



## Review: an embryo's eye view of avian eggshell pigmentation

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Current research into the evolution and adaptive function of avian eggshell pigmentation, including maculation, has focused mostly on signalling-based and structural function hypotheses but ignored the potential consequences of shell pigmentation for the developing avian embryo, especially in moderating the embryo's interaction with its light environment. The exposure of the eggs to sunlight that frequently accompanies avian incubation behaviour is one of the major evolutionary steps setting apart birds and reptiles, and coincides with the appearance of eggshell pigmentation. This suggests that shell pigments could play a major role in ensuring the successful development of the avian embryo. We propose that the effects of shell pigments on the egg contents should be considered in addition to established hypotheses of shell pigmentation such as crypsis, egg recognition or a possible structural function. This approach has the potential to identify trade-offs between different pigment functions and to resolve some of the long standing paradoxa in the evolution of eggshell colour, such as the occurrence of conspicuous blue eggs in passerines or the secondary evolution of white eggshells in cavity nesters. In particular, we identify seven hypotheses, which address how the interaction of eggshell pigments and the light environment may influence embryonic development. These hypotheses are the: thermo-regulation; UV-B protection; photo-acceleration; lateralization; circadian rhythm; photo-reactivation; and antimicrobial defence. We believe that the understanding of eggshell pigmentation will greatly benefit from taking these hypotheses into consideration when studying the functional significance of eggshell pigmentation and suggest a number of promising directions for future experimental and comparative research.

Amongst vertebrates the evolution of eggshell pigmentation seems to coincide with the emergence and diversification of birds, and this unique avian characteristic has given rise to a broad range of adaptive explanations (reviewed comprehensively by Underwood and Sealy 2002, Kilner 2006). The basic assumption of all these hypotheses is that the ancestral avian egg colour was white, as in reptile eggs (Schönwetter 1960–1992, Patent 1977, Kilner 2006). Egg pigmentation, both base colour and maculation, evolved then to provide a selective advantage through a signalling or possibly structural function (Underwood and Sealy 2002, Kilner 2006). Unlike most dinosaurs (Deeming 2006) and extant reptiles, birds (with the notable exception of megapodes, see Conclusions and perspectives) do not bury their eggs, but place them in nests that are accessible to parental incubation and usually also to sunlight (Deeming 2002a, Buschmann et al. 2006). Light intensity naturally varies from ca 1000–10 000 lux on overcast days to > 100 000 lux in direct sunlight (Janick 1972) and light intensity is reduced to 0.1–15% of these values under forest canopy (Shirley 1929). The temporary exposure to light means that the full spectrum of radiation may potentially reach the surface of the avian egg. Depending on the light filtering or intensifying quality of the nest

environment, e.g. beach or forest floor, eggs will experience light from the heating infra-red wavelengths to the potentially mutagenic ultra-violet light, with all its potentially negative effects. Consequently, a primary role of shell pigmentation in filtering light to protect the egg contents from harmful irradiation seems plausible and was indeed proposed as one of the earliest functions of eggshell pigmentation in birds (Mcaldowie 1886). However, given the paramount effect of nest predation on avian biology (reviewed by Lima 2009), Wallace (1889) at around the same time suggested that the primary determinant of avian eggshell colour was to hide the eggs from predators or parasites, and that other adaptations to the incubating micro-environment were secondary. Since then, the hypotheses exploring the signalling function of eggshell appearance to predators, parasites, mates and conspecifics have been the subject of intense research (Underwood and Creese 1976, Bakken et al. 1978, Moreno and Osorno 2003, Kilner 2006, Reynolds et al. 2009), with a recent interest in structural functions (Gosler et al. 2005, Higham and Gosler 2006, Lahti 2008).

The exact amount of light exposure of the eggs will vary greatly with the species-specific incubation constancy, the species' behaviours during incubation such as covering

unattended eggs with nest material, its nest type and location and its breeding latitude. Incubation constancy varies across avian orders between ca 45 and 100%, and with the mode of development (Deeming 2002b). Mean attentiveness is higher in species with precocial young (95.2%), than in species with altricial offspring (91.1%). Species with shared incubation show higher attentiveness (58–100%, mean 92.6%) than species with female only (45–100%, mean 76.8%) or male only (mean 89.9%) incubation (Deeming 2002b). Breeding latitude influences both the daily period of light exposure and the light intensity e.g. UV-light, owing to the varying angles at which sunlight passes through the earth's atmosphere, cloud cover and human induced particle pollution (Johnson et al. 1976, Wild 2009). Table 1 provides a general overview of the variation of eggshell pigmentation between avian orders with incubation constancy, nest type and latitude, which illustrates the scope for the modulation of the egg's light environment through structural or behavioural adaptations and identifies orders amenable to comparative research e.g. Charadriiformes with their variable systems of parental care and thus incubation constancy and their wide breeding range.

