Temperature, humidity, and vegetation density affect eggshell pigmentation across a continent.

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DECLARATION

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This thesis is written in the form of a journal article from *The Royal Society Open Science.*

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TABLE OF CONTENTS

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ABSTRACT

The distinctive evolutionary shift from white reptilian eggs to pigmented eggs in a majority of avian species, has stimulated much thought and research, but has yet to be completely explained. Pigments in eggs may serve a variety of structural or signalling functions, which are not mutually exclusive. This research focuses on the variability of Australian Magpie (*Cracticus Tibicen*) eggs, and the possible drivers of such variability. Using a dataset of 283 clutches from the Australian continent we used a range of methodologies to assess the degree of inter-clutch variation in egg pigmentation in relation to genetic divergence, environmental factors, and brood parasitism. We found little evidence for divergence between subspecies, however the Tasmanian subspecies did differ significantly from most of the others. The analysis of environmental parameters revealed that maximum temperature and the interactions between maximum temperature, relative humidity and leaf area cover explained variation for background colour patterning. The presence of a brood parasite in about one third of the magpie's distribution was related to a small degree, not significantly so, to the variation in colour and patterning. The results from our study add to the body of evidence that environmental drivers have an impact on pigment deposition.

KEYWORDS: Antimicrobial, brood parasite, egg colouration, maculation, signalling, solar radiation, structural.

INTRODUCTION

Laying eggs is the chief means by which animals achieve the twin requirements of developing zygotes – the provision of food and protection. Birds, however, have a gone a step further than most, by additionally adorning their eggs with a remarkable diversity of colour and patterns. For example, the colour of eggs can vary from green/blue, through white, to pink or brown, and can be decorated with black or brown speckles, streaks or blotches distributed locally or across the egg (1). This variation in base colour and patterning (patterning hereafter referred to as maculation) arise from deposition of varying concentrations of two pigments - protoporphyrin and biliverdin, with brown colouration stemming primarily from the deposition of the former and blue/green resulting from the latter (2). Because, ancestrally, avian eggs are thought to have been white (3), variation in colour and maculation is likely to have evolved in response to one or more selective pressures (4). Hypotheses that advocate such pressures broadly fall into structural or signalling approaches (reviewed in 3,5). The suggested structural functions of pigmentation include egg strengthening, shock absorption, protection against extremes in temperature and solar radiation, as well as protection against microbes (6–8). By contrast, suggested signalling functions include the use of colour to camouflage eggs from predators (9,10), to signal female quality (11–13), and as a marker enabling parents to distinguish their eggs from those laid by brood parasites (14). There is significant support for each of these hypotheses, but these mostly originate from small-scale studies within a single geographic location or population. Considering a species across its whole range can add valuable insight that may be easily overlooked by studies at the smaller scale. For example, it would allow consideration of: genetic variation within a species, parasitised and unparasitised populations and higher variation in many of the environmental parameters that are thought to be significant. Importantly, selection acts across the full range of a species and not necessarily just within the bounds of a small population, or at a scale detectable in a small study system. The environmental heterogeneity across a species range will lead to important environmental parameter variability (e.g. minerals, climate). Differences in these parameters may have significant impacts on the species, for example, the accessibility to primary resources to produce strong and healthy eggs.

The primary constituent of an avian eggshell is calcium (in the form of calcium carbonate) (15). Food is the main source of calcium for egg formation, and when calcium

sources are low, birds have been found to forage on mortar, grit and digested rodent bones found in raptor pellets. The protoporphyrin pigment (in the form of maculation) has been found to be more prominent on eggshells when there is significant lack of calcium in the environment (7). This compensation is due to protoporphyrin's shock absorbing structure within the shell matrix, reminiscent of solid-state lubricants used in engineering (6). The intensity of maculation has been shown to correlate with thinner sections of the eggs and to increase when calcium in soil is limited (16,17). Soil calcium has been demonstrated to have links to nutrient availability by reflecting the amount of calcium in seed and invertebrates in that area, thus if an area is found to have high soil calcium, birds should be getting proportionate amounts through their normal diet (18). This allows us to use soil calcium as a proxy to diet-derived calcium availability (19).

An alternative structural property of egg pigmentation is its effects on temperature regulation and solar radiation. Although bird embryos are relatively tolerant to ecologically colder temperatures (reviewed in 20), for most embryos of passerine species, prolonged exposure to temperatures over 40.5°C is lethal (21). In the solar radiation hypothesis, egg colouration is suggested to serve a role in protecting the contents of eggs from overheating and solar irradiation (22–24). Recent research has revealed that protoporphyrin and biliverdin can reflect near infra-red radiation allowing better temperature control and reduced water loss (8,16). Additionally to heating egg contents, direct sunlight can damage DNA through radiation in the UV-B spectrum (290-320nm), resulting in the potential death of the embryo (8,25). Protoporphyrin and biliverdin act as an efficient adaptation for eggshells exposed to harmful radiation, due to their conjugating bonds that can absorb UV-B radiation. Research thus far has only performed experimental studies on poultry eggs and found that UV transmittance through the shell was lower in browner eggs (26).

Under the bacterial defence hypothesis, the pigments in avian eggs have evolved as a natural defence against bacterial infection. Indeed protoporphyrin has been shown to have anti-microbial properties (27). Eggshell pigmentation works alongside light exposure to keep embryos safe from microbial infections across the shell (reviewed by 8) the combination of utilising UV radiation to kill bacteria and keeping the eggs at a safe temperature is reliant on the photodynamic antimicrobial properties of shell pigments (8). For example, when brown, blue and white eggs were exposed to light, the brown eggshells were much more effective at killing bacteria. (27). Warm and humid environments are often home to higher proportions of microbes and bacteria, and in locations at risk of high microbial infection, selection may favour

the use of protoporphyrin due to these anti-microbial properties (8). While blue eggshells were not as effective as brown, they were more efficient at killing bacteria than white eggshells. The antimicrobial effect of all eggshell colours was only apparent in light conditions with natural UV radiation, indicating the necessity for light to trigger the pigments bacteria-killing chemical cascade (27).

Another component of the environment for a bird is the presence or absence of brood parasites. A particularly popular hypothesis is that variation in egg colour and maculation is driven by selection on distinguishing one's own egg from a parasite's, e.g. cuckoos (3,5,28,29). An evolutionary arms race occurs between cuckoos and their hosts due to the high cost of brood parasitism. Cuckoos have evolved methods to deceive hosts into rearing their young and in turn, hosts are expected to evolve new and improved defences against brood parasites (30). Egg colouration and maculation can be used by parasites to mislead hosts (31). However, hosts are also under selection to spot and reject odd looking eggs and may also shift the colour or patterning of their eggs away from that of their brood parasite (14). Indeed, this evolutionary mechanism is seen in introduced populations of birds where brood parasites are absent, the selection for heavy pigment deposition, intra-clutch variation and maculation significantly relaxed (32). Interspecific differences in colour and patterning of eggs have been shown to be a relatively labile trait (3), which cannot be used as a reliable trait for systematic ordering due to the strong adaptive and functional roles of pigments (8,18). It is likely that at least some divergence across species may reflect changes that occurred as subspecies were formed, as is the case for variation in traits such as plumage and song, and therefore in considering variation across broad geographical scale, it is pertinent to consider the possibility that subspecies may vary significantly from one another.

While evidence exists for each of the hypotheses outlined above, most tests have typically examined a single population or a relatively limited geographical area that precludes tests of all hypotheses (13,17,33–37). Consequently, we have a relatively limited appreciation of the relative importance of these hypotheses on large geographic scales with a broad range of climatic conditions. Fewer studies have attempted to look at multiple theories simultaneously, and yet that approach can potentially evaluate the relative significance of adaptive explanations for the variation in patterning and colouration that is frequently observed across and within a species. Comparing the relative importance of each factor is challenging due to the covariation in many of the variables, and it could be argued that previous patterns found might be the result

of confounds. For example it would be expected for climatic variables such as temperature and humidity to covary and perhaps others such as calcium will also covary across the range.

Here we attempt to evaluate egg colouration and maculation in the Australian magpie (*Cracticus tibicen*). This species has a high degree of variation in egg appearance and we use museum collections of clutches collected from known locations throughout their ca. 5 million km^2 range encompassing most of the Australian continent (See Figure 1). This range encapsulates eight sub-species, as well as extremes in climate, and corresponding variation in habitat from tropical and temperate rainforest through woodland, grassland and desert. Further, four of the subspecies are parasitised by the Channel-billed cuckoo (*Scythrops novaehollandiae*). Thus, throughout their range, Australian magpies experience significant variation in all key parameters outlined in the hypotheses above, namely: temperature and solar radiation, conditions for microbial presence, calcium availability, and the incidence of brood parasitism.

We consequently have two main aims. First, we quantify the variation in colour and maculation of eggs collected across the substantial range of the Australian magpie and investigate whether any of the variation can be explained at the subspecies level. Second, we then go on to test the hypotheses outlined below:

- The solar radiation hypothesis proposes that eggs will be browner in hot areas with high solar radiation. This combination is most common in the semi-arid and arid biomes such as the grasslands and deserts.
- By contrast, the antimicrobial defence hypothesis predicts that areas with likely higher microbial abundance (i.e. for example, the humid areas of the tropics), will have more brown and blue eggs (rather than white).
- The structural integrity hypothesis predicts that eggs should have a greater concentration of protoporphyrin in the form of maculation and possibly base colour in areas of low soil calcium. When calcium sources are low, eggs are thinner and weaker and hence will be reflected through additional strengthening with increased protoporphyrin.
- Finally, the brood parasitism hypothesis predicts that the four subspecies in the current range of the channel-billed cuckoo will show greater variance in egg colouration and maculation between females.

MATERIALS AND METHODS

Egg samples

We visited the Victoria Museum in Melbourne and the Australian National Wildlife Collection in Canberra to access historical egg specimens. Both collections were housed in the dry vertebrates' collections, in dark storage cabinets. The collection dates of the egg specimens ranged from 1862 to 1999. Maculation scores, standardised photographs and spectral measurements were taken for 283 Australian magpie clutches. The Australian magpie has eight subspecies and we sampled clutches for all subspecies (*Cracticus t. eylandtensis* n= 14, *Cracticus t. longirostris* n= 9, *Cracticus t. dorsalis* n= 34, *Cracticus t. hypoleuca* n= 28, *Cracticus t. tyrannica* n= 62, *Cracticus t. terraereginae* n = 58, *Cracticus t. tibicen* n=60, *Cracticus t. leuconota* n= 18). A distribution map extracted from Hanzab (38) and the Atlas of Living Australia (39) was digitised using Arc GIS (40) and locations of each clutch was extracted and cross-checked against the subspecies allocated by the museum labelling. In cases where the subspecies name was missing or incorrect, it was rectified using this process. Museum accession numbers for each clutch as well as geographical and taxonomic data are reported in SM Table 1.

Photography

Clutches were photographed in a standardised 40cm x 40cm studio photography light cube tent, on a specially designed egg holding surface. This surface was mounted with a DKC -PRO Digital Kolor Kard (colour reference card to process white balance and colour reflectance in the photographs) using a Canon 7D with a Sigma 18-250mm Lens (settings of 1/125, F10, ISO 100 in RAW format) in Victoria and a Canon Eos 7d with Canon Macro EfS 60mm ultrasonic focal lens in Canberra. All pictures were taken 30cm from the surface of the eggs (optimal distance for digital extraction) (41) using a tripod and remote photo launcher for stability.

