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# The function and evolution of egg colour in birds

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THE FUNCTION AND EVOLUTION OF EGG COLOURATION IN BIRDS

by

Daniel Hanley

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**DECLARATION OF CO-AUTHORSHIP**

I hereby declare that this thesis incorporates material that is result of joint research. All data chapters were written with the guidance of my supervisor, Dr. Stéphanie Doucet who provided valuable feedback and editorial input during the writing process. In addition, Chapter 2 was financially supported by Dr. Stéphanie Doucet and was published in Behavioral Ecology and Sociobiology. Chapter 3 was prepared as a manuscript for submission to the Journal of Applied Ecology and has been invited for resubmission. Chapters 4 and 5 are the joint effort of Dr. Stéphanie Doucet, Dr. Phillip Cassey and myself. Dr. Phillip Cassey provided 31% of the data required for this project and intellectual guidance. The key ideas, primary contributions, experimental designs, data analysis were my own, while the interpretation was a product of a joint effort with Dr. Stéphanie Doucet.

I am aware of the University of Windsor Senate Policy on Authorship and I certify that I have properly acknowledged the contribution of other researchers to my thesis, and have obtained written permission from each of the co-author(s) to include the above material(s) in my thesis.

I certify that, with the above qualification, this thesis, and the research to which it refers, is the product of my own work.

**ABSTRACT**

Animal colouration generally evolves via natural or sexual selection, or some combination of the two. From a naturalist's perspective, the diversity of colour exhibited by avian eggs is particularly interesting, because much of this diversity has not been thoroughly explained by either mode of selection. Until recently, a sexual selection mechanism for the evolution of egg colour was not known, and natural selection did not appear to be acting on some egg colours, most notably the unspotted white and blue-green eggs laid in open nests. The goal of my dissertation is to investigate the functional significance and selective pressures facing the evolution of egg colour. In Chapter 2, I investigate whether egg colour serves as signal of female quality. I find little support for this hypothesis and suggest that future research should examine other explanations for the evolution of egg colour. In Chapter 3, I find that environmental contaminants have a significant influence on egg colour. This has important implications for employing eggshell pigmentation as a non-destructive bio-indicator. In Chapters 4 and 5, I conduct large-scale comparative analyses that involve the reconstruction of a super-tree including representatives of all but one avian order. In Chapter 4, I find that predation is negatively related to ultraviolet chroma in open nests, and eggshell brightness is positively related to predation pressure in species using open nests above the ground. In addition, the risk of brood parasitism is greatest in species with a high proportion of blue-green chroma, but nest attendance is higher for these nests, suggesting that parents may behaviourally mitigate the risks of parasitism. I also find greater variation between clutches in species that experience high rates of parasitism; this presumably makes spotting a brood parasitic egg easier. In Chapter 5, I find that within cavity nests, selection is acting to increase

eggshell brightness. I also find suggestive evidence that eggshell pigments could be adapted to protect the embryo from harmful solar radiation. In Chapter 6, I document and describe eggshell phosphorescence, a previously undocumented property, and suggest that this property is due to porphyrin within the eggshell.

**DEDICATION**


To my parents Frank and Nadine Hanley. Thank you for a wonderful life.

scientist. Your kindness, support, and enthusiasm provided the fuel necessary for me to

reach my goals.

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# CHAPTER 1 – GENERAL INTRODUCTION

**Sexual reproduction**

Sexual organisms are derived from the unification of parental gametes (Gegenbaur 1859; Kökkiker 1899, as cited in Mayr 1982). One gamete, known as the ovum, is larger and generally less mobile than its smaller, highly motile counterpart, known as sperm. This difference in gamete size, known as anisogamy, is maintained by the combined effect of competition of two or more sperm attempting to fertilize the ovum (sperm competition), and an increased likelihood of fertilization if one gamete is numerous and small (Parker 1982). This distinction has important implications for parental investment. Specifically, males with motile gametes (sperm) invest in quantity, while females with larger immobile gametes (eggs) invest more in the quality of the gamete (Trivers 1972). This difference between the sexes provides an opportunity for the female to provision the cell with more than just a haploid set of genes. Once fertilization occurs, the developing zygote uses maternal resources allocated to the ovum. Since females have the opportunity to provision their offspring with resources, they have some options available in terms of how they will allocate those resources across progeny. The differential allocation hypothesis suggests that a female mated to a high quality partner should increase her maternal investment (Burley 1986). Such maternal investment has been found in the zebra finch (*Taeniopygia guttata*), where females add more testosterone to their eggs when mated to more attractive males (Gil et al. 1999). However, these types of decisions about maternal allocation need not necessarily be in response to the perceived attractiveness of her mate. Females may also choose to invest more or less based on environmental conditions and to enhance the competitive ability of certain chicks (Schwabl 1996a, b; Royle et al. 2001).

Females incur a number of costs associated with egg production, which can limit when and how often a female will become fertile (Monaghan and Nager 1997; Monaghan et al. 1998).Oviparity, or the production of eggs outside the body, restricts females to depositing eggs under only certain favourable conditions. For example, many conditions are too harsh or unstable for the development of external eggs (Andrews and Mathies 2000). As females invest heavily into the production of the eggs themselves, they may face limitations on the number of eggs, quality of these eggs, or frequency with which they lay (Monaghan and Nager 1997). Birds, in particular, display an interesting array of investment strategies, ranging from raising a single brood, raising multiple broods per year, raising offspring in two separate nests, leaving eggs to hatch from the heating action of decomposing debris, and even laying their eggs within the nests of conspecifics (intra-specific brood parasitism) or heterospecifics (inter-specific brood parasitism), therby evading their parental care responsibilities, with variable investment by the male within these strategies (Kendeigh 1952; Verner and Willson 1969).

Another important yet understudied female investment strategy lies in the deposition of pigments into eggshells, which produces a vast array of colours and patterns across the class Aves (Kennedy and Vevers 1976; Kilner 2006; Walters 2006). My dissertation research will investigate the functional significance and evolution of avian egg colouration.

**Formation of the avian egg**

As with most vertebrates, female birds are born with all of the gametes (oöcytes) that they will use throughout their reproductive lifespan. However, ovum maturation does

not occur until the proper hormonal triggers have begun the egg formation process. Although there are large interspecific differences in when females reach their age at first reproduction (Møller 2006; Wasser and Sherman 2009), the process of egg formation is remarkably similar between species (Romanoff and Romanoff 1949). In birds for example, environmental cues such as variation in day length are important hormonal triggers for egg formation (Bentley et al. 2000; Visser and Sanz 2009). One hormone integral to ovum development is the follicle stimulating hormone (Romanoff and Romanoff 1949; Onagbesan et al. 1999). This hormone, in conjunction with an insulin-like growth factor, is responsible for the rapid growth of follicular ova, and the timing of these processes are tied to a species-specific breeding cycle. Ova develop sequentially and the length of this process depends on the size of the bird and the size of the clutch it will lay (ranging from 4-5 days in Passeriformes to 16 days in Sphenisciformes). Prolactin levels increase at the beginning of egg laying and inhibit further egg production, which presumably corresponds to a transition from laying to incubation behaviour (Burke and Dennison 1980).

Prior to ovulation, while ova are still attached to the ovary, a vascularised follicle surrounds the primordial oöcytes and allows for the addition of yolk. Through this process oöcytes develop into ova, which are attached to the ovary by a small structure known as the peduncle. The liver-produced proteins and lipids that form the yolk are then transferred through the blood and accumulate in the yolk sac via receptor-mediated endocytosis (Romanoff and Romanoff 1949; Hirayama et al. 2003). In some species, this increase in ovum mass represents a greater than 1000% increase from its original size (Harris 1964). When the ovum has reached full size, ovulation occurs. At the time of

ovulation, the peduncle is cleaved at its base, known as the stigma, and is released from the ovary into the oviduct. The region of the oviduct that receives the ovum is known as the infundibulum, and it pulses back and forth towards each successive ovum. By the time the follicle breaks, the ovum is within the infundibulum where fertilization will occur (for a more complete review, Romanoff and Romanoff 1949).