Only two pigments, protoporphyrin and biliverdin, appear to be involved in creating the remarkable intra- and interspecific diversity of avian eggshell coloration either individually or in combination (Kennedy and Vevers 1976, Gorchein et al. 2009). Both pigments are tetrapyrroles and are derived from the same precursor molecule, most likely haem (Wang et al. 2009), however they differ in their absorbance spectra. Protoporphyrin absorbance peaks are at 411 and 557.2 nm resulting in a brownish-red human visible colour (Poole 1965), while biliverdin absorbance maxima are at 375 and 665 nm producing a blue-green chroma to the human eye (Falchuk et al. 2002, Siefferman

et al. 2006). Both shell pigments occur in the calcite and the cuticular layer of the eggshell and are generally deposited during the latest stages of egg formation (Schönwetter 1960–1992, Poole 1965, Roberts 2004). In addition to the spectral qualities of the pigments, recently, attention has been drawn to a potential antimicrobial (Bulmer et al. 2008, Ishikawa et al. 2010) or structural function (Solomon 1991, Gosler et al. 2005).

For most species assessed, the colour seen on the inside of the egg, when the shell is lit from the outside, has been reported to correspond to the base colour visible at the egg surface (Schönwetter 1960–1992). However, no study has yet quantified the relationship between light reflectance from the shell surface and light transmission through the shell for the eggs of wild bird species. Similarly no data on the reduction of intensity the light experiences when passing through the shell and the layers of albumen, both decreasing during embryonic growth, are available.

In this review we aim to highlight the potential effects of eggshell pigmentation on embryonic development. Base-colour pigments are likely to control the light that reaches the embryo by blocking light of harmful wavelengths but admitting beneficial wavelengths. Circumstantial evidence supports this idea. Although the avian shell is made up of >95% calcite (Nys et al. 2004) the occlusion of light from the egg contents is usually not complete (Fig. 1), even in thick-shelled and pigmented eggs (Westerskov 1950). In addition eggs with black or at least very dark green pigmentation, as in the emu *Dromaius novaehollandiae*, or some *Chalcites* spp. cuckoos, are extremely rare (Eisenmann 1953, Schönwetter 1960–1992, Langmore et al. 2009). This observation suggests that the reflection and admission of light are both important aspects of avian eggshell pigmentation. A role in moderating the light that reaches the embryo may not be restricted to base-colour

Table 1. General overview of the variation of egg pigmentation following Schönwetter (1960–1992) and factors potentially influencing light exposure of the clutch across avian orders. Mean percent incubation constancy adapted from Deeming (2002b). Main breeding latitudes were derived from del Hoyo et al. (1992–2011) for each order and are defined by their variation in day length throughout the year as equatorial (E), with little variation, temperate (T) with distinct seasonal variation and polar (P) with near constant light in summer.

Order	Egg pigmentation	Mean% incubation constancy	Nest type	Breeding latitude
Struthioniformes	white	> 95	open	E
Rheiformes	pigmented	> 95	open	E, T
Casuariiformes	pigmented	> 95	open	E, T
Apterygiformes	white	> 90	cavity	T
Tinamiformes	pigmented	> 90	open	E
Sphenisciformes	white	> 95	open	P
Podicipediformes	white	> 85	open	E, T, P
Gaviiformes	pigmented, maculated	> 95	open	P
Procellariiformes	white	> 95	open or cavity	E, T, P
Pelecaniformes	white to pigmented	> 95	open or cavity	E, T, P
Ciconiformes	white to pigmented	> 90	open	E, T
Anseriformes	white or pigmented	> 85	open or cavity	E, T, P
Falconiformes	white to pigmented, maculated	> 95	open or cavity	E, T, P
Galliformes	white to pigmented, maculated	> 90	open	E, T, P
Gruiformes	pigmented, maculated	> 90	open	E, T
Charadriiformes	pigmented, maculated	> 90	open	E, T, P
Colombiformes	white	> 95	open or cavity	E, T
Strigiformes	white	> 95	open or cavity	E, T, P
Caprimulgiformes	pigmented, maculated	> 95	open or cavity	E, T
Apodiformes	white	> 70	open or cavity	E, T
Trogoniformes	white	> 85	cavity	E
Coraciiformes	white	> 95	cavity	E, T
Piciformes	white	> 85	cavity	E, T, P
Passeriformes	white to pigmented, maculated	> 75	open or cavity	E, T, P



Figure 1. Blown eggshells (from left to right) of stock dove *Columba oenas*, corncrake *Crex crex*, red-legged partridge *Alectoris rufa*, and lesser black-backed gull *Larus fuscus*, photographed in incandescent light (top panel) and in the dark, lit only by single red diode placed centrally inside each eggshell to demonstrate the intensity of light transmission through natural eggshells of thickness between ca 150  $\mu\text{m}$  in the corncrake and ca 350  $\mu\text{m}$  in the gull.

pigments, however. Maculation such as the widespread ‘halos’ or ‘wreaths’ around the blunt pole of the egg may create a specific light pattern inside the egg which could provide directional information to the embryo during its development or during hatching. To date no conceptual framework exists to explore the effects of shell pigmentation on the embryonic light environment. Below we highlight seven specific hypotheses that can guide future research into the evolution of avian eggshell pigmentation, which takes into account not only the egg environment but also the effect of pigmentation on the developing embryo.