Reflectance measures

Avian vision differs to ours, and our analysis needed to account for both visual signals available to birds and the physical properties that result from different reflectance and

absorbance properties. For this reason, colour analysis was performed with the use of spectrometry. The use of spectrometry in animal colouration studies has become widespread and readily adopted; this method generates a reflectance spectrum of the surface measured, is objective, repeatable and can be used in a variety of analyses (9). With the spectrometer, we were specifically interested in measuring the background colouration of the egg rather than the maculation on top of that background. In the Victoria Museum, every egg in a clutch was measured for colour, whereas in ANWC just one egg per clutch was measured. To measure background colour measurements were taken from the pointed end of the egg, the median line of the egg (central part), and the base of the egg, avoiding heavily maculated areas. Spectral measurements were all taken by a single observer in each collection (LON in Victoria and SCG in ANWC). All clutches were processed for eggshell reflectance using a USB2000 + Miniature Fiber Optic spectrophotometer (Ocean Optics Inc., Dunedin, FL, USA), a xenon light source PX-2 (Ocean Optics Inc.) with a fibre-optic cable held at a 90° angle to the shells' surface and reflectance data were recorded using the AvaSoft 7 program (Avantes, Eerbeek, Netherlands). Measures were taken in dark conditions to avoid any interference from artificial lighting, and reflectance measures were taken relative to a white reflectance standard.

Maculation scoring

To assess the variation in maculation, the type of pattern on each egg was allocated from four categories, "spots", "lines", "blotches" and "mottle" and in cases where more than one type of pattern featured, the most prominent one was chosen and recorded. Maculation was then recorded using the 'IDS' system, a previously published method developed to assess an average score of patterning in Great tit (*Parus major*) eggs (7,16,18). The categorical scoring system comprised of scoring the maculation size (whereby 1= "small", 2= "medium", 3= "Large"); intensity (1= "faint", 2= "pale", 3= "medium", 4= "intense"); and distribution (the approximate percentage of markings in one half of the egg, whereby 1 = >90%, 2= 75-90%, 3= 50-75%, 4= <50%). Some slight modifications were made to the method to match the pattern types of magpie eggs, as seen in Figure 2. All eggs were assessed by a single observer (KLL). The subjective nature of the IDS system led it to being considered less representative of maculation than the digital image analysis (see below), and was thus not used for the main analysis, however, results can be found in SM Tables 3-6.

Digital Image analysis

All the photographs were processed using an automated image-processing tool SpotEgg to provide a non- subjective analysis of patterning (42). This program was used with MATLAB ver. 2012b and scored information in the pictures about egg maculation (number, size, distribution and shape of spots), colouration (red/ green/ blue colour space, referred to as RGB hereafter) and egg dimension. The SpotEgg tool allows for configuration settings by species, using a representative egg to automatically detect pattern data on the remaining images. Due to the highly variable maculation across the total sample of eggs, the images were manually separated into three distinct classes of patterning, defined as "spots", "blotches" and "clear", and each of these three categories were scored separately using SpotEgg, with a different configuration for each (details in SM Table 2). This allowed the tool to run as systematically as possible, allowing over or under detection of pattern, rather than altering the configuration setting for each clutch independently. Due to the restraint of the RGB data not accounting for avian photoreceptors, it was not included in the main analysis, however results can be found in SM Tables 3-6. The total area of maculation of the eggs' surface area was the main variable retained from this technique for our analysis.

Environmental Data

To assess the relationship between the environmental variables and variability in egg colouration and maculation, across the Australian continent, we calculated maximum temperature (T_{max}, °C), relative humidity (%), and calcium (pH tests) at a 1° x 1° grid cell resolution (~100km x 100km). Average annual T_{max} (0.05° x 0.05° grid cell resolution), and humidity (0.1° x 0.1° grid cell resolution) were downloaded from the Australian Water Availability Project (43) via http://www.bom.gov.au and resampled to 1° x 1° resolution using the raster package (44). T_{max} data were annual averages based on standard 30 year climatology (between 1961-1990) and relative humidity data were average annual humidity at 3pm from 1976-2005 (45) .

Calcium levels in the soil were extracted from the Soil and Landscape grid of Australia in the form of 0-5cm deep pH tests ($CaCl_2$, 3" resolution) carried out throughout Australia (46), and resampled to a 1° x 1° grid cell resolution. Leaf Area Index (LAI) measurements for 16-day intervals during the period February 2000 to 2016 were obtained via the TERN AusCover portal and were produced from tiles originally downloaded from USGS (47,48), and were averaged across the whole period. The LAI represents the amount of vegetation in an area in

relation to bare ground (49) and in areas with lower values of LAI, nests are more likely to be exposed to direct sunlight. These data were also aggregated to a 1° x 1° grid cell resolution. Maps of the environmental variables with corrected resolution can be found in Figure 3.

Statistical Analyses

All analyses were carried out in R 3.3.2 and R Studio version 1.0.136 (50). In addition to base functions we used the packages *maptools* (51), *raster* (44), and *visreg* (52) for data extraction, manipulation, and visualization. Statistical tests were considered significant at an alpha-level of $p \leq 0.05$.

To assess if the background colour varied in relation to our hypotheses, we carried out the analysis with the use of the *pavo* package in R. This tool allowed us to organise, visualise and analyse spectral readings (53). Using this package, the readings were aggregated into a single value per egg. The processing functions within the package allowed the aggregated spectra to be loess-smoothed by a factor of 0.05 (see (53) for further details). A model was created based on different quantum catches at each photoreceptor for avian vision (54) (using avg.uv for average avian UV system). This model utilised the D65 "standard daylight" background illuminant as Australian magpies are open cup nesters. The quantum catch outputs from this model were assessed through principal component analysis (PCA), and the first and second principal components were used in further analyses (PC1 and PC2 hereafter). Due to the zero-sum constraint, the PCA technique built into the *robComposition* package was used (55), which performs an isometric log-ratio transformation. Analysis of variance (ANOVA) and equality of variance (Levenes Test, package *car*) (56) were run in between background colour against subspecies to assess the degree of variation. Due to subspecies being a categorical variable, we used a Tukey's pair-wise comparison, from the package "*multcomp*" (57), to identify any significant differences between subspecies.

PC1 and PC2 were analysed against environmental data to determine if the components were spatially autocorrelated. The effect of positive spatial autocorrelation would result in similar values clustering together in a map, and with negative autocorrelation, dissimilar values would cluster together (58). This was performed by calculating Moran's *I* statistic with the package *spdep* (59,60) and spatial autocorrelation was not detected.

To test our hypotheses we used mixed-effect models using the package *lme4* (61). The packages *lmerTest* (62) and *MuMIn* (63) were used to calculate p-values and r^2 values,

respectively. Before testing these hypotheses, we performed an ordinary least squares (OLS) regression model to test if background egg colour varied with the age of eggs, because it has been suggested that the long-term storage of eggs in the museum may reduce the reflectance in comparison to fresh eggs (34,64). We found no relationship between background colour and the age of eggs (p= 0.58) thus age was dropped from all subsequent analysis. Additionally we used OLS regression followed by a Tukey's pair-wise comparison, performed using "*multcomp*" (57), to test if there was significant difference in background colour based on the individual who took spectrometry measures. We found no significant difference (p= 0.31) thus it was dropped from further analyses. Clutch ID was retained as the only random variable in each model to control for the multiple eggs sampled from each female. Our response variables consisted of the spectroscopy outputs (Background colour as PC1) and the SpotEgg output (Total Area of maculation).

Linear mixed-effect models were used to investigate if the brown pigment may be used for its anti-bacterial properties in warm and humid locations. We fitted our models with an interaction between temperature and relative humidity as a fixed effect and clutch identity as a random effect. To test if background colour or maculation act as a protection against solar radiation, we used the maximum temperature and amount of leaf cover for each location. Absorbance and reflectance properties of pigments would be expected to change on the basis of how hot and exposed to sunlight they are. We fitted a model with the interaction between LAI and T_{max} as fixed effects and clutch identity as a random effect. To test if a genetic variation of subspecies is driving differences in egg colouration and maculation, we fitted our models with subspecies as a fixed effect and clutch identity as a random effect. To assess if background and maculation may reflect calcium availability, we fitted a mixed-effect model with calcium as a fixed effect and clutch identity as a random effect. The effect of parasitism on egg colouration was assessed by fitting a mixed-effect model with presence of parasitism as a fixed effect and clutch identity as a random effect.

RESULTS

Background Colour

We looked at variation in background colour of wavelengths 300-700nm. This represents both the ultra-violet and visible sections of the spectrum. The two prominent reflectance peaks were both in the visible spectrum at 500nm and 630nm, differing notably from each other and these curves represent the blue and brown pigment respectively, as shown in Figure 4. The PC1 and PC2 components explained 69% and 29% of the variance respectively and 98% of the variation in background colour across all samples. PC1 was negatively related to the variation in wavelength, whilst PC2 ran orthogonally to that variation. Higher PC1 values were associated with blue reflectance curves (Biliverdin pigment) and lower values corresponded to brown reflectance curves (Protoporphyrin) as shown in Figure 5. There was significant variation in PC1 across sub-species (One-way ANOVA $F_{7, 622}= 11.5$, $p< 0.001$, $R^2= 0.11$) and PC2 (One-way ANOVA $F_{7, 622}= 8.71$, $p< 0.001$, $R^2= 0.088$). The Tukey's pairwise comparison indicated that *C. Hypoleuca* (Tasmanian subspecies) significantly differed to five of the other seven subspecies (see Table 1,2). The variance of homogeneity test (Levene's test) showed no significant difference in the variation in background colour across all subspecies (PC1: $F_{7, 622}= 1.79$, $p= 0.087$; PC2: $F_{7, 722}= 1.064$, $p= 0.38$).

Maculation

The maculation scores produced by SpotEgg indicated that the average area of maculation on our Australian magpie eggs was 40.23 % (±22.73) of the total surface. There was significant variation in maculation scores from SpotEgg across sub-species (Kruskal-wallis chi-squared test= 23.62 df= 8, p <0.01). Whilst there were significant differences between the subspecies, the overlap between them does not allow the discrimination between them on the basis of patterning. The Dunn post hoc test indicated similar results, as seen in SM Table 7.

Genetic Hypothesis

The Tukey's pairwise comparison indicated that background colour and maculation in *C. Hypoleuca* (Tasmanian subspecies) significantly differed to five of the other seven subspecies (see Table 1-3). The remaining six subspecies however all varied as much as each other.

Solar radiation Hypothesis

We found evidence that T_{max} has an influence on background colour ($F_{1,268.45}$=13.416, $p< 0.05$), explaining 5% of the variation (See Figure 6). Eggs were laid with more protoporphyrin (brown pigment) as temperature increased. 77% of the variation however was explained when including Clutch ID, suggesting it explains much of the variation. LAI and the interaction between LAI and T_{max} did not influence the for background colour ($p= 0.63$, $p= 0.76$ respectively), as shown in Table 4a. The total area of maculation significantly decreased with increased LAI ($F_{1,262.74}= 0.17$, $p< 0.001$), maculation also decreased with higher T_{max} ($F_{1,272.77}= 2.65$, $p< 0.01$), and increased with the interaction of higher LAI and T_{max} ($F_{1.352.64}= 8.34$, $p< 0.01$), as shown in Table 4b. The fixed variables (LAI and T_{max}) explained 3% of the variation but including the random variable (clutch ID) explained 85% of the variation.