The structure of the avian oviduct allows a female to store sperm for long periods of time prior to fertilization (Birkhead and Møller 1992; Das et al. 2008). The sperm is stored in sperm storage tubules that are located at the junction of the vagina and uterus, situated at the opposite end of the female's reproductive tract to the site of fertilization (Bobr et al. 1964). During the laying period, sperm must be continuously secreted from the sperm storage tubules so that it can travel to the infundibulum where fertilization occurs (Baskt 1998). This mechanism facilitates insemination even if females have not mated at the exact moment that would allow both the sperm and ovum to coincide within the infundibulum.

Once fertilization has occurred, the ovum moves further along the oviduct into the magnum, where the egg undergoes the process of albumen addition. There are actually four dehydrated layers of albumen, including the familiar layer of white twisted-looking strands that is found on either end of the yolk. This layer comprises strands known as the chalazae, which take this form because the ovum is slowly rotated as this layer is secreted around it. More specifically, the chalaza attached to the pointed end of the egg is longer, thicker, and more firmly attached to the albumen, and it is twisted in a counter clockwise direction. The chalaza at the blunt end of the egg is twisted in a clockwise direction as it is applied. While the egg rotates, this serves to tighten the chalazae and keep the

blastoderm oriented upwards and within the geometric center of the egg (Romanoff and Romanoff 1949; Rahman et al. 2007). After the chalazae are added, the remaining three layers of albumen are added over top. The egg continues to move away from the infundibulum into the isthmus where the porous inner- and outer- membranes are added. The inner membrane is a fine mesh of keratin fibres, while the outer membrane is composed of a coarser mesh of keratin fibres. The inner keratinized membrane often appears pinkish, and is the reason why some white eggshells appear to have a pinkish hue. These porous membranes allow for gas and liquid exchange after the egg is laid.

It is the permeability of these membrane layers which allows the egg to take on its characteristic shape. The albumen enclosed within these membranes becomes hydrated at this stage, through a process known as plumping. Now the egg has its ultimate shape and a firmer surface onto which the shell will adhere. In this form, the avian egg is reminiscent of the eggs of some closely related taxa within Chelonia (turtles, tortoises, and terrapins) and Lepidosauria (scaled lizards) (Ewert 1979). The membrane-bound egg then moves to the uterus where the process of shell formation begins.

The next step of complete calcification and pigmentation makes bird eggs unique. The evolution of shell calcification is believed to have been linked to selection pressures caused by soil microbes because the common ancestor of birds and reptiles were likely at risk of microbial invasion (Packard and Packard 1980). This hypothesis proposes that the calcified shell reduces permeability, and therefore provides greater protection for developing embryos. Nonetheless, there remains a great diversity in the degree of shell calcification found in reptiles (Ewert 1979; Packard and DeMarco 2004) and an investigation of the evolutionary origins of calcification would be enlightening.

Within the uterus, eggshell pigments are added to the shell. This process results in the diversity of colours exhibited by avian eggs, which forms the basis of the chapters to follow. Cone-shaped calcium carbonate structures are first laid over the outer membrane, and these ultimately form what is known as the mammillary layer of the egg. This layer has the important function of providing calcium necessary for bone formation to the developing embryo (Dieckert et al. 1989). After this layer has been laid, a layer known as the palisade (or spongy) layer is placed over it. This layer is created by the interweaving of collagen-like fibres and calcite, resulting in the hard dense layer which characterizes the outer surface of avian eggs (Romanoff and Romanoff 1949). It is within this palisade layer that the eggshell ground colouration is added. Here, when I refer to eggshell ground colouration, I mean the colour that uniformly covers the shell's surface. Ground colouration is created by two pigments that may be found independent of one another or in combination: proto-porphyrin, which produces brown colours, and biliverdin, which produces blue-green colours (Romanoff and Romanoff 1949; Kennedy and Vevers 1976; Miksik et al. 1994; Miksik et al. 1996; Gorchein et al. 2009). Although these two pigments may also be circulating in the blood, those found within the shell originate from within the shell gland (Baird et al. 1975; Zhao et al. 2006). Recent research suggests that the mechanism behind biliverdin deposition more specifically involves transportation of biliverdin from the shell gland into the uterus fluid; in blue-green eggs, biliverdin in the shell gland was transferred to uterine fluid and then to the shell surface, while in white eggs, biliverdin was produced in the shell gland but was not present in the uterine fluid (Liu et al. 2010). This implies that once within the fluid, pigments may be easily intermixed with the calcium matrix. The process of interspersing pigments within the

calcium matrix begins after the formation of the palisade layer, and therefore pigments are rarely found within the mammillary layer (Romanoff and Romanoff 1949). However, there are exceptions to how far pigments penetrate into the shell, even within a single species (personal observation).

Many avian eggs also possess another layer known as the cuticle; however, this layer is not present in all species (e.g., gulls, Romanoff and Romanoff 1949). When present, this layer is comprised of two membranes and covers the entire shell surface, including numerous pores in the shell. This outer layer is gas permeable, which allows gas exchange necessary to sustain the developing embryo, and is the last feature added to the egg before laying. The properties of this layer determine the apparent texture of the eggshell (glossy, chalky, etc.).Within this layer, another form of porphyrin-based pigmentation is applied, which creates the familiar brown streaks, spots, and other markings found atop the ground colouration in a variety of species. This layer is thickest where the pigments are deposited and is otherwise even across the unspotted areas (Romanoff and Romanoff 1949). Some species, especially those with absent or thin cuticles, will create spots by intermixing pigments within the calcium matrix, known as shell pigments, while the spotting found within the cuticle is known as cuticular pigment (Romanoff and Romanoff 1949).

Interestingly, spots are placed specifically where the shell is thinnest (Gosler et al. 2005), which has been hypothesized to be due to a shared carrier protein between porphyrin and calcium (Solomon 1997). Such a mechanism would allow porphyrin to be carried to the shell whenever calcium is lacking.The deposition of pigment where the shell is thinnest potentially adds to the structural integrity of the eggshell (Gosler et al.

2005). However, researchers have yet to determine the mechanism that allows pigments within this proteinaceous cuticle layer to bind to specific shell areas. For example, the pigments forming dark eggshell spots could initially be evenly distributed throughout the cuticle layer and then become concentrated at thin parts of the shell. The thin parts of the shell would then act as sinks for pigment concentration, leading to a patchy distribution of pigmentation in the cuticle layer. More research on dark eggshell spotting is also warranted because dark spots appear to have different photo-electric properties than lighter speckling (Chapter 6), even though they should be produced by the same pigments (Kennedy and Vevers 1976). More precise analytical approaches will be necessary to fully characterize the pigment composition of avian eggs. This point is timely because current extraction protocols do not necessarily isolate pigments found in specific areas of the egg; they usually homogenize pigments throughout the shell.

**Pigment composition of avian eggs**

Although researchers have been in almost unanimous agreement about the general composition and origin of eggshell pigments since the late 1800's (Sorby 1875), the specific composition of pigments has long been debated (Liebermann 1878; Sorby 1878) and remains contentious (Lang and Wells 1987; Gorchein et al. 2009). What is certain is that there are two main pigment classes involved in colouring birds' eggs: porphyrins and verdins (Kennedy and Vevers 1976; Miksik et al. 1994; Miksik et al. 1996). These are biologically important pigments, and are intimately connected to the heme biosynthesis pathway, which is necessary for the formation of chlorophyll in plants and haemoglobin in nearly all vertebrates (Moore 1998; Ponka 1999; McDonagh 2001). Porphyrin is

comprised of four pyrrole subunits, arranged in a ring with substitutions around this ring perimeter (Figure 1A; McGraw 2006). This molecule is constructed by the binding of identical colourless monopyrrole units. Chain-link polymerization of these pyrroles creates the highly planar, conjugated double bond system which produces the brilliantly coloured and highly photo-sensitive porphyrin (Needham 1974). Porphyrin has multiple absorption peaks (Figure 2) and on the surface of avian eggs this pigment appears brown to reddish brown. In addition, porphyrin is the precursor to numerous important natural colourants including chlorophyll and heme, a precursor to hemoglobin that is integral to the oxygenation of living tissues (Ponka 1999). The difference between heme and chlorophyll begins with the addition of an iron ion (in the case of heme), and a magnesium ion (in the case of cholorphyll). The porphyrin that precedes the addition of a metal ion is known as proto-porphyrin IX. The majority of investigations have only found the iron-less proto-prophyrin in avian eggshells (Kennedy and Vevers 1976; Miksik et al. 1994; Miksik et al. 1996; Gorchein et al. 2009). However, some researchers have detected other forms of natural porphyrins (Sorby 1875; With 1973; Baird et al. 1975), prompting questions about the possible presence of other forms of porphyrin in the eggshell (Lang and Wells 1987; Gorchein et al. 2009). In some cases, the detection of other natural porphyrins may be the result of experimental contamination (Gorchein et al. 2009).