One crucial assumption is common to all these hypotheses and will likely prove an intriguing and productive field of research in itself: the assumption that light reaches the embryo. This idea seems self-evident especially for open-nesters (Fig. 1). It requires, however, that 1) the nest is accessible to light, 2) the parents do not constantly cover the eggs with their own body or with nest-material during recess; and 3) that eggshell, membrane and albumen are all permeable to light. The extent, to which each of these conditions are met, has not been quantified for any species and the duration, timing, spectral composition and intensity of embryonic light exposure in the course of natural incubation all remain poorly understood. As detailed information on these aspects of the embryonic light environment emerge, the hypotheses presented below are likely to be refined and expanded.

### Thermo-regulation hypothesis

Most research into the effects of eggshell pigmentation on embryonic development in wild birds has focussed on pigmentation as a means to regulate the temperature of the egg contents by reflecting sunlight (Bakken et al. 1978, Bertram and Burger 1981, Westmoreland et al. 2007,

Lahti 2008, Magige et al. 2008). As bird eggs are more tolerant to lower than to higher temperatures (reviewed by Webb 1987), the main thermoregulatory function of pigments is proposed to be the prevention of overheating the egg contents rather than protection from cooling e.g. during incubation recesses by the participating parent. Generally, any eggshell pigmentation reduces the reflection of infra-red wavelength light compared to the light reflection of pure white eggshells and thus increases the risk of overheating the egg by irradiation (Bakken et al. 1978, Magige et al. 2008). However, the protoporphyrin and biliverdin pigments employed in eggshell coloration differ from the carotenoids and melanins found in other avian tissues by providing a higher near infra-red reflectance (Bakken et al. 1978) and thus a reduced risk of overheating the egg contents. It is interesting to note that the lack of melanin in the eggshell is not due to an inherent difficulty with melanin deposition in calcite structures. In fact, melanins are regularly deposited (alongside porphyrins) in the calcite shells of marine and terrestrial molluscs, where they too may have a thermoregulatory function (Comfort 1951). Eggshell pigmentation thus seems to be specifically designed to achieve its ecological function e.g. camouflage, without causing a catastrophic rise in egg temperatures (Bakken et al. 1978). However, the thermoregulatory cost of egg pigmentation is not universal. For instance, the eggs of three cup-nesting New World black-bird species (Icteridae) that differed markedly in pigment intensity, showed similar increases in temperature with light exposure (Westmoreland et al. 2007). This finding highlights the importance of the nest environment in regulating temperatures but also suggests an additional role of eggshell pigments (Westmoreland et al. 2007).

### UV-B protection hypothesis

Independent of heating the egg contents, sunlight could also harm the avian embryo through radiation in the UV-B (290-320 nm) spectrum, which can damage DNA (De Gruijl et al. 2001). If this damage occurs early on during embryonic development, the flawed DNA sequence will be replicated by mitosis throughout embryonic growth causing deformities or even leading to the death of the embryo. The equatorial position and upward exposure of the germinal spot in the egg (Mcaldowie 1886) might exacerbate this risk. Since UV damage is cumulative, the detrimental effects of UV-B radiation increase steadily with the duration of the exposure over the course of the incubation period. UV-B radiation is a ubiquitous environmental agent and accordingly living organisms have evolved effective adaptations to it (Thoma 1999, Ries et al. 2000, Blaustein and Belden 2003). One of these adaptations is to incorporate, in the light exposed tissue, pigments containing conjugated bonds, which absorb UV-B radiation. Protoporphyrin and biliverdin (but also melanins and carotenoids) perform this function and likely play an important role in protecting organisms from increased UV-B levels at the earth’s surface and potential tissue and DNA damage (Kerr and Mcelroy 1993, Blaustein et al. 1994, Madronich et al. 1998, Ries et al. 2000).

The calcite shell alone can block UV-B light only partially (Shafey et al. 2004) and the remaining UV-B transmission can have fatal consequences for embryonic development. For instance chick mortality in domestic fowl *Gallus gallus* increased, when eggs were exposed to UV-B radiation for longer than 3 h d<sup>-1</sup> (Veterany et al. 2004). To date, transmission of light below 400 nm through the eggshell has been studied experimentally only in chicken eggs (Shafey et al. 2004). The findings of this study support a UV-B protective function of shell pigmentation: UV transmittance was lowest in the eggs with the highest intensity of brown pigmentation. Only small differences in light transmission were found within eggs of homogenous pigmentation and these differences may be due to variation in pore density in different egg regions (Peebles and Brake 1986, Shafey et al. 2004, Bamelis et al. 2008).

The importance of the protective function of pigmentation is likely to increase with decreasing shell thickness, as at least in artificially manufactured calcium carbonate layers, thinner layers and those with lower crystal density, allow greater UV-light transmission (Larena and Villar 2001). Eggshell thickness in the majority of wild bird species (e.g. waders and passerines) is considerably lower than that of the domestic fowl (Schönwetter 1960-1992), putting the eggs and embryos of those species at a greater risk of UV-B damage. For these thin-shelled species UV-B-absorbent eggshell pigmentation, could play a key role in shielding the egg contents from harmful radiation and ensuring successful embryonic development.