Bacterial Hypothesis

Background colour was not impacted by an increase in T_{max} or relative humidity ($p= 0.6$). However, total area of maculation, as calculated from SpotEgg significantly increased with the interaction of T_{max} and relative humidity ($F_{1, 351.29}= 4.47$, $p> 0.05$). Although the results were significant, only 2% of the variation in maculation was explained by these climatic variables while the model including the random factor (clutch ID) explained 83%, as shown in Table 5a,b.

Calcium Hypothesis

We found no evidence that background or maculation could be used as a correlate of calcium availability ($p= 0.49$, $p= 0.27$ respectively), as shown in Table 6 a,b.

Parasitism hypothesis

Our results indicated that there was a trend for background colour to be bluer in subspecies in the range of the cuckoo, however it was not statistically significant (p= 0.08). Maculation showed no trend between subspecies within the range of the cuckoo and those outside its range (p= 0.83). Results are presented in Table 7 a,b.

DISCUSSION

We used an extensive comparative dataset of environmental factors and egg features to investigate the variation in Australian magpie egg colouration and maculation. We found that whilst subspecies differed from one another, only *C. Hypoleuca* (Tasmanian subspecies) significantly differed from five of the seven other subspecies. We found that higher temperature significantly affected both background colour and maculation; increased T_{max} leads to browner eggs (lower PC1), and a decrease in maculation. We found a significant decrease in maculation in areas with increased leaf cover and the interaction between LAI and T_{max} lead to an increase in maculation. These results offer support for an environmental effect on egg colouration and maculation. There was no significant pattern between egg colouration or maculation with changes in soil calcium concentration. There was a trend for bluer eggs in parasitised populations, although this was not statistically significant. There was a very clear result that the random effect of clutch ID explained a huge amount of the variation. This is presumably due to the fact that eggs within the same clutch were highly similar to one another and markedly different between clutches (and likely different females).

Our results indicate that in conditions of higher maximum temperatures, the background colour of Australian magpie eggs is browner. Related research has suggested that in moderate light environments where sunlight filters through vegetation, survival can be helped by blue pigments due to their ability to absorb and reflect solar radiation rather than transmitting it to the interior of the egg, and the sensitive developing embryo (24) . Lahti (24) found Village weavers that had escaped the brood parasite selection pressures through relocation, laid increasingly more blue-green coloured eggs when exposed to higher sunlight levels. While our results differ, the conditions are dissimilar. The weavers, by contrast to the magpie have an enclosed nest and eggs are not exposed to direct sunshine. In magpies, the only

protection between the embryo and direct sunlight are the shell, incubating females, and any leaf cover. Whilst magpies do place their nests among branches and leaves, they typically nest fairly high in a tree and almost certainly their nests will be exposed to the sun throughout the day. One possibility for future research would be to explore the possibility that both brown and blue pigments in the eggshell are effective at protecting the embryos from solar radiation. To do this, another measure of colour could be used whereby we measure the distance from white. This would tell us whether eggs in hot climates have a higher amount of pigments than those in cooler places, rather than looking the continuum from blue to brown as we currently have.

Maculation results indicate that with higher leaf cover and temperatures, egg maculation covers a larger surface area of the eggs. Very little is known about the physical advantages of increased maculation. One possibility would be that the maculation, formed of the protoporphyrin (brown) pigment plays a significant role in embryo protection in hot and shaded areas. Hot and shaded areas in particular biomes such as the tropics may be relatively humid, which in turn may increase the bacterial presence within the nests (65), maculation increases in areas of high temperature and relative humidity. Previous research on the anti-bacterial properties of protoporphyrin pigments has thus far focused on background colour, not maculation, however this could be a new avenue for research that may prove to be fruitful. Ishakawa et al. (27) investigated bacterial survival on different coloured eggs and found that when exposed to light (as most natural eggs would be other than cavity nesters), brown pigments had the most effective anti-bacterial properties. By finding an increase of maculation in hot and humid areas, our results may suggest that spotting, alongside background colour, could serve an antimicrobial function. It has been suggested that ground-nesting birds, whose eggs tend to make use of a lot of protoporphyrin, may be linked to the higher density of microbial activity in ground-dwelling birds, and higher susceptibility to bacterial infestation (1). Thus, our results could lend some support to the hypothesis that brown pigmentation is used in part as a photodynamic antibacterial defence against UV-B radiation (26,27), however they only explain a marginal proportion of the variation across a continent.

We found no evidence that calcium content in the soil was reflected in maculation load on the eggs of the Australian magpie. Studies by Gosler (7,16,17) found that in his study site (Whytham woods, UK), the amount of maculation could be used as a correlate of calcium content in the soil. Differences in our study may be down to the difference in sheer size of the study side. The rich soils in a European wood contrast to the arid and homogenous soils of the Australian continent. Indeed, there was a 415-fold range of variation in soil calcium within

Wytham woods; while an effective study site it is challenging to replicate the conditions elsewhere (18). Additionally, it is possible that soil contents are not the best representation of calcium intake from Australian magpie, due to their diet being omnivorous and perhaps ingesting much higher proportions of calcium through feeding on a variety of carrion as well as vertebrate and invertebrate prey (66).

It is unclear why so much variation occurs within Australian magpie subspecies. Whilst the *C. Hypoleuca* differed, it is found in Tasmania so this difference may be related to the Bass Strait acting as a geographical barrier between Tasmania and the mainland; indeed, there are many other species that show extensive divergence across the Bass Strait. The extensive polymorphism within the remainder of sub-species on the mainland, however, remains unexplained. Studies have successfully shown that higher deposition of pigments (in the blue-green chroma) can indicate females' capacity to control free radicals despite the cost of removing this antioxidant from their body (67), and in turn can lead to higher paternal care (11,68). The intensity of blue pigments has been demonstrated to reflect the proportion of antibodies deposited in the yolk, suggesting that egg colour can be used to predict female and chick condition (12). Although we cannot shed light on this matter, further research on the possibility of colour being used as a correlate of female condition would be of interest. This would be of particular interest for magpie, as they are facultative cooperative breeders, gaining maximal help is of importance (69). Whether different colours reflect condition of females or are linked to their genetics have very different consequences on the way colours may vary and how selection will act on them. Our results indicated that clutch ID explained an extensive proportion of the variation. We neglected to take into consideration that the similarities between eggs in a single clutch and differences between clutches would drive such explanatory power, and it can be assumed that it is driven by the female identity. Collias (70) demonstrated that egg background was best explained by Mendelian inheritance of two autosomal loci and both parents of the laying female contribute to the phenotype of the next generation.

The geographic distribution of the Australian magpie and channel-billed cuckoo allowed us to perform a comparative analysis in between parasitised and non-parasitised subspecies. Our results suggest that parasitised subspecies tend to use more blue backgrounds than those that are unparasitised, yet our statistical result was above the significant threshold of 0.05 (p= 0.08), and the effect size was very small. A great deal of research looking at brood parasitism has shown that the presence of parasites is a major factor leading to increased egg variation within species (3,5). A reason for the intra-specific variation in parasite hosts is to

allow females to discriminate between her eggs and those of the parasite (71). This has been well supported in a variety of comparative studies of parasitised allopatric and sympatric populations (32,72–74).The primary host of the channel-billed cuckoo is the Pied currawong (*Strepera graculina*) (75) and as shown in SM Table 8, their eggs and those of the cuckoo (sampled visually in ANWC collection) seem very similar (brown background with brown maculation). However, whilst the cuckoo and host eggs we sampled were largely brown we do not really know the frequency of brown or blue eggs in the cuckoo, and of course the eggs held in the museum collection may be a non-random sample as presumably, close matching eggs could easily be overlooked. The trend for Australian magpies to use biliverdin could be associated with an adaptive response and discriminative tactic to differ from the channel-billed cuckoos brown and maculated eggs. Theoretical analyses have suggested that the evolutionary arms race between host and parasite will come to the stable conclusion once polymorphic eggs are produced by host or parasite alike (76).

Our environmental datasets and egg collection characteristics are extensive, however, like every study there are drawbacks. The historical nature of the egg specimen did result in having to dilute the resolution of our environmental variable to 100km x 100km scale. This resolution loss can be considered as a shortcoming for variables such as leaf area cover or calcium due to the weaker variation in these variables, and thus the possible loss of accuracy. For variables such as temperature, this dilution in resolution is less of an issue; temperature over such a scale on average will not fluctuate excessively, and the general deficiency of micro-climates around Australia allows us to decrease the spatial resolution. Some parameters in the analyses were non-significant, which may be explained by the reduced resolution. It is possible that such a variable needs be utilised on a more refined scale. It must be noted that the level of correlation between environmental traits makes looking at related hypotheses challenging. The main reason is the covariation in many of them, which makes such tests difficult to do and would require thousands of samples to adequately parse out any differences. In fact, studies undertaken on variables such as calcium and climate are often highly correlated and therefore it is possible previous patterns might be the result of the kind of confounds that we ourselves could do nothing about. Lastly, the present day distribution of the channel-billed cuckoo is well quantified in terms of scientific literature and citizen science (for example 39). However, this distribution may have changed over evolutionary time, which could affect the outcome of the parasitism analysis.

CONCLUSION

These results give us an insight into the complexity of pigment use. This research has asked why there is so much disparity in colouration and patterning of avian eggs, and by looking at a single species over a broad distribution, has attempted to narrow down the causal drivers of such differences. By looking over a wide area we show how correlated different variables can be, and how this could be a problem for previous studies considering just one or two of them as well as something to consider in future research. Our main finding is that even though there is huge variation in temperature, relative humidity, calcium content and leaf cover, across the Australian continent they explain very little of the extensive phenotypic variation in this species. This may suggest that perhaps these factors may be somewhat overrated and we are missing other crucial factors or that we still don't really understand why some species have huge amounts of polymorphism in egg colour and patterning. This study has not only improved our knowledge about this species and the causes of continent-wide egg variation but aided the progress on the eternal question of why avian eggs have evolved to be so variable

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TABLES

TABLE 1: Tukey multiple comparisons of means between Principal Component 1 (PC1) background colour values and subspecies of Australian magpies (*C. Tibicen*). Eight subspecies and 283 clutches were samples from preserved museum samples. Significance is indicated by asterisks. Name in bold are those that differed most frequently.
95% family wise confidence level.