The second pigment found in avian eggs is biliverdin, which produces blue-green colouration. Researchers have been aware of this pigment's role for more than a hundred years (Sorby 1875); however, biliverdin in avian eggs was known as oöcyan until 1945 when it was confirmed to be identical to biliverdin (Romanoff and Romanoff 1949). This

pigment is formed through the oxidation of heme, a process which releases both an iron ion and a single molecule of carbon monoxide (Galbraith 1999). Biliverdin is an open-chain tetrapyrrole molecule (Figure 1B), and along with its derivatives, is known to have powerful antioxidant capacities (Stocker et al. 1987; Kaur et al. 2003). Biliverdin is characterized by two major absorption peaks in the 375-384 nm and 665-670 nm ranges (Figure 2; Ding and Xu 2002; Falchuk et al. 2002).

**Genetic determination of eggshell pigments**

For either natural or sexual selection to act on a trait, variation within the trait needs to be heritable (Darwin 1871). Heritability, or the proportion of variation in a trait attributable to an organism's genes rather than environmental conditions, can be calculated to determine if a trait meets this basic criterion for selection (Boag and Grant 1978). Considering the wealth of empirical and theoretical studies on egg colouration (Underwood and Sealy 2002; Kilner 2006; Cherry and Gosler 2010), there has been surprisingly little research on the environmental and genetic control of egg colour. Nevertheless, our knowledge of the heritability of egg colour is expanding, and we are beginning to understand at least generally how several different forms of pigmentation are inherited. The heritability of white and brown colours has been well studied in poultry (Wei et al. 1992; Francesch et al. 1997; Zhang et al. 2005); however, less effort has focused on blue-green egg colour. It has been proposed that blue shell colouration is under simple autosomal dominance (Punnett 1933; Stevens 1991) that involves independent pairs of alleles at two loci (Collias 1993), although this may be an over-simplification. Collias (1993) suggested a two allele system, and categorized egg colours

as "white," "emarld," or "turquoise." Although this work carefully describes what was known about eggshell pigmentation at the time, these colour classifications do not currently have an adequate pigment strategy to explain them, nor was there any attempt to use an analytical approach to quantify them. If future work should find other pigments in avian eggs, this genetic control mechanism may provide an adequate explanation. More careful genetic studies outlined a similar system in the Japanese quail (*Coturnix japonica*) (Ito et al. 1993). An eggshell colour mutation, known as celadon, entered a captive population and produced blue-green eggshells. This mutation was controlled by an autosomal recessive gene (*ce*) and is located on a different locus than the gene controlling white eggshells in Japanese quail (*we*). These loci are not linked, but the phenotypic expression of *ce* is masked by the expression of *we* (Ito et al. 1993). In combination, these two studies provide evidence for a two-allele system for the genetic control of egg colour.

A recent five-year study has established heritability measures for blue-green eggshell colour in a population of pied flycatchers (*Ficedula hypoleuca*), and has shown that in this population, within-clutch standard deviation in blue-green chroma and egg brightness were the most heritable aspects of eggshell colouration (Morales et al. 2010). In addition, investigations into the inheritance of eggshell spotting has shown that this trait is sometimes linked to the female W chromosome (Gosler et al. 2000), while in other cases it is not (Mahler et al. 2008). These investigations establish that there is a genetic component to egg colouration on which selection may operate, despite there also being a significant environmental component (Avilés et al. 2007; Jagannath et al. 2008; Morales et al. 2011).

**Objective colour measurement**

Although there are numerous methods for quantifying colour (Andersson and Prager 2006; Montgomerie 2006), and many different colour spaces in which colours may be modeled (Wyszceki and Stiles 1982; Endler and Mielke 2005), I will restrict this discussion to the field of spectroscopy, which is the technique I used in the following chapters. Spectroscopy involves the quantification of light emitted from surfaces. The reflectance of a surface is defined as the ratio of reflected light to incident light across a range of wavelengths (Wyszceki and Stiles 1982). In behavioural sciences, reflectance is often expressed as a percentage relative to a white standard. The wavelengths of light are measured in nanometers (nm). A perfectly white object should reflect at 100% across all wavelengths, and the reflectance of other achromatic colours should be similarly even across all wavelengths but at increasingly lower reflectance levels as you progress from white through grey to black. Throughout this dissertation I use a WS-1 Spectralon-based white standard, which provides 96% reflectivity between 300 – 400nm, and 99% between 400 – 700nm (Ocean Optics, Dunedin, FL).

Reflectance is generally measured with a device known as a spectroradiometer. This device measures radiometric quantities across a wavelength range (Wyszceki and Stiles 1982). A spectrophotometer measures both the reflectance and transmission of light, while simultaneously examining the radiant power of an object at each wavelength relative to incident light. There has been confusion about the terminology regarding the equipment commonly employed by researchers measuring the reflectance of animal surfaces. The data collected by a spectroradiometer is compared to a reference light

source, and then percent reflectance across the wavelength range can be determined from these data. These conversions are conducted automatically with most end-user applications (Ocean Optics, Dunedin, FL). Andersson and Prager (2006) provide a good general rule of thumb: if your instrument "measures the spectral composition of the radiation as a function of wavelength, it is a spectroradiometer" (p. 50). However, changes in how spectrometers operate, modern charge-couple device (CCD) spectrometer technology, and integration with computer software seems to be blurring the line between spectroradiometer and spectrophotometer. This is most likely why companies such as Ocean Optics and many researchers opt for the more generic term spectrometer, which is the term I use throughout this dissertation.

Throughout this dissertation I used an external light source which provides full spectrum light through a bifurcated fibre optic cable. This cable comprises six separate fibre optic cables, with the light being delivered through the outer five cables of the bundle. The inner fibre optic cable carries the reflected light back to the spectrometer. This returning light enters the unit and then is redirected to a diffraction grating. The grating of this component is specifically adjusted for each unit, and essentially separates the light much the way a prism would. This refracted light then is focused on a mirror which shines the light on the CCD photo-diode array. These diodes are photosensitive and the light that falls on this array is registered as voltage differences across the elements of the array. These data are simultaneously assessed by the integrated software installed on the computer operating the spectrometer, and reflectance (as well as other output) may then be visualized.

**Colour**

Our concept of colouration is necessarily anthropogenic. However, if we hope to understand the function and evolution of colour signals across diverse taxa, it is necessary to have a broader and more generally applicable appreciation of colouration (Endler 1990; Bennett et al. 1994). In the past, perceptual biases dictated how researchers quantified variation in colour, and these biases influenced theories on animal colour perception (Bennett et al. 1994). This illustrates an important point, that colour is more than just the spectral properties emitted by an object, it is actually a physiological experience for the receiver (Wyszceki and Stiles 1982). A good, psychologically-grounded definition of colour should take this into account. One such definition is that colour is the perceptual ability of an observer to discriminate two equivalently illuminated structures of equal size and shape by differences in the spectral composition of reflected light alone (Wyszceki and Stiles 1982). This definition makes proper measurement difficult, and only recently have our technical abilities caught up with our conceptual knowledge-base.

In terms of natural pigments, most colours are produced through the transfer of electrical charges from one ion to another. This operates under the general umbrella of molecular orbital theory and applies to molecules with alternating single and double bonds (Needham 1974). Generally, larger molecules with multiple rings, or those possessing side groups, have extended pi orbitals, which define the combined wave characteristics of the electrons comprising the molecule (Nassau 1997). These molecules exhibit absorption properties in the human-visible range. These properties are shared by porphyrin and biliverdin as well as most natural pigments (Needham 1974), and the

difference between the structures of porphyrin and biliverdin explain the variation in their absorption spectra. In addition, these differences in orbitals, conjugation, and resonance explain differences in the luminescence properties of these two pigments. This point will be elaborated on more thoroughly in Chapter 6. Human perception has traditionally been used to classify which molecules are considered pigments. For example, although simple benzene rings can be excited in the ultraviolet range (Nassau 1997), these are not considered pigments because humans lack the ability to detect ultraviolet light. Nevertheless, these molecules may be important for organisms with different perceptual abilities (see Avian Vision section, below).