Evidence that UV-B exposure acts as a selective pressure in the evolution of shell pigmentation in wild birds is provided by a study of two introduced populations of village weavers *Ploceus cucullatus* (Lahti 2008). The introductions led to the establishment of weaver populations in a habitat without their brood parasite, a cuckoo with mimetic eggs. As a consequence, intraspecific variation in egg pigmentation between the clutches of different females declined in the introduced weaver populations compared to the source, in <200 years (Lahti 2008). Instead, blue egg pigmentation has now become more prevalent and stronger across females of the introduced populations than it is in the source populations and this increase in blue coloration correlates with the intensity of solar radiation at the two introduction sites. Interestingly this effect occurred even though the closed weaver nests already provide protection from direct sunlight (Lahti 2008). In species where shell pigmentation is weak or lacking, its protective role may be taken by skin pigmentation (de Gruijl et al. 2001). This could, for example, provide a novel explanation for the jet black embryos and hatchlings of some tropical bird species (Maurer et al. 2010, Langmore et al. 2011).

### Photo-acceleration hypothesis

Extensive experimental research on captive bred poultry has established that exposure of the egg to incandescent or fluorescent light during incubation increases the speed of embryonic development, even when controlling for temperature. This suggests that light mediates developmental acceleration through (currently unknown) molecular

mechanisms e.g. improved protein transduction (Matsushita et al. 2004). No effects (either positive or negative), on the survival, size, or quality of the hatchling were found in species with either plain eggs, as in chickens and turkey *Meleagris gallopavo*, or with maculated eggs of Japanese quail *Coturnix japonica* and bobwhite *Colinus virginianus* (Shutze et al. 1962, Siegel et al. 1969, Walter and Voitle 1973, Coleman and McNab 1975, Ghatpande et al. 1995, Rozenboim et al. 2003, Archer et al. 2009). Eggshell pigmentation is likely to influence this photo-acceleration by moderating both the intensity and the wavelength of the light reaching the embryo (Shafey et al. 2004). However, little research has addressed light intensity (Ghatpande et al. 1995, Shafey et al. 2005) or wavelength specifically (Shutze et al. 1962, Lauber 1975, Shafey and Al-Mohsen 2002, Rozenboim et al. 2003, 2004) and only two studies assessed the effect of pigmentation on photo-acceleration of embryonic development directly (Coleman and McNab 1975, Shafey et al. 2005).

Light intensity can play an important role for the speed of development of the avian embryo and hatchability, at least in the domestic fowl (Ghatpande et al. 1995, Shafey et al. 2005). However, few conclusions can be drawn with regards to the optimal light intensity for embryonic development in the wild as the studies used continuous lighting and the results are ambiguous. In a study applying continuous white fluorescent light during the first 40 h of incubation the maximum acceleration of embryonic development was produced at intensities between 1500 and 3000 lux (Ghatpande et al. 1995). By contrast, in a comparative study of two light intensities of continuous green fluorescent light, similar light intensities of 1430 to 2080 lux had no effect on hatching time or chick weight. Surprisingly at these intensities green light even led to reduced hatchability when compared to eggs exposed to green light at lower intensities of 900 to 1380 lux (Shafey et al. 2005).

Two studies assessed the effect of shell pigmentation on embryonic growth directly using Japanese quail and domestic fowl eggs with different intensities of brown pigmentation (Coleman and McNab 1975, Shafey et al. 2005). Due to their variation of shell appearance in pigment intensity, but not spectral composition of the reflected light, the conclusions from these studies are restricted to the role of pigmentation in moderating the intensity rather than the wavelength of the light reaching the embryo. Generally lighter pigmentation seemed to facilitate photo-acceleration of embryonic development. Depigmented Japanese quail eggs, for instance, developed faster than pigmented eggs, when exposed to light and this effect was apparent from the first day of incubation (Coleman and McNab 1975). In chicken eggs of three different intensities in brown pigment, only the lightest-coloured eggs showed increased hatchability after exposure to green light for eggs laid by young chickens, while other growth and development parameters remained unaffected (Shafey et al. 2005). However, light of high intensities (1430–2080 lux) reduced hatchability for all but the darkest-coloured eggs. This effect of pigmentation was consistent, even though light transmission through the shell was small (Shafey and Al-Mohsen 2002, Shafey et al. 2004) and varied with shell porosity (Shafey et al. 2002).

A broader range of studies is available for the effect of wavelength, and green light (ca 545 nm) seems to be the most effective in stimulating embryonic growth and development (Lauber 1975, Shafey and Al-Mohsen 2002), and post-hatch growth (Rozenboim et al. 1999) in chickens. Green light exposure at the egg stage was also more effective than white light (and the no-light control), in creating a greater body weight in female but not male turkey embryos and young chicks (Rozenboim et al. 2003). Interestingly, the wavelength of greatest stimulation lies within the region of least absorbance and highest reflectance reported for the blue-green eggshell pigment biliverdin (Falchuk et al. 2002, Siefferman et al. 2006).