Subspecies			Estimate	Std. Error	Z value	Pr(>|z|)	
Longirostris	-	**Hypoleuca**	-1.16 $^{e-01}$	2.60^{e-02}	-4.472	<0.001	***
Hypoleuca	-	Dorsalis	7.69^{e-02}	1.90^{e-02}	4.043	0.0013	**
Tyrannica	-	Longirostris	9.31 $^{e-02}$	2.37^{e-02}	3.925	0.002	**
Tyrannica	-	Dorsalis	5.39^{e-02}	1.58^{e-02}	3.41	0.014	*
Hypoleuca	-	Eylandtensis	7.36^{e-02}	2.39^{e-02}	3.073	0.04	*
Leuconota	-	**Hypoleuca**	-7.69^{e-02}	2.29^{e-02}	-3.351	0.017	*
Tibicen	-	**Hypoleuca**	-5.97^{e-02}	1.74^{e-02}	-3.431	0.013	*
Terraereginae	-	Longirostris	7.65 $^{e-02}$	2.28^{e-02}	3.358	0.016	*
Eylandtensis	-	Dorsalis	3.32^{e-03}	2.30^{e-02}	0.14	1.00	
Leuconota	-	Dorsalis	1.57^{e-05}	2.20^{e-02}	0.001	1.00	
Longirostris	-	Dorsalis	-3.92^{e-02}	2.51^{e-02}	-1.56	0.76	
Terraereginae	-	Dorsalis	3.73^{e-02}	1.58^{e-02}	2.35	0.25	
Tibicen	-	Dorsalis	1.72^{e-02}	1.61^{e-02}	1.065	0.96	
Leuconota	-	Eylandtensis	-3.30^{e-03}	2.63^{e-02}	-0.12	1.00	
Longirostris	-	Eylandtensis	-4.25 $^{e-02}$	2.90^{e-02}	-1.46	0.81	
Terraereginae	-	Eylandtensis	3.40 $^{e-02}$	2.15^{e-02}	1.58	0.75	
Tibicen	-	Eylandtensis	1.38 $^{e-02}$	2.17^{e-02}	0.64	1.00	
Tyrannica	-	Eylandtensis	5.06 $^{e-02}$	2.15^{e-02}	2.35	0.25	
Terraereginae	-	Hypoleuca	-3.96^{e-02}	1.72^{e-02}	-2.307	0.27	
Tyrannica	-	Hypoleuca	-2.30^{e-02}	1.72^{e-02}	-1.341	0.87	
Longirostris	-	Leuconota	-3.92^{e-02}	2.82^{e-02}	-1.39	0.85	
Terraereginae	-	Leuconota	3.73 $^{e-02}$	2.04^{e-02}	1.828	0.58	
Tibicen	-	Leuconota	1.71^{e-02}	2.06^{e-02}	0.832	0.9	
Tyrannica	-	Leuconota	5.39 $^{e-02}$	2.04^{e-02}	2.645	0.13	

Tibicen	-	Longirostris	5.63 $^{e-02}$	2.39^{e-02}	2.356	0.25
Tibicen	-	Terraereginae	-2.01^{e-02}	1.39^{e-02}	-1.451	0.82
Tyrannica	-	Terraereginae	1.66 $^{e-02}$	1.35^{e-02}	1.227	0.92
Tyrannica	-	Tibicen	3.67^{e-02}	1.38^{e-02}	2.654	0.13

TABLE 2: Tukey multiple comparisons of means between Total area of maculation values (from SpotEgg) and subspecies of Australian magpies (*C. Tibicen*). Eight subspecies and 283 clutches were samples from preserved museum samples. Significance is indicated by asterisks. Name in bold are those that differed most frequently.
95% family wise confidence level.

Subspecies			Estimate	Std. Error	Z value	Pr(>|z|)	
Longirostris	-	**Hypoleuca**	-1.16^{e-01}	2.60^{e-02}	-4.47	<0.001	***
Hypoleuca	-	Dorsalis	7.69 $^{e-02}$	1.90^{e-02}	4.043	0.0012	**
Tyrannica	-	Longirostris	9.31 $^{e-02}$	2.37^{e-02}	3.92	0.002	**
Tyrannica	-	Dorsalis	5.39 $^{e-02}$	1.58^{e-02}	3.41	0.013	*
Tibicen	-	**Hypoleuca**	-5.97^{e-02}	1.74^{e-02}	-3.43	0.013	*
Leuconota	-	**Hypoleuca**	-7.69^{e-02}	2.29^{e-02}	-3.35	0.017	*
Hypoleuca	-	Eylandtensis	7.36 $^{e-02}$	2.39^{e-02}	3.073	0.041	*
Terraereginae	-	Longirostris	7.65 $^{e-02}$	2.28^{e-02}	3.36	0.016	*
Eylandtensis	-	Dorsalis	3.32 $^{e-03}$	2.30^{e-02}	0.14	1.00	
Leuconota	-	Dorsalis	1.57 $^{e-05}$	2.20^{e-02}	0.001	1.00	
Longirostris	-	Dorsalis	-3.92^{e-02}	2.51^{e-02}	-1.56	0.76	
Terraereginae	-	Dorsalis	3.73 $^{e-02}$	1.58^{e-02}	2.35	0.25	
Tibicen	-	Dorsalis	1.72 $^{e-02}$	1.61^{e-02}	1.065	0.96	
Leuconota	-	Eylandtensis	-3.30^{e-03}	2.63^{e-02}	-0.12	1.00	
Longirostris	-	Eylandtensis	-4.25^{e-02}	2.90^{e-02}	-1.46	0.81	
Terraereginae	-	Eylandtensis	3.40 $^{e-02}$	2.15^{e-02}	1.58	0.75	
Tibicen	-	Eylandtensis	1.38 $^{e-02}$	2.17^{e-02}	0.64	1.00	
Tyrannica	-	Eylandtensis	5.06 $^{e-02}$	2.15^{e-02}	2.35	0.25	
Terraereginae	-	Hypoleuca	-3.96^{e-02}	1.72^{e-02}	-2.31	0.27	
Tyrannica	-	Hypoleuca	-2.30^{e-02}	1.72^{e-02}	-1.34	0.87	
Longirostris	-	Leuconota	-3.92^{e-02}	2.82^{e-02}	-1.39	0.85	

Terraereginae	-	Leuconota	3.73 $^{e-02}$	2.04^{e-02}	1.83	0.58
Tibicen	-	Leuconota	1.71 $^{e-02}$	2.06^{e-02}	0.83	0.99
Tyrannica	-	Leuconota	5.39 $^{e-02}$	2.04^{e-02}	2.64	0.13
Tibicen	-	Longirostris	5.63 $^{e-02}$	2.39^{e-02}	2.36	0.25
Tibicen	-	Terraereginae	-2.01^{e-02}	1.39^{e-02}	-1.45	0.82
Tyrannica	-	Terraereginae	1.66 $^{e-02}$	1.35^{e-02}	1.23	0.92
Tyrannica	-	Tibicen	3.67 $^{e-02}$	1.38^{e-02}	2.65	0.13

TABLE 3: Results of mixed effect model whereby **a)** looks at the explanatory power of different subspecies of Australian magpies on background colour using Principal component 1 (PC1) and **b)** looks at the effect of subspecies on maculation scores from SpotEgg. Eight subspecies and 283 clutches were samples from preserved museum samples. The R^2m reports the R^2 of the model with just fixed effects while the R^2c reports the R^2 of the full model including random variables. Significance is indicated by asterisks.

SUBSPECIES HYPOTHESIS

a) Background colour PC1~ Subspecies

	Estimate	SE	df	t	Pr(>|t|)	
Intercept	-3.30 $^{e-02}$	1.26 $^{e-02}$	2.47 $^{e+02}$	-2.621	0.0093	**
Eylandtensis	3.32 $^{e-03}$	2.3 $^{e-02}$	2.44 $^{e+02}$	0.14	0.88	
Hypoleuca	7.69 $^{e-02}$	1.90 $^{e-02}$	2.59 $^{e+02}$	4.043	6.97^{e-05}	***
Leuconota	1.56 $^{e-05}$	2.2 $^{e-02}$	2.84 $^{e+02}$	0.001	1.0	
Longirostris	-3.92 $^{e-02}$	2.51 $^{e-02}$	3.65 $^{e+02}$	-1.561	0.12	
Terraereginae	3.73 $^{e-02}$	1.58 $^{e-02}$	2.57 $^{e+02}$	2.35	0.019	*
Tibicen	1.71 $^{e-02}$	1.61 $^{e-02}$	2.$^{53\ e+02}$	1.065	0.29	
Tyrannica	5.39 $^{e-02}$	1.58 $^{e-02}$	2.51 $^{e+02}$	3.41	0.00076	***
	Variance	Std. dev				
R^2m	0.11					
Clutch ID	0.0047	0.068				
Residuals	0.0012	0.034				
				R^2c	0.82	

b) Maculation scores ~ Subspecies

	Estimate	SE	df	t	Pr(>|t|)

Intercept	42.72	3.46	257.3	12.36	<2 e-16	***
Eylandtensis	-26.21	6.31	254.5	-4.15	4.48 e-05	***
Hypoleuca	-4.24	5.2	266.9	-0.82	0.41	
Leuconota	-8.04	5.98	286.7	-1.35	0.18	
Longirostris	-14.7	6.71	394.2	-2.19	0.029	*
Terraereginae	0.55	4.33	267	0.13	0.9	
Tibicen	-11.1	4.41	262.5	-2.52	0.012	*
Tyrannica	-2.79	4.33	261	-0.64	0.52	
	Variance	Std. dev				
R^2m	0.1					
Clutch ID	361.35	19.01				
Residuals	69.48	8.34				
				R^2c	0.85	

TABLE 4: Results of mixed effect model whereby **a)** looks at the explanatory power of maximum temperature (T_{max}) and leaf area cover (LAI) on background colour using Principal component 1 (PC1) and **b)** looks at the same environmental variable on maculation scores from SpotEgg. 283 clutches were samples from preserved museum samples; T_{max} were average annual averages based on 30 year climatology; LAI were obtained from 16 day intervals for a 16 year period. All variables were aggregated to a 1^o x 1^o grid cell resolution. The R^2m reports the R^2 of the model with just fixed effects while the R^2c reports the R^2 of the full model including random variables. Significance is indicated by asterisks.

SOLAR RADIATION HYPOTHESIS

*a) Background colour PC1~ T_{max} * LAI*

	Estimate	SE	df	t	Pr(>|t|)
Intercept	1.11 e-01	5.18 e-02	3.24 e+02	2.14	0.033
LAI	-1.8 e-02	3.78 e-02	3.28 e+02	-0.48	0.63
T_{max}	-4.67 e-03	1.96 e-03	3.45 e+02	-2.38	0.018
LAI:T_{max}	4.87 e-04	1.61 e-03	3.37 e+02	0.30	0.76
	Variance	Std. dev			
R^2m	0.051				
Clutch ID	0.0051	0.071			

Residuals	0.0012	0.034			
				R^2c	0.82

*b) Maculation scores ~ T_{max} * LAI*

	Estimate	SE	df	t	Pr(>|t|)
Intercept	80.21	13.91	344.6	5.76	1.89 $^{e-08}$
LAI	-29.53	10.16	347	-2.91	0.0039
T_{max}	-1.73	0.52	370.8	-3.3	0.00107
LAI:T_{max}	1.24	0.43	360.6	2.89	0.004
	Variance	Std. dev			
R^2m	0.031				
Clutch ID	386.57	19.66			
Residuals	69.41	8.33			
				R^2c	0.853

TABLE 5: Results of mixed effect model whereby a) looks at the explanatory power of maximum temperature (T_{max}) and relative humidity on background colour using Principal component 1 (PC1) and b) looks at the same environmental variable on maculation scores from SpotEgg. 283 clutches were samples from preserved museum samples; T_{max} were average annual averages based on 30 year climatology; relative humidity were average annual humidity at 3pm over 30 years. All variables were aggregated to a 1^o x 1^o grid cell resolution The R^2m reports the R^2 of the model with just fixed effects while the R^2c reports the R^2 of the full model including random variables. Significance is indicated by asterisks.