Numerous terms are used to describe colour such as hue, saturation, chroma, and brightness. These are complicated by the colloquial usages of colour terms that are also used in a technical sense (MacAdam 1997). Hue represents the perception of predominant wavelengths of colour (such as red, blue, yellow, etc.). Saturation and chroma can be thought of as the degree of purity of the colour, while brightness refers to its value on a white to black scale (Kelber et al. 2003). In the human visual system, any colour can be explained by two chromatic (hue, saturation) and one achromatic (brightness) aspect of colour. Variation in colours is detected by the combined output of photoreceptors known as rods and cones. These receptors are activated at different thresholds of light. Rods are active in low light and are the predominant photoreceptors used in scotopic conditions such as at night, whereas cones are activated at high light levels often experienced in full daylight (Jacobs 1981; Kelber et al. 2003). Furthermore, cones possess pigments, known as photopigments, which have specific absorptance characteristics. The absorptance properties of the photopigments allow cones to

differentially absorb light across the spectrum based on the photopigment that they possess, and these differences can be used to classify different cone types. To discriminate between colours, a viewer must possess at least two distinct cone types (Jacobs 1981; Wyszceki and Stiles 1982; Kelber et al. 2003); however, possessing multiple cone types does not necessarily equate to possessing colour vision (Chen et al. 1984; Chen and Goldsmith 1986). In addition to these reception prerequisites, the perception of colour is also dependent on subsequent neurological stages (Jacobs 1981). Careful physiological, neurological, and behavioural experimentation are necessary to determine if an animal has colour vision (Jacobs 1981; Kelber et al. 2003). Such experimentation has improved our understanding of both mammalian and avian colour vision and has contributed significantly to the study of animal behaviour (Vorobyev et al. 2001; Goldsmith and Butler 2003, 2005).

**Visual systems of avian nest predators**

An appreciation for the visual abilities of potential predators has important implications for avian egg colour (Ricklefs 1969; Bosque and Bosque 1995; Cain et al. 2006). Aside from birds, mammals and reptiles are important nest predators of birds (Ricklefs 1969; Bosque and Bosque 1995; Weatherhead and Blouin-Demers 2004; Sinclair et al. 2005; Cain et al. 2006). Snakes may arguably be the most important avian nest predators in some parts of the world (Weatherhead and Blouin-Demers 2004). Although the visual system of snakes remains poorly described, the photopigments of at least one species seem to be primarily adapted for low light vision and motion detection rather than colour vision (Sillman et al. 2001). Nevertheless, colour may still be an

important cue in prey detection, especially when used in combination with other signal reception modalities (de Cock Buning 1983). Mammals also rely heavily on non-visual signaling modalities (Alberts 1992), although colour has been shown to act as an important visual cue in this group (Wells and Lehner 1978; Jacobs 1993) and is therefore worthy of being addressed. Variation in mammalian colour vision is quite high because mutations within the opsin gene that controls photopigment expression are common (Kelber et al. 2003). Unfortunately, little of this diversity has been subjected to rigorous examination among mammals. Even when information on spectrally distinct cone types is available, mammalian visual abilities have not often been examined behaviourally. We do have a general understanding of some commonalities in colour vision across this class. Generally, mammals are classified as dichromats, meaning that they have only two cone types, and this distinction results in marked differences from our own trichromatic vision. When considering the six mammalian families representing the most important avian nests predators (Sinclair et al. 2005), there is variation in the sensitivity of both cone types (Canidae: 429 and 555 nm, Felidae: 450 and 555 nm, : 444 and 543 nm in tree squirrels, 436 and 518 nm in ground squirrels, Muridae: 360 and 512 nm, Procyonidae: unknown and 560, Didelphidae: unknown and 560; *reviewed in,* Jacobs 1993). In dichromats, the spectral sensitivities of both photopigments dictate which colours are differentiable. Primates are also common nest predators; however their visual systems vary across the order, and even within a species between sexes. Colour vision is important for successful foraging in a number of primate species, and trichromacy is thought to be an adaptation for this lifestyle in some primates (Mollon 1989; Osorio and Vorobyev 1996). Old world primates tend to be trichromatic, and new world primates

tend to be dichromatic or trichromatic or a combination of both (Jacobs et al. 1996; Kelber et al. 2003). These colour vision abilities may explain the relatively high occurrence of primate induced nest predation (Olmos 1990; Tarwater 1998; Robinson and Robinson 2001). Birds also possess excellent colour vision and are another important source of avian nest predation, and the colour of nest contents appears to be an important factor regulating this pressure (Blanco and Bertellotti 2002; Castilla et al. 2007).

**Avian vision**

Birds possess four spectrally distinct photopigments and have tetrachromatic vision (Bennett et al. 1994; Church et al. 2001; Hart 2001a; Maddocks et al. 2001; Bennett and Thery 2007). In birds, all four photopigments are involved in colour vision (Church et al. 2001). These photopigments are sensitive over a wide spectral range from approximately 320 to 700 nm (Chen et al. 1984; Church et al. 1998; Withgott 2000; Hunt et al. 2001; Ödeen and Håstad 2003). The four classes of avian photopigments are sensitive over different wavelength ranges, which include long-wave-sensitive (LWS; $\lambda$max 543 - 571 nm), medium-wave-sensitive (MWS; $\lambda$max 497 - 509 nm), short-wave-sensitive (SWS; $\lambda$max 430 - 463 nm), and either violet-sensitive (VS; $\lambda$max 402 - 426 nm) or ultraviolet-sensitive (UVS; $\lambda$max 355 - 376 nm). Although there are interspecific differences in the wavelength of maximum sensitivity for these visual pigments (Hart 2001b), the absorption characteristics of these photoreceptors are generally similar across all birds (Hart et al. 2000; Cuthill 2006). In addition to these photopigments, birds (as well as some fishes, amphibians, and reptiles) possess oil droplets that absorb lower wavelengths and effectively narrow the cone sensitivity curves. This reduces the overlap

between cone type sensitivities, which ultimately improves discriminability between

colours (Bowmaker et al. 1997; Hart 2001b).

**Illustrating the diversity of avian egg colour**

The colour of birds' eggs has captured the interest of artists, philosophers, and scientists

for millennia (Stagiritis 350 BC; Wallace 1889; Purcell et al. 2008). When examining the

diversity of colours and forms of patterns found across species (Figure 3), it is no wonder

why people have been drawn to this trait. Although this diversity is believed to be

produced by only two pigment classes (Kennedy and Vevers 1976; Gorchein et al. 2009),

the dramatic variation in egg colour between species suggests that the mechanisms of

colouration remain to be fully explained. Colours on the surface of avian eggs reflect

many hues within the human visual range (400 – 700 nm). Reflectance spectra for species

that differ in visually perceived egg colour illustrate that the reflectance properties

between these eggs are indeed quite different. The blue-green colouration commonly

found in avian eggs is generally similar across species; this colour varies most often in

terms of chroma, with some species (Figure 3B) exhibiting higher and narrower

reflectance peaks than other species (Figure 3A). As mentioned earlier, the ground

colouration can comprise a combination of biliverdin and porphyrin, which can result in

olive, brown, or blue-green colours (Figure 3C). Although green eggs are rare, some

species such as the elegant crested tinamou (*Eudromia elegans*) exhibit remarkably green

eggs (Figure 3D). These spectral curves have a fundamentally different spectral shape

than those of blue-green eggs (Figures 3A -B), and of blue-green eggs created through a

mixture of blue-green and brown pigments (Figure 3C). The light buff brown colour

produced by fine speckling creates the overall impression of a light brown colour (Figure 3E). Although the deep brown colour found in the eggs of *Nothura boraquira* are fairly uncommon (Figure 3F), these colours do occur. Unlike many other egg colours, the deep chocolate brown colours found in this species are very dark and result in relatively low reflectance across the spectrum.