The findings in poultry research infer an important possible role of eggshell pigmentation on the embryonic development of wild birds and highlight promising areas for comparative and experimental research. Eggshell pigmentation could be selected to adjust both the intensity and the wavelength of the light reaching the embryo in order to achieve fast development and increased growth of the chicks. The implications for a species' breeding biology could be substantial. Faster development and growth of the entire clutch could decrease the risk of nest predation by reducing incubation and nestling periods. Interestingly, blue-green eggs are particularly common in small passerines (Lack 1958, Schönwetter 1960–1992), where nest predation is a substantial cause of reproductive failure (Bosque and Bosque 1995). Crucially, the internal colour shining through the shell generally corresponds to the external base-colour (Schönwetter 1960–1992). This opens up the intriguing possibility that biliverdin based pigmentation creates a light environment that accelerates the development of the avian embryo. Even if eggshell pigments do not generate a specific beneficial intensity or wavelength, they might still promote photo-acceleration by allowing light exposure in general, while blocking harmful infrared and UV wavelengths. Similarly, any benefits of light exposure for embryonic development may present an alternative explanation for the secondary evolution of unpigmented eggshells in cavity nesting species (Kilner 2006). White eggshells would allow what little light enters the cavity to easily reach the embryo, while simultaneously making eggs more detectable in the nest to the returning parent (Underwood and Sealy 2002) and avoiding the cost of pigment production, even though it seems to be negligible (Wang et al. 2009).

In addition to these whole clutch effects, pigmentation may also affect individual eggs differentially and could influence hatching asynchrony caused by incubation delays after the completion of the clutch (Magrath 1990). In gulls, for instance, the second egg (in *Larus delawarensis*) or third egg (in *Larus glaucescens*) of a three-egg clutch is regularly lighter than those of its siblings (Verbeek 1988, Hanley and Doucet 2009). This could accelerate the later chicks' development and allow for more synchronous hatching.

Finally, variation in eggshell pigmentation may facilitate adaptation to changing environmental conditions. Egg pigmentation has been shown to vary with rainfall, and eggs laid in wetter breeding seasons (when rainfall and cloud cover should reduce the light intensity) were generally lighter than those laid in drier years (Arendt 2004, Avilés et al. 2007). This change in egg colour could thus

compensate for the lack of sunlight and ensure that the embryo receives sufficient light exposure to maintain embryonic growth and development at similar rates in dry and in wet (or light and dark) years.

### Functional asymmetry and lateralization hypothesis

Light exposure does not only influence the speed of development in the avian embryo, it also plays an important role in establishing the functional asymmetry of the bird. Chicks hatched from eggs exposed to light performed better in a number of behavioural tasks than those incubated in the dark and showed a range of structural asymmetries in the brain (reviewed for domestic fowl by Rogers and Krebs 1996, Daisley et al. 2009). Four aspects of lateralization are of particular importance with respect to eggshell pigmentation.

- 1) For lateralization to occur, it is crucial that some light reaches the embryo. Light elicits a response in chicken embryos as early as three days into incubation (Rogers 1995) but it exerts its greatest influence on lateralization once the embryo has turned its head to the left side (thus occluding the left eye) in the final stages of embryonic development (day 17 onwards). Lateralization, as photo-acceleration, thus provides an intuitive explanation for the secondary development of white eggshells in cavity nesting species, especially those with strongly developed lateralization e.g. parrots (Rogers and Workman 1993).
- 2) Only minimal light exposure is required to establish this lateralization (Rogers 1982, Bobbo et al. 2002, Buschmann et al. 2006). Widespread natural incubation patterns with only short parental recesses (Skutch 1976, Deeming 2002b), would therefore be sufficient to generate the lateralization of the embryo.
- 3) The performance in behavioural tasks can be determined by the wavelength of the light the egg was exposed to during incubation (Rogers and Krebs 1996). Chicken hatchlings that received white light during their embryonic development were better able to distinguish between pebbles and seeds using their right eye, than those that were exposed to green light, while chickens that were exposed to red light or no light fared worse again.
- 4) Functional asymmetry is sex specific (Rogers et al. 1993, Daisley et al. 2009). Chickens are better at social tasks, for instance gauging a conspecific's reaction to a potential food source, when using their left eye (Daisley et al. 2009) and this effect is stronger in males than in females.

Light induced lateralization and in particular its relationship with the wavelength of the light to which the egg was exposed to during incubation has been addressed specifically only in studies on the white or brown-shelled eggs of domestic fowl (Rogers and Krebs 1996). Here the immense variability of egg pigmentation in wild birds, (e.g. Fig. 1, Cassey et al. 2009) provides an opportunity to explore the effect of eggshell colour on morphological and behavioural lateralization comparatively and experimentally.