BACTERIAL HYPOTHESIS

*a) Background colour PC1 ~ T_{max} * relative humidity*

	Estimate	SE	df	t	Pr(>|t|)
Intercept	-3.34 $^{e-02}$	1.11 $^{e-01}$	3.11 $^{e+02}$	-0.3	0.76
T_{max}	-4.008 $^{e-04}$	4.19 $^{e-03}$	3.40 $^{e+02}$	-0.096	0.92
R. Humidity	1.74 $^{e-03}$	2.036 $^{e-03}$	3.17 $^{e+02}$	0.86	0.39
T_{max}:humidity	-4.53 $^{e-05}$	8.59 $^{e-05}$	3.35 $^{e+02}$	-0.53	0.6
	Variance	Std. dev			
R^2m	0.052				
Clutch ID	0.0051	0.072			

Residuals	0.0012	0.034				
				R^2c	0.82	

*b) Maculation scores ~ T_{max} * relative humidity*

	Estimate	SE	df	t	Pr(>|t|)	
Intercept	94.05	30.29	328.9	3.10	0.0021	**
T_{max}	-2.59	1.13	366.5	-2.29	0.023	*
R. Humidity	-1.04	0.55	336	-1.88	0.061	
T_{max}:humidity	0.049	0.023	359.8	2.11	0.035	*
	Variance	Std. dev				
R^2m	0.02					
Clutch ID	393.46	19.84				
Residuals	69.35	8.33				
				R^2c	0.85	

TABLE 6: Results of mixed effect model whereby a) looks at the explanatory power of calcium content in the soil on background colour using Principal component 1 (PC1) and b) looks at the explanatory power of calcium content in the soil on maculation scores from SpotEgg. 283 clutches were samples from preserved museum samples; Calcium levels in soil were extracted from 0-5cm deep pH tests ($CaCl_2$). Calcium values were aggregated to a 1^o x 1^o grid cell resolution. The R^2m reports the R^2 of the model with just fixed effects while the R^2c reports the R^2 of the full model including random variables. Significance is indicated by asterisks.

CALCIUM HYPOTHESIS

a) Background colour PC1 ~ calcium

	Estimate	SE	df	t	Pr(>|t|)
Intercept	-0.026	0.032	262.53	-0.8	0.42
Calcium	0.0047	0.0068	261.57	0.7	0.49
	Variance	Std. dev			
R^2m	0.0018				
Clutch ID	0.0053	0.073			
Residuals	0.0012	0.034			

				R^2c	0.82	
b) Maculation scores ~ calcium						
	Estimate	SE	df	t	Pr(>|t|)	
Intercept	27.75	8.64	273.29	3.21	0.0015	**
Calcium	2.022	1.83	272.51	1.1	0.27	
	Variance	Std. dev				
R^2m	0.0046					
Clutch ID	399.0	19.98				
Residuals	69.5	8.34				
				R^2c	0.85	

TABLE 7: Results of mixed effect model whereby **a)** looks at the explanatory power of presence or absence of a parasite on background colour using Principal component 1 (PC1) and **b)** presence or absence of a parasite on maculation scores from SpotEgg. 283 clutches were samples from preserved museum samples; presence and absence of parasitism was determined using distribution maps. The R^2m reports the R^2 of the model with just fixed effects while the R^2c reports the R^2 of the full model including random variables. Significance is indicated by asterisks.

PARASITE HYPOTHESIS						
a) Background colour PC1~ parasite						
	Estimate	SE	df	t	Pr(>|t|)	
Intercept	-0.015	0.008	290.4	-1.87	0.062	
Parasite Yes	0.017	0.0099	299.53	1.76	0.08	
	Variance	Std. dev				
R^2m	0.0099					
Clutch ID	0.0053	0.073				
Residuals	0.0012	0.034				
				R^2c	0.82	
b) Maculation scores ~ parasite						
	Estimate	SE	df	t	Pr(>|t|)	
Intercept	37.59	2.22	304.7	16.96	$<2^{e-16}$	***
Parasite yes	-0.58	2.66	317.1	-0.22	0.83	

	Variance	Std. dev		
R^2m	0.00016			
Clutch ID	401.0	20.024		
Residuals	69.5	8.34		
			R^2c	0.85

FIGURES

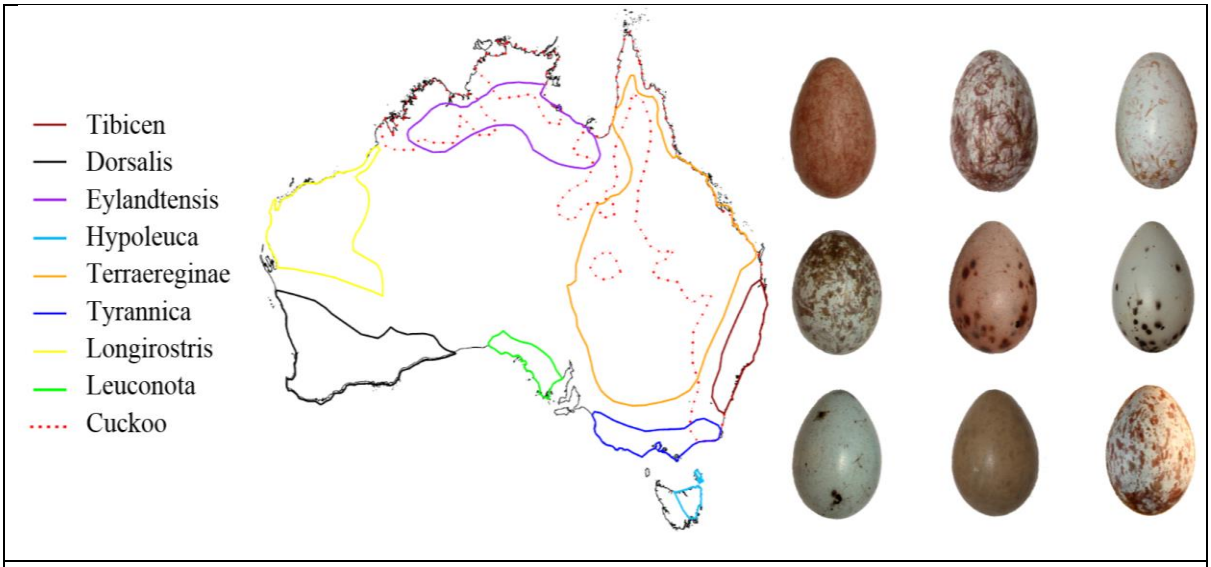


FIGURE 1: *Left:* Australian magpie (*Cracticus Tibicen*) subspecies and Channel-billed cuckoo (*Scythrops novaehollandiae*) distribution. Map digitised from Hanzab and the Atlas of Living Australia (38,39). *Right:* Example of intra-specific variation within the same species. Photographs taken by author. All samples were from preserved museum collections. 283 clutches were analysed (*Tibicen*= 60, *Dorsalis*= 34, *Eylandtensis*= 14, *Hypoleuca*= 28, *Terraereginae*= 58, *Tyrannica*= 62, *Longirostris*= 9, *Leuconota*= 18).

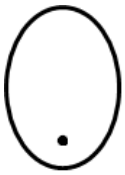
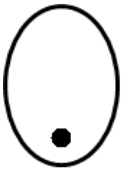
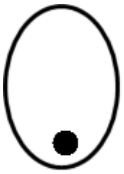
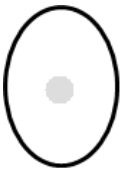
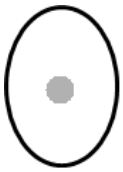
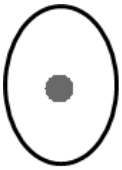
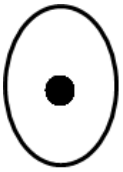
	SPOTS (S)	LINES (L)	BLOTCHES (B)	MOTTLED (M)
CHOOSE PROMINENT FEATURE				
SCORE	**1**	**2**	**3**	**4**
SIZE Scored 0-3	 **1= Small**	 **2= Medium**	 **3= Large**	
INTENSITY Scored 1-4	 **1= Faint**	 **2= Pale**	 **3= Medium**	 **4= Intense**
DISTRIBUTION Scored 1-4 Approximate percentage of markings in one half, typically the blunt end	 **1= >90%**	 **2= 75-90%**	 **3= 50-75%**	 **4= <50%**

FIGURE 2: Egg Scoring guidelines modified from previously published 'IDS' system (18). Top row represents different types of patterning found on eggs. Following rows represent increasing value from score of 1 to 4 in size (S), intensity (I) and distribution (D). All scoring was performed by a single observer to maintain consistency.

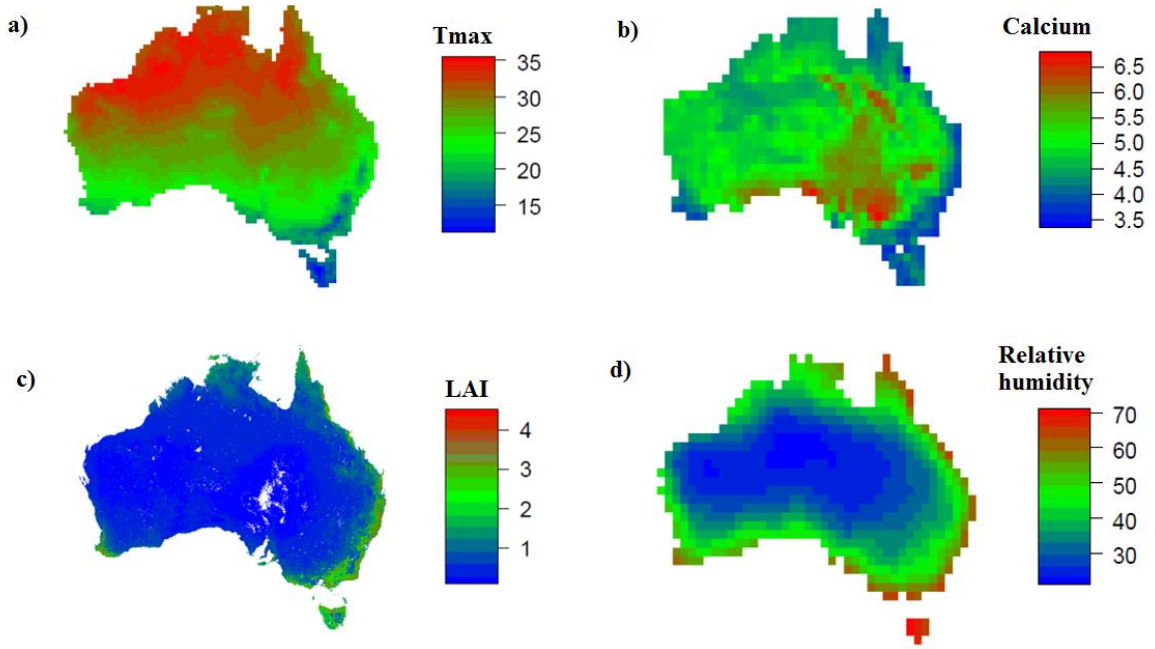


FIGURE 3: Maps showing variation in a) maximum temperatures **(**T_{max}) based on 30-year daily average. b) Calcium levels extracted from pH soil tests 0-5 cm deep, c) leaf area index (LAI) based on average 16-year 16-day intervals, d) relative humidity (%) based on a 30-year daily average for the Australian continent. Maps have been resampled to a 1^{o} x 1^{o} grid cell resolution.