**Concluding remarks**

Avian egg colouration is remarkably variable across species (Walters 2006). This variation is primarily produced by the differential contribution of two related pigment classes that are integrated into the eggshell matrix while the eggs are *in utero* (Romanoff and Romanoff 1949). In this dissertation, I adopt a comprehensive approach to understanding variation in egg colour, ranging from the properties of the pigments themselves to the evolutionary factors influencing the evolution of egg colouration. Here, I provide a brief summary of the chapters that follow.

In Chapter 2, I test the hypothesis that blue-green egg colour may indicate female quality in the ring-billed gull (*Larus delawarensis*). To this end, I combine an observational and experimental approach to evaluate multiple assumptions of the sexual signalling hypothesis: 1) blue-green egg colour is limiting, 2) high quality females produce more chromatic eggs, 3) egg colour reflects offspring quality, 4) males exhibit post-mating sexual selection based on this proposed egg colour signal.

In Chapter 3, I examine egg colouration in a non-signalling context in a related gull species, the herring gull (*Larus argentatus*). Specifically, I used the world's longest-running environmental monitoring program examining changes in environmental

contaminants, and their influence on avian populations of the Great Lakes. This investigation is the first of its scale to examine the relationship between egg colour and contaminants.

In Chapter 4, I investigate the role of egg colouration as a potential cue or signal to conspecifics and heterospecifics. This large-scale comparative analysis involved the reconstruction of a super-tree that includes representatives of all avian orders (except sandgrouse, Pteroclidiformes). In this chapter I examine the hypothesis that egg colours influence predation levels and found that in open-nesting species, predation pressure was positively related to eggshell brightness. I also examine the blackmail hypothesis we recently proposed (Hanley et al. 2010), which suggests that females lay colourful eggs to coerce males into providing additional care. For this hypothesis to operate, risk needs to be associated with certain egg colours and parents need to compensate for this risk. In addition I examine the sexual signalling hypothesis suggesting that egg colour indicates female quality and the sensory bias hypothesis suggesting that egg colour is selected based on inherent colour preferences. Moreover, I examine the possibility that egg colour enhances egg recognition in the context of brood parasitism and dense coloniality.

In Chapter 5, I use the same comparative information to investigate whether broad environmental and ecological factors, such has habitat type and the form of nest, are important selective agents for the evolution of egg colour. In particular, I test whether eggs have been selected to be brighter in cavity nests, if egg pigments have evolved to protect eggs from microbial invasion, and whether egg pigments provide protection from direct solar radiation. Together with chapter 4, this research represents the largest comparative examination of the evolution of egg colour to date.

In Chapter 6, I provide the first documentation that avian eggshells phosphoresce, and provide evidence to suggest that porphyrin within the shell matrix is the source of eggshell phosphorescence. This property does not appear to negatively influence eggshell reflectance; however, future egg colour research should utilize light sources that include ultraviolet irradiance because this would best approximate natural lighting conditions. Phosphorescence has both applied and evolutionary implications. Specifically, this property may be diagnostic of the presence of proto-porphyrin within an egg, and is likely related to the photo-dependent anti-microbial properties recently discovered in porphyrins (Ishikawa et al. 2010).

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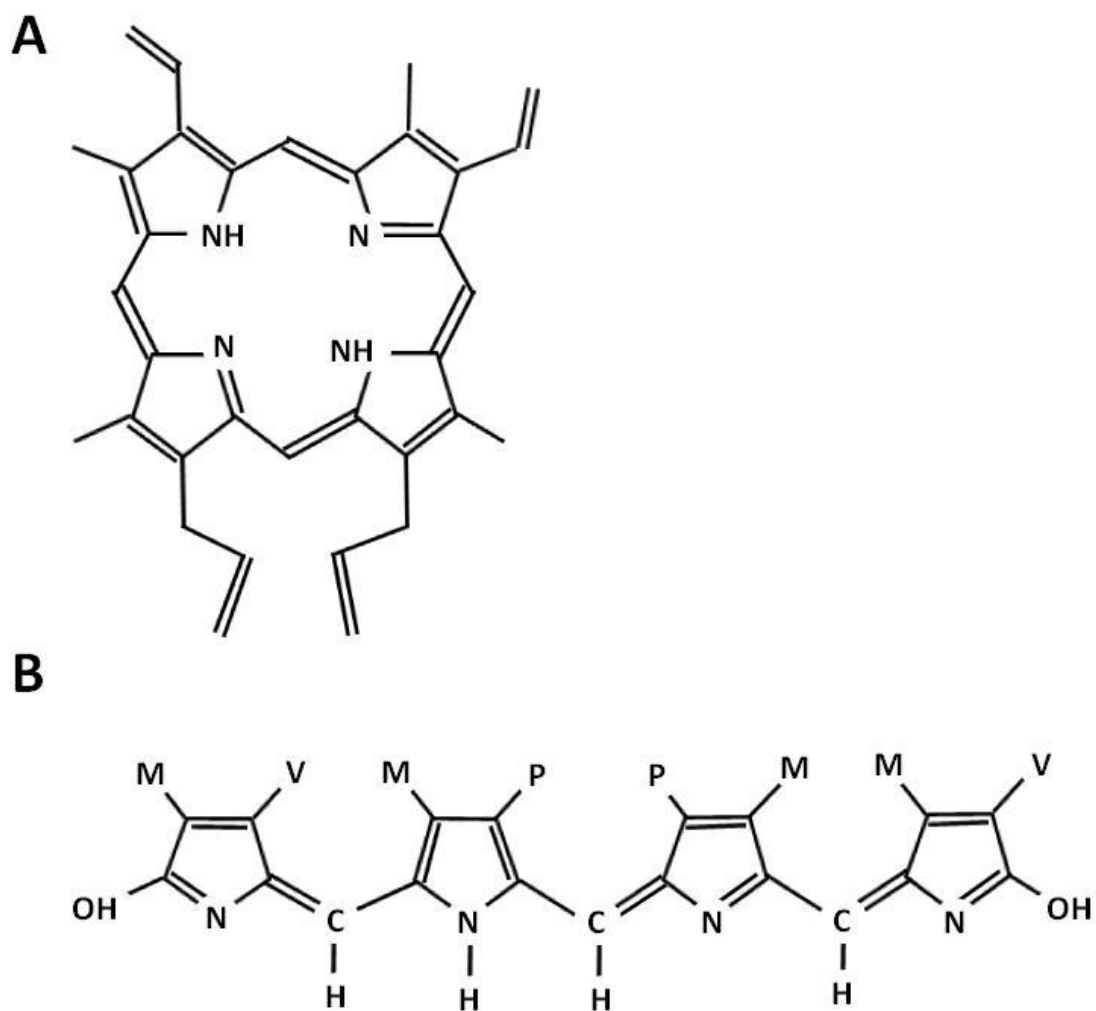
**Figure 1. 1**

The molecular structure of A) proto-porphyrin and B) biliverdin. These figures were