## Circadian rhythm hypothesis

Day–night rhythms, mediated by high concentrations of the hormone melatonin at night and low levels by day, are a universal feature of living organisms (Schultz and Kay 2003). Birds have evolved one of the most complex circadian clocks in the animal kingdom to regulate this rhythm (Bell-Pedersen et al. 2005). The circadian rhythm in birds is established early on in the embryonic development, but unlike mammal embryos, which can simply synchronize with their mother's circadian clock via the maternal melatonin oscillation in the shared bloodstream, avian embryos require an external cue or 'zeitgeber' to establish this rhythm (Mcnabb et al. 1998, Nichelmann et al. 1999, Zeman et al. 1999). The most important of these zeitgebers for the avian embryo is the regular change between light exposure and darkness, which eggs experience during recess and contact periods of incubation (Zeman et al. 1999, Bell-Pedersen et al. 2005). Other rhythmic changes occurring during incubation, e.g. the presence or absence of the parental heartbeat or temperature cycles, by contrast seem to have little impact on the embryo's circadian rhythm (Gwinner et al. 1997, Nichelmann et al. 1999). Melatonin production starts early on in the incubation period, in chicken embryos from day 10 of incubation onwards (Mcnabb et al. 1998). A day night rhythm of melatonin production is established after 16 d of the 21 d incubation period in the precocial chicken (Chabot and Menaker 1986) and one day before hatching in the altricial, cavity-nesting European starling *Sturnus vulgaris* (Zeman and Gwinner 1993).

The importance of light exposure for the synchronization of embryonic development with the photoperiod implies a crucial role of eggshell pigmentation in the establishment of a circadian clock of pre- and peri-natal chicks of both precocial and altricial species (Zeman and Gwinner 1993, Zeman et al. 1999). Specifically, shell pigmentation must not completely occlude light from the egg as the circadian rhythm established in the embryo determines the timing of hatching and the hatchlings day–night rhythm until at least three days after hatching (Zeman and Gwinner 1993, Zeman et al. 1999).

In vitro experiments on chicken embryo pineal gland cell cultures clarify the potential role of eggshell pigmentation in the regulation of circadian rhythms. Chicken pinealocytes, the brain cells which release melatonin, were very sensitive to light with an intensity of as little 10 lux and showed a circadian activity pattern (Faluhelyi and Csernus 2007). Furthermore, embryonic pineal gland cells responded most to light of blue wavelengths (Csernus et al. 1999, Csernus and Mess 2003), as would be expected from the peak sensitivity of pinopsin (470 nm), the light sensitive molecule in the avian pineal gland (Okano et al. 1994). In addition, very short bouts of light (1 h) are sufficient to inhibit melatonin production in hatchling Japanese quail and starling (Zeman and Gwinner 1993). Starling chicks are a particularly instructive example as they fully develop melatonin oscillation under the low light conditions (10 lux) of their cavity nests and a natural incubation regime (Gwinner et al. 1997). The light blue-coloured eggshell, which should admit light of the most stimulating wavelength freely, possibly aids this process

(Schönwetter 1960–1992, Okano et al. 1994) and may provide yet another selective advantage explaining the evolution of white or pale blue coloured eggs in a cavity nester (Kilner 2006). It appears reasonable to assume that, despite obvious similarities, the mechanisms underlying the circadian clock in adults and embryos are not identical. Only additional research on developing embryos can reveal to what extent their circadian clock is influenced by light and how differences in eggshell pigmentation modulate this influence by filtering light.

## DNA repair through photo-reactivation

The avian embryo could benefit directly from light exposure through the process of photo-reactivation, i.e. the light-dependent repair of DNA lesions caused by UV-B exposure (Thoma 1999). Intriguingly, photo-reactivation has been found in species with external reproduction, namely amphibians, reptiles and birds, but has not yet been discovered in any mammal (Thoma 1999). Eggshell pigmentation functioning as a light filter could play an important role in this process. While UV-B radiation is damaging to DNA, light at 350–400 nm (UV-A) is instrumental in the repair of UV-B induced damage to the DNA (cis-syn pyrimidine dimers and pyrimidine-pyrimidone photoproducts) by delivering the energy for the photolyase repair process (Thoma 1999). Ideally eggshell pigmentation should therefore block the harmful UV-B radiation but let the beneficial wavelengths of 350–400 nm pass, so that the damage to the developing embryo's DNA can be minimised. In vitro experiments on chicken embryo fibroblasts show maximum repair through photo-reactivation after exposure to light of 365 nm wavelength at 10 Joule m<sup>-2</sup> for only 30 min (Van De Merwe and Bronk 1981). Interestingly, more dimers were repaired if cells were kept in the dark prior to the treatment with 365 nm wavelength light (Van De Merwe and Bronk 1981), possibly because some damaged sites are inaccessible to photolyase immediately after exposure to damaging UV-B radiation (Pendry 1983). The incubation pattern of most birds that alternates periods of coverage and exposure of the clutch (Skutch 1976, Deeming 2002b) readily matches this rhythm of the repair function of photolyase. Future work would greatly benefit from analysing the light transmission through differently pigmented eggshells of open nesting wild bird species in the damaging and beneficial UV wavelengths between 280–350 nm and 350–400 nm, respectively.

## Antimicrobial defence hypothesis

Finally, light exposure may play a role in the defence of the egg contents against microbial infections via the shell surface through three different mechanisms, all of which depend on or are facilitated by eggshell pigmentation: 1) exposure to UV-radiation which kills bacteria and fungi (Fargues et al. 1997, Davies-Colley et al. 1999, Chavez et al. 2002); 2) photodynamic antimicrobial activity of shell pigments (Ishikawa et al. 2010); 3) keeping the eggshell dry and at a constant temperature e.g. through safe exposure to sunlight (Bruce and Drysdale 1994, Messens et al. 2005). These three mechanisms complement each

other to generate an effective barrier to microbial infections across the shell.