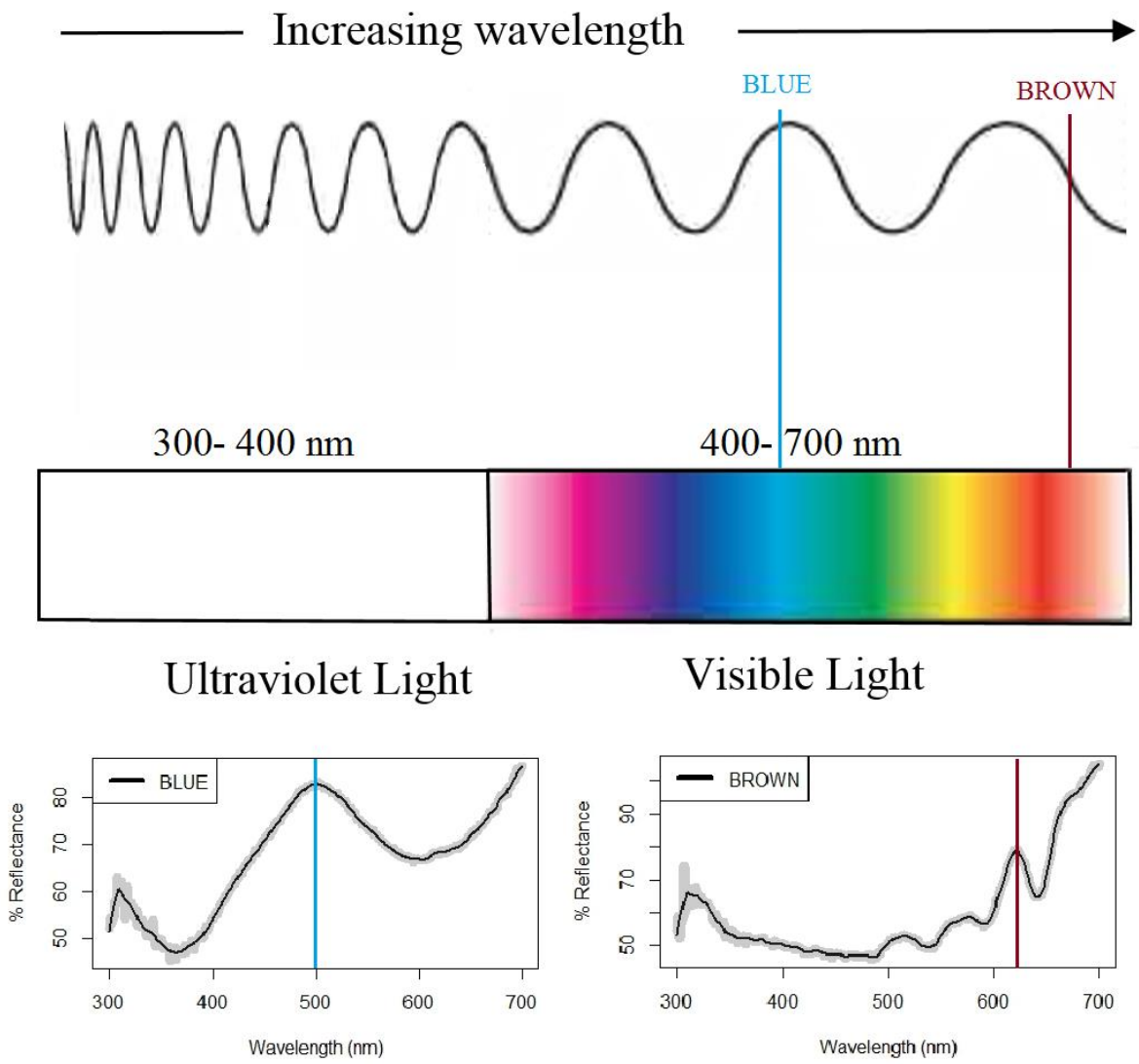


FIGURE 4: Above a schematic representation of the colour spectrum between wavelengths of 300-700nm (range taken for our spectrometry readings. Below, examples of reflectance curves returned from the spectrophotometer. On the left, the blue with main peaking curve at 500 nm and on the right brown with main sharp peak at 630 nm. Their location on the spectrum can be found represented by respectively coloured lines. Both curves have been smoothed to remove noise using smooth loose function= 0.05.

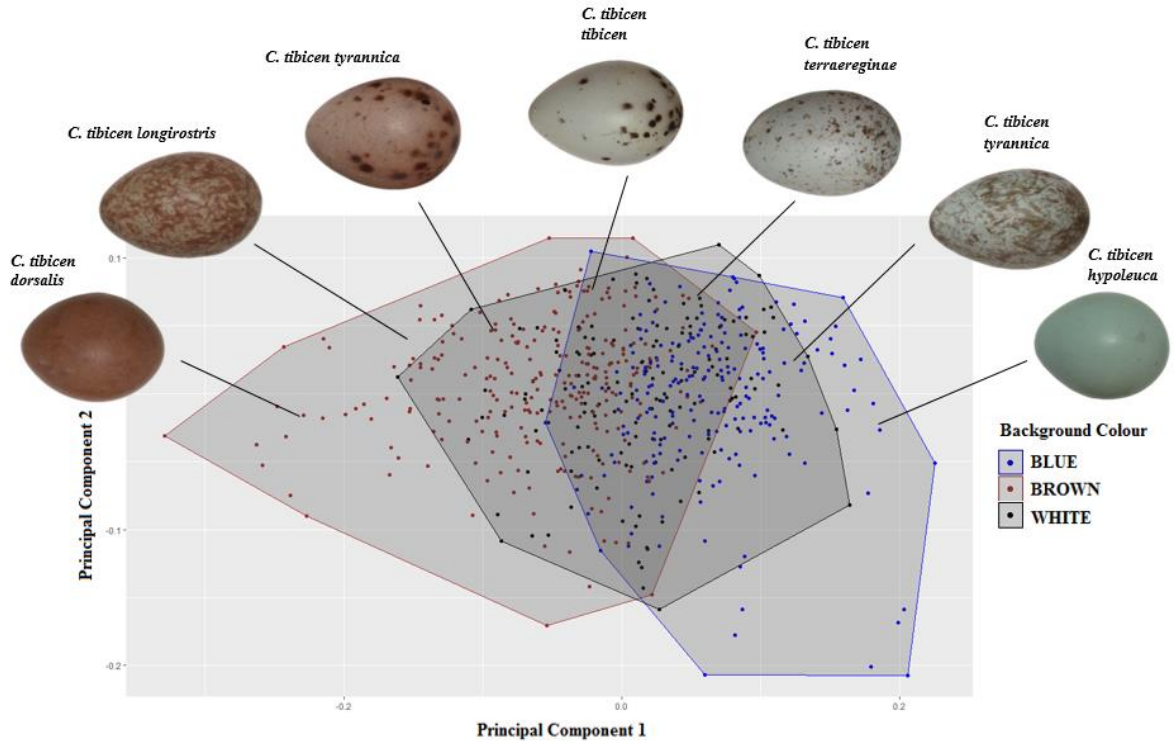


FIGURE 5: Example of Australian magpie egg variety and their location in colour space when analysed with Principal component analysis (both PC1 and PC2). Data represent 283 clutches of eggs. Background colours (Blue, Brown, White) were visually marked in the museum and plotted to visualise where colours fell within the matrix.

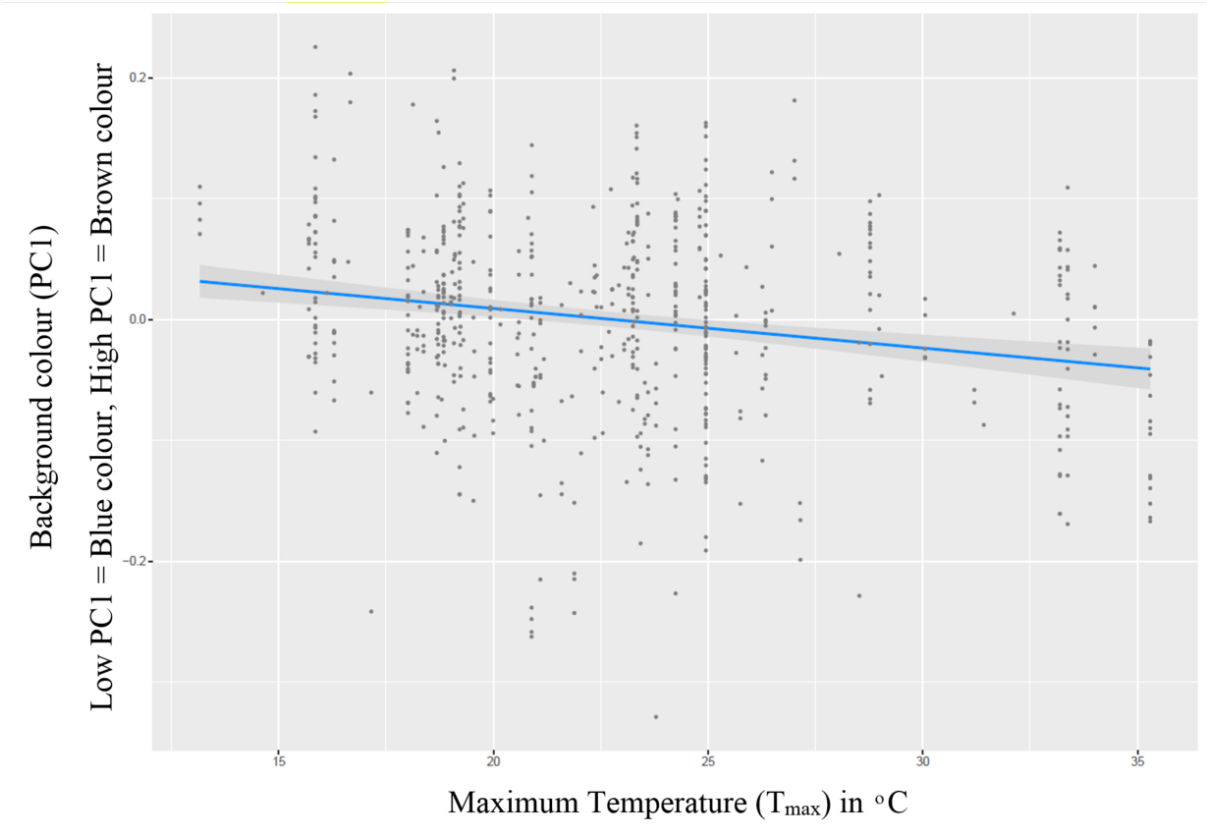


FIGURE 6: Relationship between eggshell background colour (in the form of principal component 1scores) against maximum temperature (T_{max}), The direction of change indicates a lower PC1 value with higher T_{max} (High PC1 = Blue, Low PC1= Brown). Results based on analysis of 283 clutches of Australian magpie eggs from museum collections.