produced with XDrawChem v 1.9.9 (Herger 2010).
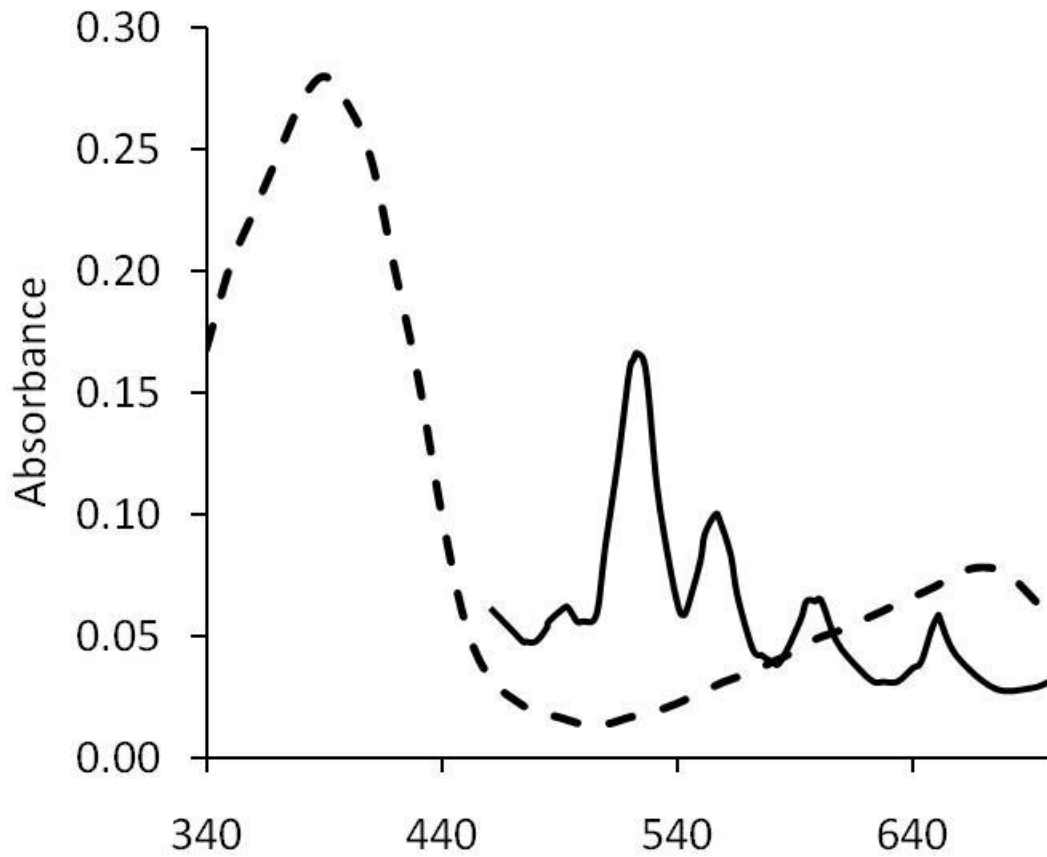
**Figure 1. 2**

The absorptance spectra of proto-porphyrin (solid line), and biliverdin IXα (dashed line).

These data are redrawn from Ding and Xu (2002), and Scalise and Durantini (2004).

Wavelength (nm)
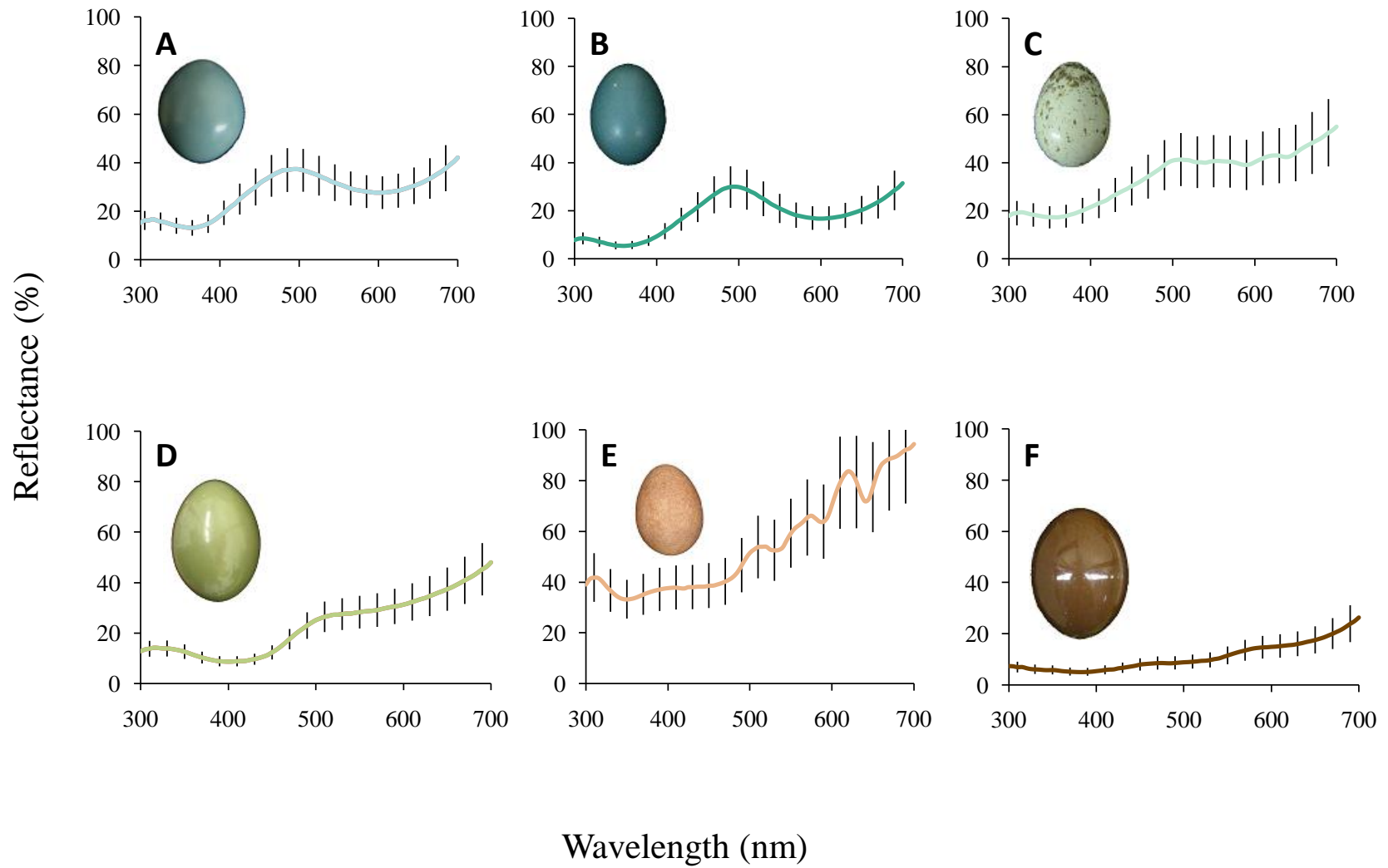
**Figure 1. 3**

Average reflectance spectra (± SE) of six species exhibiting some of the variation seen across avian eggs. This variation encompasses the bright blue-green of *Tinamus major* (A), the deep blue-green of *Dumetella carolinensis* (B), the brighter blue-green of *Corvus brachyrhynchos* (C), the grass green of *Eudromia elegans* (D), the buff brown created by fine speckling in *Campylorhynchus brunneicapillus* (E), and the deep chocolate brown of *Nothura boraquira* (F).

# CHAPTER 2: EGG COLOURATION IN RING-BILLED GULLS (*LARUS DELAWARENSIS*): A TEST OF THE SEXUAL SIGNALING HYPOTHESIS[1]

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[1] This manuscript is the product of joint research

**Chapter summary**

Although many avian eggs appear to be cryptically coloured, many species also lay vibrant blue-green eggs. This seemingly conspicuous colouration has puzzled biologists since Wallace, as natural selection should favor reduced egg visibility to minimize predation pressure. The sexual signaling hypothesis posits that blue-green egg colouration serves as a signal of female quality, and that males exert post-mating sexual selection on this trait by investing more in the nests of females laying more intensely blue-green eggs. This hypothesis has received mixed support to date, and most previous studies have been conducted in cavity-nesting species, where male evaluation of his partner's egg colouration, relative to that of other females, may be somewhat limited. Here, we test the sexual signaling hypothesis in colonially nesting ring-billed gulls (*Larus delawarensis*), where males have ample opportunity to assess their mate's egg colouration relative to that of other females. We used correlational data and an experimental manipulation to test four assumptions and predictions of the sexual signaling hypothesis: (1) blue-green pigmentation should be limiting to females; (2) extent of blue-green egg colouration should relate to female quality; (3) extent of blue-green egg colouration should relate to offspring quality; (4) males should provide more care to clutches with higher blue-green chroma. Our data provide little support for these predictions of the sexual signaling hypothesis in ring-billed gulls. In light of this and other empirical data, we encourage future studies to consider additional hypotheses for the evolution of blue-green egg colouration.

