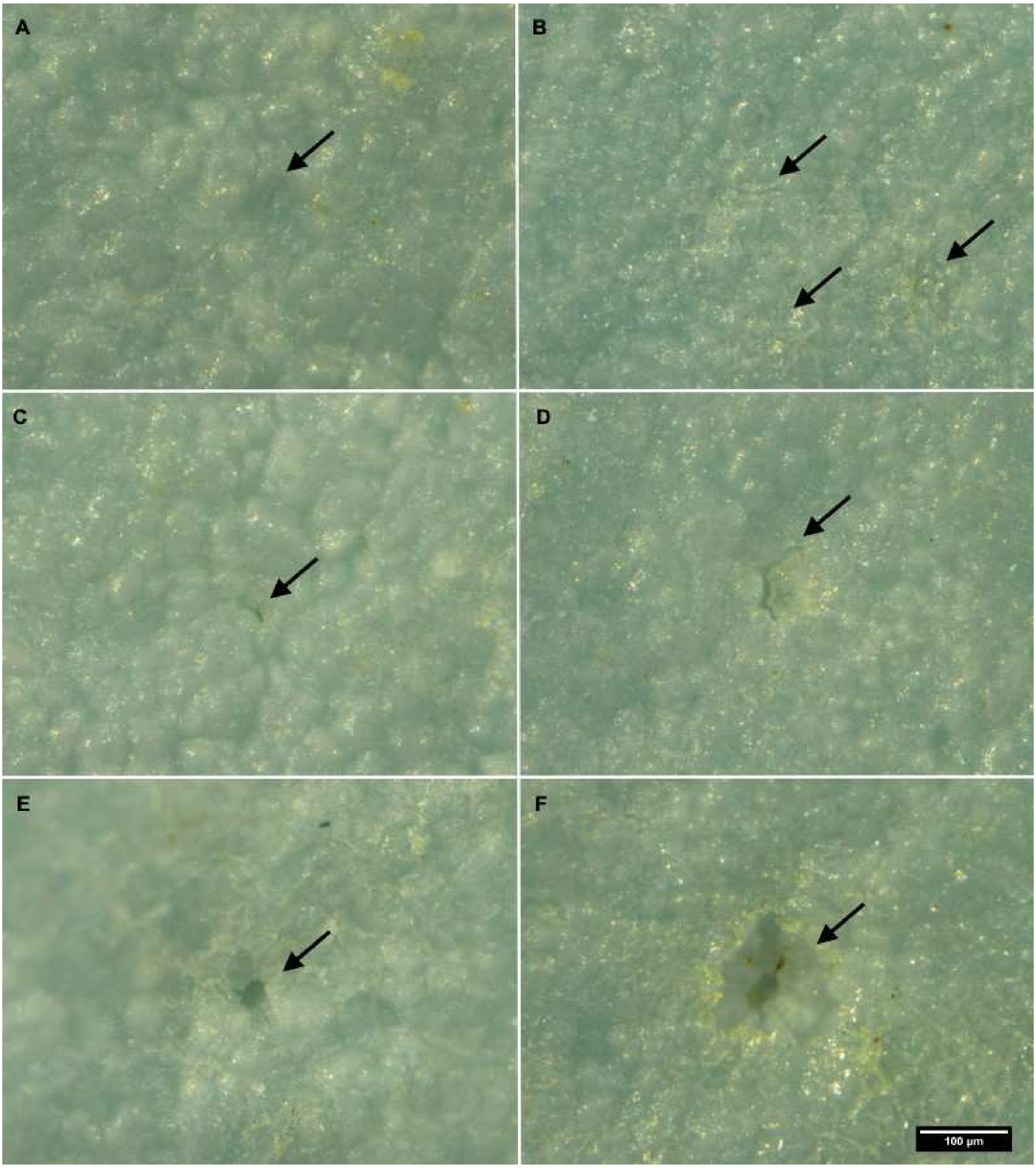

Figure S4. Natural variation in shell accessory material cover over pores. A - F show a sequence of pores starting with one that is fully covered in shell accessory material (A) to pores that have shell accessory material covering them but it is cracked to differing degrees (B-D), to pores that are open with the shell accessory material completely cracked or damaged meaning they are no longer covered (E-F). All images are from the same egg and are at the same scale – see scale bar on image F. Arrows indicate the location of visible pores.

Datasets

Below are datasets 1 and 2. These contain the data we collected and analysed in this paper. To access the data used for Table 1 please refer to the following reference:

Hoyt, D. F., Board, R. G., Rahn, H., and Paganelli, C. V. (1979). The eggs of the Anatidae: conductance, pore structure, and metabolism. *Physiological Zoology*. **52**, 438-450.

Dataset 1: The effect of debris on eggshell gas conductance and pore blockages.

ID	Clean gas conductance	Dirty gas conductance	Difference in conductance	Relative difference in conductance (%)	Pore number	Blocked pores (in channel)	Blocked pores (%)	Average trueshell thickness (μm)	Average pore length (μm)	Average thickness of debris (μm)	Average thickness of debris covering pores (μm)
G107	10.31098	10.55226	0.24128	2.34	13	3	23.08	445.249	389.342	299.312	315.299
G114	4.196583	4.768366	0.571783	13.62	11	2	18.18	413.796	351.176	218.746	155.243
G129	8.694998	7.435982	-1.259016	-14.48	12	4	33.33	384.065	324.896	179.077	155.838
G16	12.90546	9.1036	-3.80186	-29.46	32	23	71.88	425.195	376.768	473.303	470.233
G20	14.37053	10.52241	-3.84812	-26.78	40	28	70	400.731	351.007	263.407	261.079
G105	14.74378	14.22333	-0.52045	-3.53	24	13	54.17	386.198	330.678	249.206	224.340
G106	11.6527	10.32138	-1.33132	-11.42	37	14	37.84	347.584	302.236	633.628	695.597
G116	21.72172	20.22435	-1.49737	-6.89	52	26	50	408.248	361.531	198.325	207.693
G123	8.405391	6.660318	-1.745073	-20.76	39	23	58.97	440.979	357.482	221.920	264.848
G126	13.44856	7.803131	-5.645429	-41.98	35	22	62.86	360.403	326.294	301.522	268.721

N.B. Average trueshell thickness measures are not the same as average pore length values.

Dataset 2: The effect of shell accessory material removal with bleach on the percentage of pores blocked by debris in an eggshell fragment.

ID	Treatment	Blocked pores	Proportion of pores blocked	Blocked pores (%)
G107	Control	0	0	0
G107	SAM removal (Bleach)	6	0.40	40
G114	Control	2	0.133	13.3
G114	SAM removal (Bleach)	7	0.467	46.7
G129	Control	3	0.2	20
G129	SAM removal (Bleach)	7	0.467	46.7
GE2	Control	1	0.067	6.7
GE2	SAM removal (Bleach)	3	0.2	20
GE6	Control	3	0.2	20
GE6	SAM removal (Bleach)	5	0.333	33.3