SUPPLEMENTARY MATERIALS

SM TABLE 1: Museum accession numbers (Reg. Number), year and location of original collection and Museum code (VIC = Victoria museum in Melbourne, ANWC = Australian National Wildlife Collection in Canberra)

Reg. Number	Year	Latitude	Longitude	Museum	Reg. Number	Year	Latitude	Longitude	Museum
3542	1908	-31.98	151.12	VIC	E00830	1952	-35	149	ANWC
3543	1894	-29.7	152.93	VIC	E00831	1952	-35	149	ANWC
3544	1899	-36.78	145.17	VIC	E00832	1952	-35	149	ANWC
3545	1893	-29.7	152.93	VIC	E00833	1952	-35	149	ANWC
3546	1897	-23.72	149.67	VIC	E00847	1952	-35	149	ANWC
3547	1906	-31.98	151.12	VIC	E00886	1952	-35	149	ANWC
3548	1876	-34.82	149.68	VIC	E00887	1952	-35	149	ANWC
3549	1909	-31.82	151.32	VIC	E00888	1952	-35	149	ANWC
3550	1893	-29.7	152.93	VIC	E00889	1952	-35	149	ANWC
3551	1897	-23.72	149.67	VIC	E00890	1952	-35	149	ANWC
3552	1894	-29.7	152.93	VIC	E00892	1952	-35	149	ANWC
3553	1897	-23.72	149.67	VIC	E02001	1912	-32	149	ANWC
3554	1876	-34.82	149.68	VIC	E02002	1920	-32	149	ANWC
3555	1892	-29.7	152.93	VIC	E02003	1920	-32	149	ANWC
3556	1876	-34.82	149.68	VIC	E02004	1913	-31	116	ANWC
3557	1907	-31.98	151.12	VIC	E02005	1913	-32	149	ANWC
3558	1892	-29.7	152.93	VIC	E02029	1920	-32	149	ANWC
3559	1893	-29.7	152.93	VIC	E02058	1911	-31	116	ANWC
3560	1894	-29.7	152.93	VIC	E03606	1930	-27	153	ANWC
3561	1907	-31.82	151.32	VIC	E04467	1948	-34	146	ANWC
3562	1897	-23.72	149.67	VIC	E04470	1948	-34	146	ANWC
3563	1908	-19.33	146.47	VIC	E05726	1956	-35	139	ANWC

3564	1894	-29.7	152.93	VIC	E05728	1953	-35	139	ANWC
3565	1909	-31.82	151.32	VIC	E05734	1947	-35	139	ANWC
3566	1909	-31.82	151.32	VIC	E05735	1954	-34	151	ANWC
3567	1909	-31.82	151.32	VIC	E05736	1955	-34	118	ANWC
3568	1909	-32.08	150.98	VIC	E05737	1960	-33	116	ANWC
3569	1909	-31.82	151.32	VIC	E05738	1961	-33	116	ANWC
3570	1909	-31.82	151.32	VIC	E05739	1975	-29	138	ANWC
3571	1909	-31.98	151.12	VIC	E05740	1982	-35	139	ANWC
3572	1909	-17.5	145.47	VIC	E05741	1979	-29	139	ANWC
3573	1910	-31.87	141.42	VIC	E05743	1981	-29	139	ANWC
3574	1910	-31.62	144.98	VIC	E05747	1954	-35	140	ANWC
3575	1911	-17.5	145.47	VIC	E05748	1954	-35	140	ANWC
3576	1911	-29.33	148.67	VIC	E05750	1957	-35	141	ANWC
3577	1899	-33.38	149.48	VIC	E05751	1957	-35	141	ANWC
3578	1908	-33.83	151.07	VIC	E05752	1960	-32	138	ANWC
3579	1899	-33.38	149.48	VIC	E05753	1944	-38	140	ANWC
3580	1895	-33.42	149.57	VIC	E05756	1960	-35	138	ANWC
3581	1899	-33.38	149.48	VIC	E05759	1956	-33	138	ANWC
3582	1913	-16.07	136.3	VIC	E05760	1953	-33	138	ANWC
3583	1913	-16.07	136.3	VIC	E05762	1971	-35	138	ANWC
3584	1913	-16.07	136.3	VIC	E05764	1956	-35	138	ANWC
3585	1913	-16.07	136.3	VIC	E06294	1999	-33	146	ANWC
3586	1913	-16.07	136.3	VIC	E06337	1999	-35	149	ANWC
3587	1913	-18.6	136.1	VIC	E06426	1918	-37	150	ANWC
3588	1913	-18.6	136.1	VIC	E06427	1918	-37	150	ANWC
3589	1913	-18.6	136.1	VIC	E06428	1918	-37	150	ANWC
3590	1913	-18.6	136.1	VIC	E06429	1918	-37	150	ANWC
3591	1913	-18.6	136.1	VIC	E06468	1960	-37	144	ANWC
3592	1913	-18.6	136.1	VIC	E07851	1921	-43	147	ANWC

3593	1890	NA	NA	VIC
3594	1914	-32.08	150.37	VIC
3595	NA	-32.07	149.23	VIC
3596	1917	-35.17	141.7	VIC
3597	1921	-13.95	143.2	VIC
3598	1921	-13.95	143.2	VIC
3599	1906	-17.32	123.63	VIC
3600	1908	-20.68	119.65	VIC
3601	1908	-20.68	119.65	VIC
3602	1907	-34.32	118.45	VIC
3603	1908	-20.68	119.65	VIC
3604	1908	-20.68	119.65	VIC
3605	1908	-20.68	119.65	VIC
3606	1908	-20.68	119.65	VIC
3607	1907	-34.32	118.45	VIC
3608	1907	-34.32	118.45	VIC
3609	1903	-31.95	115.85	VIC
3610	1909	-30.63	116	VIC
3611	1909	-30.63	116.02	VIC
3612	1910	-30.63	116.02	VIC
3613	1912	-30.63	116	VIC
3614	1912	-30.63	116	VIC
3615	1912	-30.63	116	VIC
3616	1912	-30.63	116	VIC
3617	1912	-30.63	116	VIC
3618	1912	-30.63	116	VIC
3619	1912	-30.63	116.02	VIC
3620	1912	-30.63	116	VIC
3621	1904	-37.83	144.57	VIC
E07852	1933	-43	147	ANWC
E07853	1927	-43	147	ANWC
E07854	1928	-43	148	ANWC
E07855	1926	-43	147	ANWC
E07856	1925	-43	147	ANWC
E07857	1931	-43	147	ANWC
E07858	1921	-43	147	ANWC
E07859	1921	-43	147	ANWC
E07860	1911	-41	147	ANWC
E07861	1927	-43	147	ANWC
E07863	1924	-43	147	ANWC
E07864	1925	-43	147	ANWC
E07865	1950	-42	147	ANWC
E08398	1900	-33	145	ANWC
E08403	1905	-33	149	ANWC
E09982	1910	-24	150	ANWC
E10177	1908	-35	141	ANWC
E10178	1908	-35	141	ANWC
E10180	1915	-31	116	ANWC
E10182	1905	-36	138	ANWC
E10183	1906	-38	144	ANWC
E10184	1906	-37	142	ANWC
E10187	1906	-35	142	ANWC
E10188	1908	-43	147	ANWC
E13137	1959	-28	152	ANWC
E13138	1959	-28	152	ANWC
E13139	1962	-20	147	ANWC
E13140	1967	-20	147	ANWC
E13141	1984	-26	145	ANWC

3622	1897	-37.83	144.57	VIC
3623	1896	-37.62	144.35	VIC
3624	1893	-36.52	142.42	VIC
3625	1904	-37.83	144.57	VIC
3626	1899	-36.17	145.88	VIC
3627	1913	-34.92	141.03	VIC
3628	1918	-38.17	144.18	VIC
3629	1917	-38.17	144.18	VIC
3630	1919	-37.78	145.32	VIC
3631	1919	-37.03	141.28	VIC
3632	1898	-42.83	147.28	VIC
3633	1907	-42.43	145.62	VIC
3634	1905	-41.35	147.25	VIC
3635	1914	-42.75	147.23	VIC
3636	1919	-41.42	147.13	VIC
3637	1914	-42.53	147.2	VIC
3638	1914	-42.93	147.48	VIC
3639	1914	-42.75	147.23	VIC
3640	1908	-42.93	147.48	VIC
5246	NA	NA	NA	VIC
5247	NA	NA	NA	VIC
5255	NA	NA	NA	VIC
6752	1889	-33.63	115.15	VIC
6755	1885	-23.9	149.5	VIC
6756	1908	-20.68	119.65	VIC
6766	NA	-41.55	147.28	VIC
6774	NA	NA	NA	VIC
6783	NA	-38.08	144.27	VIC
6791	1886	-37.72	144.83	VIC

E13142	1986	-29	142	ANWC
E13391	1949	-34	150	ANWC
E13463	1926	-35	150	ANWC
E13464	1926	-35	150	ANWC
E13465	1926	-35	147	ANWC
E14139	1961	-35	149	ANWC
E14140	1978	-34	115	ANWC
E14804	1975	-30	117	ANWC
E14805	1958	-35	139	ANWC
E14806	1963	-35	139	ANWC
E14811	1972	-30	146	ANWC
E14812	1932	-34	151	ANWC
E15488	1904	-38	144	ANWC
E15489	1924	-38	144	ANWC
E15490	1919	-38	144	ANWC
E15491	1919	-38	144	ANWC
E15492	1922	-38	144	ANWC
E15493	1907	-38	145	ANWC
E15494	1922	-38	144	ANWC
E15495	1905	-36	145	ANWC
E15496	1919	-38	144	ANWC
E15497	1931	-38	144	ANWC
E15498	1922	-38	144	ANWC
E15871	1937	-22	144	ANWC
E15901	1931	-38	144	ANWC
E18168	1904	-33	149	ANWC
E18182	1926	-31	116	ANWC
E18193	1905	-36	149	ANWC
E18231	1903	-33	149	ANWC