**Introduction**

The evolution of conspicuous traits, such as elaborate displays and vibrant colours, has long interested biologists and naturalists (Darwin 1871; Wallace 1889). While theoretical models and empirical studies have provided a satisfying explanation for the evolution of sexually selected ornaments (Andersson 1994), other exaggerated traits remain perplexing. One particularly bewildering example is that of conspicuous egg colouration. In several avian species, females lay eggs that are strikingly blue-green in colour (Underwood and Sealy 2002; Moreno and Osorno 2003; Kilner 2006). This blue-green colouration is acquired through deposition of a blue-green pigment called biliverdin into the eggshell (Kennedy and Vevers 1976).

For more than a century, researchers have sought adaptive explanations for the evolution of blue-green egg colouration (Kilner 2006). A number of hypotheses have been proposed, including aposematism (Swynnerton 1916; Cott 1948), thermoregulation (McAldowie 1886; Bakken et al. 1978; Lahti 2008), egg recognition (Victoria 1972; Jackson 1992; Soler and Møller 1996), and crypsis (Lack 1958). Despite a substantial amount of research devoted to this topic, the adaptive significance of blue-green egg colouration remains a matter of debate, as these hypotheses either remain inconclusive (Underwood and Sealy 2002; Kilner 2006) or have been largely discredited (Lack 1958; Kilner 2006). Moreover, a recent comparative analysis failed to yield new insight into the adaptive significance of blue-green egg colouration, despite addressing multiple hypotheses using a comprehensive dataset spanning all of Aves (Kilner 2006).

Recently, Moreno and Osorno (2003) proposed a novel hypothesis for the evolution of blue-green egg colouration. They suggested that blue-green egg

pigmentation acts as a sexually-selected, condition-dependent signal of female quality. Moreno and Osorno (2003) reasoned that since biliverdin has been shown to have antioxidant properties (Kaur et al. 2003), females should balance the use of biliverdin for protection against free radicals and for deposition into eggshells. The sexual signaling hypothesis proposes that only high quality females can afford the costs of depositing large amounts of biliverdin during the laying period, a time of high oxidative stress. Males should in turn respond to this signal by increasing their investment in clutches with more deeply pigmented blue-green eggs (Moreno and Osorno 2003). The intraspecific assumptions and predictions arising from this hypothesis can be divided into four main categories. First, blue-green egg pigmentation should be limiting and costly to deposit. Second, degree of blue-green egg pigmentation should relate to female quality. Third, degree of blue-green egg pigmentation should relate to offspring quality. Fourth, males should exert post-mating sexual selection on this trait by providing greater paternal investment to nests with more intensely pigmented blue-green eggs.

The sexual signaling hypothesis has been investigated in a number of species, but support for the hypothesis has been mixed. For example, a positive association between male parental investment and blue-green egg colouration was documented in some studies (Moreno et al. 2004; Moreno et al. 2006b; Soler et al. 2008) but not in others (Krist and Grim 2007; Lopez-Rull et al. 2007). These findings, among others, suggest that more research needs to be undertaken to assess the general applicability of the sexual signaling hypothesis.

In this study, we investigated the sexual signaling hypothesis in ring-billed gulls (*Larus delawarensis*). This species is well suited for addressing the sexual signaling

hypothesis because both males and females care for offspring, and these birds usually nest in large, densely-packed colonies that provide ample opportunity for direct comparison of egg colour across females (Ryder 1993). Additionally, females lay variably coloured eggs, with some females laying particularly blue-green eggs and others laying eggs that are brownish in colour. This degree of variation could, in theory, facilitate assessments of relative mate quality based on egg colour. Interestingly, all tests of the sexual signaling hypothesis to date have been conducted in cavity nesters or species that defend all-purpose nesting territories. In nest cavities, low light conditions may reduce visibility and make egg colouration more difficult to assess (Aviles et al. 2006). In species that defend all-purpose nesting territories, including some cavity-nesting species, territorial intrusions may make egg colour assessments relatively costly, and the distance between nests prevents males from making direct comparisons of egg colour between females.

We tested the following four assumptions and predictions of the sexual signaling hypothesis using a combination of correlational and experimental data. (1) If blue-green pigmentation is limiting, we expected that blue-green chroma would decrease with laying order. We expected this pattern because egg laying is particularly energetically demanding in gulls (Ricklefs 1974), and because the level of a potent antioxidant is known to decrease over the laying period in a congener (Monaghan et al. 1998). (2) If blue-green pigmentation signals female quality, an important assumption of the hypothesis, we expected a positive relationship between female health and condition and the blue-green chroma of her eggs. (3) If blue-green pigmentation signals offspring quality, we predicted that chicks hatched from eggs with higher blue-green chroma would

be larger than chicks hatched from less chromatic eggs. (4) If males exert post-mating sexual selection based on blue-green egg colouration, we predicted that males mated with females who laid more chromatic eggs would invest more in those clutches.

**Materials and Methods**

*Study species and study site*

From 1 May to 14 July 2007, we studied ring-billed gulls near Windermere Basin in Hamilton, Ontario (43°15'49.30" N, 79°46'54.83" W). The ring-billed gull is a largely monogamous, colonial species. Males and females cooperate in building nests on the ground in low, open areas. Males and females share nearly equally in incubation, brooding, and feeding young (Ryder 1993). Clutches are generally complete in 3-5 days, and incubation lasts 25 days (Ryder 1993). In our study, most clutches were initiated on 4 May 2007 (mode), and hatched on 31 May 2007 (mode). Super-normal clutches are known to occur in this species (Conover et al. 1979), and these would complicate our study because these result from multiple females laying eggs into a single nest, or a male pairing with two females at a single nest. Previous work has shown that 98% of 2-3 egg clutches are from male – female pairings (Conover 1989). As a conservative means of excluding super-normal clutches, we restricted our analysis to clutches with three or fewer eggs. Therefore, our average clutch size for clutches with colorimetric data was 2.9 $\pm$ 0.5 ($n = 81$).

We captured adult gulls in circular walk-in wire mesh traps placed on nests 9.69 $\pm$ 2.6 days prior to egg hatching (see Brown 1995). For each individual captured, we recorded tarsal length, bill length, length of head from tip of bill to base of skull, length

of the exposed culmen, depth at the gonys, and wing chord (to the nearest mm), as well as

mass (to the nearest gram).  We obtained blood from adult birds by puncturing the

brachial vein with a 26 ½ gauge needle, and drawing up a small amount of blood using a

heparinized capillary tube. This blood was used to calculate heterophil to lymphocyte

ratio in females (see below). We used a standard discriminant function (Ryder 1978) to

determine sex upon first capture. This discriminant function is based on morphometric

measurements and has a validated accuracy of 95.0%. Since the male is always larger

than his female partner (Ryder 1993), we were able to confirm these classifications based

on morphometric measurements when we caught both members of a mated pair. In

addition, we confirmed these sex classifications based on visual size comparisons and

behavioral observations. To facilitate visual identification of individual birds during

behavioral observations, we applied unique combinations of coloured leg bands as well

as Nyanzol dye markings on the head or wings.

*Egg colour quantification*

Female ring-billed gulls lay eggs that range from deep brown to deep olive-green

or paler blue-green in ground colouration, with a variable amount of dark brown

maculation (Figure 1; Ryder 1993; Baicich and Harrison 1997). These eggs are visually

similar in colouration to those of herring gulls (*Larus argentatus*) and black-headed gulls

(*Larus ridibundus*), the ground colouration of which is known to result from a

combination of protoporphyrin and biliverdin pigmentation (Kennedy and Vevers 1976).

We quantified the colouration of ring-billed gull eggs using a USB 4000

spectrophotometer with a PX-2 pulsed xenon light source and a Spectralon white

standard (Ocean Optics, Dunedin, FL). For each egg, we measured reflectance on three different regions of the egg: the lower portion, the medial portion, and the upper portion. We took two measurements within each region, each of which comprised 30 readings averaged by the spectrophotometer operating software (OOIBase32), and used the mean of these readings in our analyses since colorimetric variables were highly repeatable within eggs (see below). Because maculation likely results entirely from protoporphyrin pigmentation (Kennedy and Vevers 1976), we only measured patches of ground colouration free of maculation, as blue-green pigmentation was a focus of our study. Visual inspection of reflectance spectra revealed that, as with other gulls (Kennedy and Vevers 1976), the ground colouration of ring-billed gull eggs is likely produced by a combination of biliverdin and porphyrin pigmentation. Most spectra had a series of long-wavelength peaks and troughs, as expected from the absorbance properties of protoporphyrin pigmentation (Scalise and Durantini 2004), and greenish eggs exhibited proportionally greater reflectance in the blue-green portion of the spectrum, as expected from patterns of biliverdin absorbance (Figure 1; Ding and Xu 2002; Falchuk et al. 2002).