UV-B radiation has both a fungicidal and a bactericidal function (Fargues et al. 1997, Davies-Colley et al. 1999). Commercial eggshell treatments use UV-C light for sanitation, which does not naturally reach the earth's surface and a detailed analysis of the effectiveness of UV-B light for antimicrobial treatments has therefore not yet been explored, but could of course be of considerable importance for naturally incubated eggs (Scott 1993, Kuo et al. 1997, Chavez et al. 2002). The safe application of UV-B radiation to the clutches of wild birds relies on the capacity of the eggshell to protect the embryo from the harmful effects of UV-B through pigmentation (see above). This need to finely balance UV-B exposure is demonstrated by the dosage-dependent effects of UV exposure in chicken eggs. Short UV-B exposure ( $1-2 \text{ h d}^{-1}$ ) throughout incubation led to increased hatching success and embryonic development compared to eggs incubated in darkness, but longer bouts of UV-B exposure caused increased mortality (Veterany et al. 2004).

The photodynamic antibacterial activity of eggshell pigments is more specific in its antibacterial function than UV-B (Ishikawa et al. 2010). Protoporphyrin can reduce the survival of gram positive bacteria to  $< 0.01\%$  but only, if the pigment is activated by light. Ishikawa et al. (2010) used the fluorescent light of an incubator for this purpose so the spectral sensitivity of this effect still needs to be explored. Gram-negative bacteria, on the other hand, were unaffected by protoporphyrins. Bacterial infections across the eggshell pose a considerable mortality risk to the developing embryo (Cook et al. 2003, 2005), especially before the onset of incubation (Cook et al. 2005). In species, where incubation starts with the last egg only, females could use pigmentation with antibacterial activity to protect earlier laid eggs from bacterial infections. These earlier laid eggs should then show greater protoporphyrin deposits, and thus greater antibacterial defence, than last laid eggs. The darker colour of earlier laid eggs observed in gulls and waders (Hockey 1982, Verbeek 1988), could serve to protect the embryo through its longer environmental exposure. In addition, maculation may coincide with structural variation of the shell surface e.g. indentations (Gosler et al. 2005, Maurer et al. 2011) which could provide shelter for bacterial growth. Increased protoporphyrin concentration as suggested by dark speckling may help fend off infections at these locations. For antibacterial albumen proteins, the second line of defence, differential deposition has already been shown, although it did not reflect the laying order (D'alba et al. 2010).

The gram-negative bacteria unaffected by protoporphyrin, are susceptible to desiccation (Ishikawa et al. 2010) and simple exposure to sunlight that ensures a dry and warm egg surface may be sufficient to reduce their survival. Egg pigments can facilitate this exposure by blocking or reflecting UV and infrared light (see above) or by providing camouflage (Underwood and Sealy 2002). The dryness of the egg surface is particularly important as bacteria can be drawn into the eggshell much more easily when suspended in moisture (Bruce and Drysdale 1994, Messens et al. 2005). Eggshell pigmentation can further aid this process as darker shells should heat up more quickly than

reflective, pure white eggshells. Alternatively, pigmentation could change the surface of the eggshell to prevent structurally the adhesion of water, similar to the lotus effect (Gu et al. 2003). Finally, it remains of great interest how the unpigmented eggs of grebes, which are covered with wet and rotting vegetation during incubation recess achieve protection from bacterial infection.

## Conclusions and perspectives

We have provided an embryo's eye view of avian eggshell pigmentation which produces a number of easily testable hypotheses for the evolution of the diversity of shell pigmentation in birds (Fig. 2) and highlights the need for a better understanding of the specific light environment of avian embryos in natural incubation events. In isolation these hypotheses are unlikely to explain fully, the evolution of the diverse colours and patterns of shell pigmentation observed in wild birds. Furthermore potential phylogenetic constraints either on pigment deposition or the interaction of light with the developing embryo need to be taken into account. For instance, these constraints may help explain the retention of the presumed ancestral state of white eggshells in some large families with species nesting in very different light environments such as hummingbirds or pigeons (Kilner 2006). Instead, the different functions highlighted in this review are likely to overlap with the established signalling-based or structural functions (Under-