6799	NA	NA	NA	VIC
7624	NA	NA	NA	VIC
7630	NA	NA	NA	VIC
7636	1951	-37.6	144.25	VIC
9051	1930	-36.7	144.27	VIC
9052	1920	-37.55	143.85	VIC
9053	1920	-37.55	143.85	VIC
9054	1920	-37.55	143.85	VIC
9055	1961	-33.25	115.83	VIC
9056	1966	-33.33	115.63	VIC
9057	NA	NA	NA	VIC
9059	NA	-17.27	145.6	VIC
9060	1954	-35.07	142.32	VIC
9260	1964	-17.27	145.6	VIC
9261	1934	-35.62	144.12	VIC
9263	1916	-35.12	142.8	VIC
9264	1907	-35.43	145.72	VIC
9265	1917	-36.72	141.83	VIC
9266	1910	-34.33	117.92	VIC
9267	1909	-41.4	147.13	VIC
9268	1918	-37.97	144.5	VIC
9270	1914	-38.12	147.07	VIC
9271	1914	-42.53	147.2	VIC
9272	1917	-30.63	116	VIC
9273	1905	-38.22	145.17	VIC
9274	1906	-37.7	144.47	VIC
9275	1907	-35.47	143.63	VIC
9276	1919	-30.63	116	VIC
9277	1917	-36.72	141.83	VIC
E18232	1909	-41	147	ANWC
E18233	1906	-33	149	ANWC
E18234	1905	-36	149	ANWC
16227	1923	-35.17	141.68	VIC
16229	1923	-35.17	141.68	VIC
16230	1923	-35.17	141.68	VIC
16231	1922	-37.77	145.35	VIC
16232	1922	-37.77	145.35	VIC
16233	1918	-37.8	145.03	VIC
16451	1906	-31.95	141.45	VIC
16462	1903	-37.58	141.4	VIC
16597	1930	-36.9	144.22	VIC
17140	1917	-35.5	138.7	VIC
17141	1917	-29.47	149.85	VIC
17142	NA	-30.63	116	VIC
17143	NA	-18.6	136.1	VIC
17144	1917	-42.75	147.28	VIC
17524	1929	-30.28	115.03	VIC
18227	NA	-36.62	143.27	VIC
18228	NA	-36.62	143.27	VIC
18412	NA	NA	NA	VIC
18522	NA	-37.83	144.57	VIC
18613	1897	NA	NA	VIC
18645	1889	NA	NA	VIC
13754	1898	NA	NA	VIC
13757	NA	-12.45	130.83	VIC
16225	1923	-35.17	141.68	VIC
16226	1923	-35.17	141.68	VIC
11801	NA	NA	NA	VIC

9278	1919	-37.97	144.5	VIC
9279	1912	-35.43	143.63	VIC
9280	1917	-37.85	147.08	VIC
9281	1913	-35.47	143.63	VIC
9282	1918	-37.92	144.45	VIC
9283	1917	-35.47	143.63	VIC
9284	1909	-35.25	141.1	VIC
9285	1909	-22.05	116.88	VIC
9286	1917	-35.47	143.63	VIC
9287	1911	-30.07	116.13	VIC
9288	1906	-33.38	148.02	VIC
9289	1914	-38.12	147.07	VIC
9290	1919	-37.78	145.32	VIC
9291	1919	-37.78	145.32	VIC
11808	1895	-29.7	152.93	VIC
12338	NA	NA	NA	VIC
12342	NA	NA	NA	VIC
13080	1913	-18.6	136.1	VIC
13081	1893	-23.72	149.67	VIC
13740	NA	-31.9	115.97	VIC
13741	NA	NA	NA	VIC
13742	1866	-35.3	149.67	VIC
13743	1864	-34	150.92	VIC
13744	1862	-34	150.92	VIC
13747	NA	NA	NA	VIC
9292	1918	-37.92	144.45	VIC
9293	1919	-37.97	144.5	VIC

SM TABLE 2: SpotEgg configurations settings for spot detection and linearization for three categories of eggs. Settings are determined by the training interface incorporated within SpotEgg. Reflection values refer to the colour reference card to process white balance and colour reflectance.

Setting type	Radius Filter	Sensitivity	Minimum spot size	Background fill threshold
Spot	1.01	0.080389	0.5	0.5
Blotch	0.62	0.035479	0.5	0.5
Clear	0.74	0.20872	0.5	0.5
Reflectance values using DKG5x7	0.649000, 0.346000, 0.226800, 0.137800, 0.078900, 0.037000			

SM TABLE 3: Results of a mixed effect model whereby **a)** looks at the explanatory power of leaf area cover (LAI) and maximum temperature (T_{max}) on the red/green/blue (RGB) background colour value produced by SpotEgg, and **b)** looks at the effect of the same environmental variables on principal component 1 from the pattern score (IDS system). Eight subspecies and 283 clutches were samples from preserved museum samples. The R^2m reports the R^2 of the model with just fixed effects while the R^2c reports the R^2 of the full model including random variables. Significance is indicated by asterisks.

SOLAR RADIATION HYPOTHESIS

a) RGB colour space~ LAI T_{max}*

	Estimate	SE	df	t	Pr(>|t|)
Intercept	1.61^{e-01}	4.53^{e-02}	3.16^{e+02}	3.56	0.00043***
LAI	-7.43^{e-03}	3.3^{e-02}	3.19^{e+02}	-0.23	0.82
T_{max}	1.41^{e-03}	1.71^{e-03}	3.4^{e+02}	0.83	0.41
LAI:T_{max}	5.15^{e-04}	1.4^{e-03}	3.31^{e+02}	0.37	0.71
	Variance	Std. dev			
R^2m	0.051				
Clutch ID	0.004	0.06			
Residuals	0.0008	0.03			
				R^2c	0.82

b) IDS Pattern score pc1 ~ LAIT_{max}*

	Estimate	SE	df	t	Pr(>|t|)
Intercept	-0.73	0.71	305.8	-1.04	0.3
LAI	0.66	0.51	310.8	1.27	0.2
T_{max}	0.027	0.027	317.9	1.00	0.32
LAI:T_{max}	-0.028	0.022	314.7	-1.27	0.2

	Variance	Std. dev			
R^2m	0.005				
Clutch ID	0.84	0.91			
Residuals	0.33	0.57			
				R^2c	0.72

SM TABLE 4: Results of a mixed effect model **whereby a)** looks at the explanatory power of maximum temperature (Tmax) and relative humidity on the red/green/blue background colour value produced by SpotEgg, and **b)** looks at the effect of the same environmental variables on principal component 1 from the pattern score (IDS system). Eight subspecies and 283 clutches were samples from preserved museum samples. The R^2m reports the R^2 of the model with just fixed effects while the R^2c reports the R^2 of the full model including random variables. Significance is indicated by asterisks.

BACTERIAL HYPOTHESIS

a) RGB colour space ~ Tmax relative humidity*

	Estimate	SE	df	t	Pr(>|t|)
Intercept	9.32 $^{e-02}$	9.75 $^{e-02}$	3.01 $^{e+02}$	0.96	0.34
Tmax	2.63 $^{e-03}$	3.66 $^{e-03}$	3.35 $^{e+02}$	0.72	0.47
R.Humidity	7.13 $^{e-04}$	1.78 $^{e-03}$	3.07 $^{e+02}$	0.4	0.69
Tmax:R.Humidity	7.65	7.49 $^{e-02}$	0.1	0.92	
	Variance	Std. dev			
R^2m	0.05				
Clutch ID	0.004	0.063			
Residuals	0.0008	0.03			
				R^2c	0.82

b) IDS Pattern score pc1 ~ Tmax relative humidity*

	Estimate	SE	df	t	Pr(>|t|)
Intercept	-5.01 $^{e-01}$	1.51 $^{e+00}$	2.95 $^{e+02}$	-0.33	0.74
Tmax	3.19 $^{e-02}$	5.73 $^{e-02}$	3.14 $^{e+02}$	0.56	0.78
R.Humidity	1.53 $^{e-02}$	2.76 $^{e-02}$	3.00 $^{e+02}$	0.56	0.58
Tmax:R.Humidity	-9.41 $^{e-04}$	1.17 $^{e-03}$	3.12 $^{e+02}$	-0.8	0.4
	Variance	Std. dev			
R^2m	0.02				
Clutch ID	083	0.91			
Residuals	0.33	0.57			
				R^2c	0.85

SM TABLE 5: Results of a mixed effect model looking at a) the explanatory power of calcium levels in the soil on the red/green/blue background colour value produced by SpotEgg, and **b)** looks at the effect of calcium on principal component 1 from the pattern score (IDS system). Eight subspecies and 283 clutches were samples from preserved museum samples. The R^2m reports the R^2 of the model with just fixed effects while the R^2c reports the R^2 of the full model including random variables. Significance is indicated by asterisks.

CALCIUM HYPOTHESIS

a) RGB colour space ~ calcium

	Estimate	SE	df	t	Pr(>|t|)
Intercept	1.71^{e-01}	2.75^{e-02}	2.51^{e+02}	6.22	2.08^{e-09}***
Calcium	5.55^{e-03}	5.92^{e-03}	5.5^{e+02}	0.95	0.34
	Variance	Std. dev			

R^2m	0.0034				
Clutch ID	0.004	0.063			
Residuals	0.0008	0.028			
				R^2c	0.83

b) IDS Pattern score pc1 ~ calcium

	Estimate	SE	df	t	Pr(>|t|)
Intercept	0.39	0.41	261.81	0.94	0.34
Calcium	-0.093	0.087	260.28	-1.065	0.29
	Variance	Std. dev			
R^2m	0.005				
Clutch ID	0.82	0.91			
Residuals	0.33	0.58			
				R^2c	0.85

SM TABLE 6: Results of a mixed effect model whereby a) looks at the explanatory power of the presence or absence of a parasite on the red/green/blue (RGB) background colour value produced by SpotEgg, and b) looks at the effect of the presence or absence of a parasite on the principal component 1 from the pattern score (IDS system). Eight subspecies and 283 clutches were samples from preserved museum samples. The R^2m reports the R^2 of the model with just fixed effects while the R^2c reports the R^2 of the full model including random variables. Significance is indicated by asterisks.

PARASITE HYPOTHESIS

a) RGB colour space ~ Parasite

	Estimate	SE	df	t	Pr(>|t|)
Intercept	1.86^{e-01}	6.99^{e-03}	2.78^{e+02}	26.66	<2 e-16***

Parasite yes	1.54^{e-02}	8.4^{e-03}	2.88^{e+02}	1.84	0.067
	Variance	Std. dev			
R^2m	0.01				
Clutch ID	0.0039	0.062			
Residuals	0.0008	0.028			
				R^2c	0.83

b) IDS Pattern score pc1 ~ parasite

	Estimate	SE	df	t	Pr(>|t|)
Intercept	0.16	0.11	283.45	1.48	0.14
Parasite yes	-0.3	0.13	287.24	-2.3	0.022*
	Variance	Std. dev			
R^2m	0.016				
Clutch ID	0.81	0.9			
Residuals	0.33	0.57			
				R^2c	0.71

SM TABLE 7: Dunn test for comparison of pattern score and subspecies with Holm's stepwise multiple comparison adjustment. All values are adjusted p-values, those significant (*) reject the null model. Subspecies in bold are parasitized.

	Dorsalis	**Eylandtensis**	Hypoleuca	Leuconota	Longirostris	**Terrae reginae**	**Tibicen**
Eylandtensis	0.0000*						
Hypoleuca	0.0033*	0.030					
Leuconota	0.0037*	1.000	1.000				
Longirostris	0.507	0.031	1.000	0.868			
Terrae reginae	0.0001*	0.0057*	0.895	1.000	1.000		

Tibicen	0.0000*	0.133	1.000	0.983	0.901	0.913	
Tyrannica	0.0081*	0.0003*	0.900	0.529	0.480	1.000	0.068

SM TABLE 8: Additional background colour observations from Museum clutches of cuckoo hosts eggs and parasitic cuckoo eggs. Of two hosts, one (in bold) has variable egg colouration. Assessment was done on a total of 157 eggs from ANWC.

			Background Colour			
Species name	Scientific name		Brown	White	Blue	Total
Pied currawong	*Strepera graculina*	*Host*	30	2	0	32
Torresian crow	***Corvus orru***	***Host***	**6**	**15**	**9**	30
Channel- billed cuckoo	*Scythrops novaehollandiae*	*Parasite*	91	4	0	95

END OF THESIS