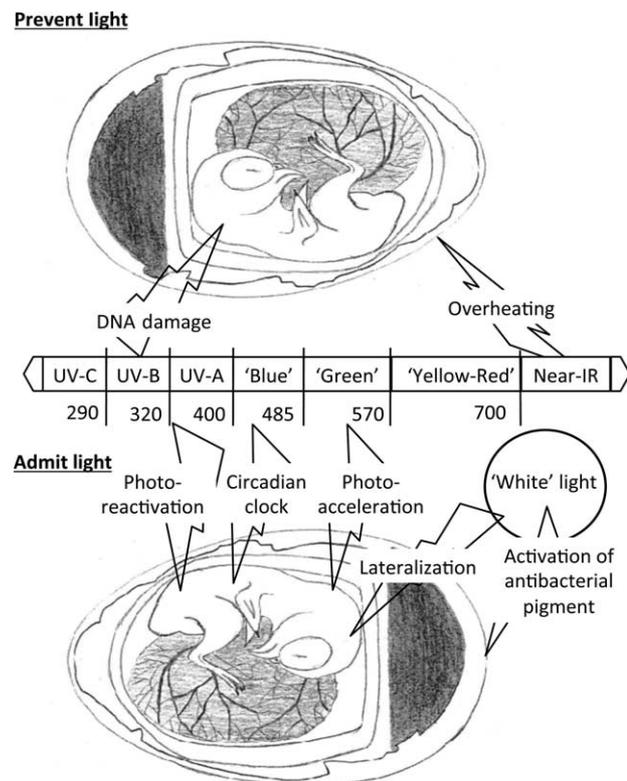


Figure 2. The potential dual role of eggshell pigmentation selectively in preventing and allowing light of distinct wavelengths to reach the avian embryo in order to create a beneficial light environment for its development.

wood and Sealy 2002, Gosler et al. 2005, Kilner 2006) and with each other. As a consequence we should expect that in most species the expressed pigmentation of the eggshell is the result of a tradeoff between different adaptive functions, some of which will benefit the embryo directly, as the ones proposed here, while others benefit the egg, the clutch or the parent. We propose that such a multifaceted approach to shell pigmentation is essential to explain shell appearance, where one-dimensional explanations struggle, for example in clarifying the adaptive function of blue shell pigmentation (Lack 1958, Reynolds et al. 2009) or the secondary evolution of white eggs in cavity nesters (Underwood and Sealy 2002, Kilner 2006).

In summary, we strongly believe that a more inclusive approach to eggshell appearance is needed to clarify the ecological function and evolution of avian eggshell pigmentation. Given the central role of the egg in the reproductive cycle of birds, this knowledge will allow us to gain a deeper understanding of the intricate adaptations of wild birds to their specific environment and their nest-habitat. We suggest that five areas should take priority in future research on the role of shell pigmentation in embryonic development:

First, it is important to quantify the light transmittance spectra and light intensities inside the eggshells across a range of eggshells with different pigmentation and porosity. To date we know that both pigmentation and eggshell porosity or thickness or both influence light transmission, at least in domestic fowl eggs (Shafey et al. 2002, 2004), but their respective contributions are unclear. The methodologies to measure light transmission through the shell, porosity (as water vapour conductance) and eggshell thickness are in place (Green 2000, Shafey et al. 2004, Portugal et al. 2010), but data on wild bird eggs are missing.

Second, recent advances in electronics and in particular the arrival of inexpensive, wavelength-specific miniature diodes, allows a suite of experiments to be conducted in the controlled light environment of a nest box (Gwinner et al. 1997). These experiments could manipulate, the wavelength duration and timing of light exposure to mimic different incubation scenarios or geographic locations. Such studies on the effects of the exposure of eggs to light of different intensity and wavelengths could clarify the adaptive significance of eggshell structure and pigmentation for embryonic development.

Thirdly, comparative and experimental research on the effects of light exposure on eggs in open cup nests can reveal the role of eggshell pigmentation in the interaction between the embryo and its light environment e.g. the levels of UV-B irradiation or rainfall (Avilés et al. 2007, Lahti 2008). The human induced changes in the structure of most bird habitats and the ongoing or proposed alteration of the light spectrum on the earth's surface (Madronich et al. 1998, Keith et al. 2010) makes this an important area of research with respect to bio-monitoring and conservation.

Fourthly, megapodes (Megapodiidae) differ from all other bird species in their incubation behaviour. Like many reptiles, megapodes bury their eggs in mounds of soil or rotting vegetation, where they develop under the exclusion of light (unlikely to occur even in very dark pigmented eggs like those of *Chalcites* cuckoos) and without the egg turning essential for most bird eggs (Booth and Thompson

1991, Deeming 1991). Their fully independent chicks hatch from an unspeckled cream or pink coloured egg (Schönwetter 1960–1992), after an exceptionally long and variable incubation period that can last for 50–90 d in the malleefowl *Leipoa ocellata* (Booth 1987). This unusual form of incubation requires significant modifications to the standard avian developmental processes (Booth and Thompson 1991), and as a consequence research on megapode embryonic development promises to greatly advance our understanding of avian reproduction (Jones and Birks 1992, Jones 1999). As such the experimental investigation of the effects of light exposure on the developmental speed and quality of megapode embryos clearly forms an exciting field of research.

Finally, research on poultry species has played an important role in advancing our understanding of eggshell pigmentation, but has so far been restricted mainly to the domestic fowl (Shafey et al. 2004). Future studies, using tightly controlled light intensities and wavelengths and applying natural patterns of illumination should try to take advantage of the diversity of poultry species and their various natural and mutated eggshell pigmentation (Ito et al. 1993). For instance, no study has yet compared the effect of light on blue-shelled vs brown-shelled eggs of the domestic fowl (Wang et al. 2009). A broader view of eggshell pigmentation could influence a more efficient production and use of eggs in the poultry and pharmaceutical industries (Tree et al. 2001, Clavijo et al. 2002).

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