We summarized variation in egg colour using two colorimetric variables (Montgomerie 2006). We calculated blue-green chroma as the proportion of reflectance in the blue-green portion of the spectrum (450-550 nm). Similarly, we calculated red chroma as the proportion of reflectance in the red (600-700 nm) portion of the spectrum. We chose narrow ranges for these two variables to encompass the maximum reflectance generated by biliverdin (Ding and Xu 2002) and porphyrin (Scalise and Durantini 2004) pigmentation. Since pigment deposition has a subtractive influence on reflectance, it is unlikely to mask the independent effects of other pigments unless it absorbs strongly

across all wavelengths. In addition, average clutch blue-green and red chroma were not correlated (r = - 0.12, N = 80, $p$ = 0.28, $CI_{0.95}$ = -0.33 to 0.10), suggesting that these two variables revealed different information about egg colouration. We did not include other colorimetric variables, such as hue, brightness, and other measures of chroma, as these tended to be correlated with either blue-green or red chroma and were therefore redundant (all $p$ < 0.0001 for either blue-green or red chroma). Blue-green and red chroma were highly repeatable across the different parts of each egg (blue-green chroma: r = 0.84, $p$ < 0.0001; red chroma: r = 0.71, $p$ < 0.0001; Lessells and Boag 1987) and we therefore used an average value for each egg in our analyses. Based on a subset of 25 eggs measured at two different times, our measurements blue-green and red chroma were very highly repeatable (0.97 and 0.94, respectively, both $p$ < 0.0001; Lessells and Boag 1987).

In most of our analyses, we used the mean colouration of each female's entire clutch.  To ensure that averaging egg colouration within clutches was reasonable, we calculated the repeatability of egg colouration within clutches (Lessells and Boag 1987). If egg colouration reveals female quality, colouration should be repeatable within clutches (Moreno et al. 2004; Krist and Grim 2007). Red and blue-green chroma were significantly repeatable within clutches (repeatabilities: 0.53 and 0.64 respectively, both $p$ < 0.0001), indicating that egg colouration was more variable among than within clutches. This interclutch variation in egg colouration is striking to humans (pers. obs.), and is presumably detectable by the refined colour discrimination abilities of birds (Cuthill 2006).

*Assessing laying order effects*

To determine whether blue-green egg pigmentation might be limiting to females, we compared egg colouration to position in the laying sequence while controlling for nest identification (ID). We monitored laying order by marking the blunt end of each egg with an indelible marker. In most cases, the egg was marked on the day it was laid with its number in the sequence. We used only eggs whose positions in the laying sequence were known in our analyses of laying order effects.

*Assessing female and offspring quality*

As a measure of female quality, we calculated the body condition of each female as size-adjusted body mass using the following equation: mass / (tarsus length + bill length) (Kitaysky et al. 1999; Verboven et al. 2003; Buck et al. 2007). We used tarsus and bill length as measures of structural size since, unlike wing length, these remain constant over the breeding season (Kitaysky et al. 1999). Similar measures of female condition have been shown to relate to immunocompetence, reproductive success, and offspring quality in this (Boersma and Ryder 1983; Meathrel and Ryder 1987), and other gull species (Alonso-Alvarez and Tella 2001; Verboven et al. 2003). Additionally, we calculated heterophil to lymphocyte ratio as a measure of immune stress in females (Davis 2005). We stained blood smears created in the field using a Hema 3 staining kit (Fisher Scientific), and viewed these under oil immersion at 1000X magnification. We counted the numbers of heterophils and lymphocytes until approximately 10,000 red blood cells had been viewed to obtain a heterophil to lymphocyte ratio (H:L ratio). Heterophils are phagocytosing cells of the innate immune system, and lymphocytes

consist primarily of T- and B-cells of the acquired immune system (Norris and Evans 2000). In birds, H:L ratio tends to increase in response to stressors such as disease, parasites, social stress, and starvation (Ots and Hõrak 1996), and represents an integrated measure of immune stress (Salvante 2006).

We calculated two related measures of offspring quality. First, we calculated the fresh egg mass of each egg using Hoyt's (1979) formula ($W = K_w \cdot LB^2$). We measured the length (L) and breadth (B) of each egg on the day its colour was measured, and used the shape-dependent constant calculated by Hoyt (1979) for western gulls (*Larus occidentalis livens*), $K_w = 0.53$, as the shape of their eggs closely approximates that of ring-billed gull eggs. Egg size has been shown to relate to offspring quality and survival in many species (Grant 1991; Hipfner and Gaston 1999), including gulls (Parsons 1970; Lundberg and Väisänen 1979), even when controlling for parental quality (Bolton 1991). Second, we weighed chicks within several hours of hatching as an additional measure of offspring quality. Our sample size is more limited for this analysis as the risk of nest abandonment prohibited our obtaining more complete hatchling weight data. Chick mass has also been shown to relate to health and survival in a number of species (e.g., Moss et al. 1981, Grant 1991).

*Assessing male investment and experimental manipulation*

To investigate whether egg colouration influenced paternal care, we monitored male investment in relation to egg colour at control nests and cross-fostered nests in the same colony. Our control nests consisted of 40 unmanipulated nests. However, any apparent influence of egg colouration on male investment in these control nests could

result as a by-product of males responding to another female trait that is correlated with egg colour, or as a consequence of assortative mating between high quality females that lay intensely coloured blue-green eggs and high quality males that provide high levels of parental care. Therefore, in our experimental treatment, we conducted full clutch swaps for 15 pairs of nests on the day the third egg was laid. This ensured that any correlation between egg colour and male care would be driven by the egg colour *per se*. We chose this experimental design because we wanted to assess male responses to real eggs that exhibited natural variation in colouration. Although some studies have used artificial eggs or painted eggs, it is often difficult to mimic the appropriate spectral shape of egg pigments using these techniques, especially in the ultraviolet range. We assume that our experimental manipulation presented males with differently coloured eggs because original egg colour was not correlated with cross-fostered egg colour for either blue-green ($r = -0.27$, $n = 11$, $p = 0.43$, $CI_{0.95} = -0.79$ to $0.47$) or red chroma ($r = 0.25$, $n = 11$, $p = 0.45$, $CI_{0.95} = -0.49$ to $0.78$). Only 12 of the 30 fully swapped nests and 15 of the 40 controls survived to hatching, were visible for observation after hatching, or were not excluded as super-normal clutches. At one of the cross-fostered nests, the original eggs were depredated at their new location before we had the opportunity to measure their colour.

To determine degree of paternal investment, two observers performed 30-minute observation bouts on focal nests from an observation blind constructed in a central location within the colony. In addition to provisioning offspring, which represents direct investment in parental care, males may also invest in offspring indirectly. We therefore recorded nestling feeding visits, length of brooding bouts, threats towards neighbors

(direct lunge at a neighbor), and long call rate as indicators of male parental investment. Long calling, which is characterized by a gull lowering its head and rapidly throwing it back to shoulder level while calling, is a known threat display and is also used in pair formation (Ryder 1993). We only included provisioning visits in our analyses if chicks ingested food. We standardized investment rates by the number of chicks in each nest. Parental feeding rate is known to decrease during the nestling period (Ryder 1993), and we therefore focused our observations on the first 11 days after hatching to minimize this effect  (3.97 ± 1.56 observations per nest, range between 2 – 7). We also tested for relationships between nestling age and paternal investment within this age class. When controlling for nest ID, hatchling age was not predictive of male feeding rates ($F_{27,75}$ =1.22, $R^2 = 0.30$, $p = 0.25$; *hatchling age*: $p = 0.41$), brooding lengths ($F_{27,75} = 1.03$, $R^2 = 0.27$, $p = 0.45$; *hatchling age*: $p = 0.03$), or threatening rates ($F_{27,75} = 3.52$, $R^2 = 0.56$, $p < 0.0001$; *hatchling age*: $p = 0.11$). Male long call rate did significantly increase with nestling age ($F_{27,75} = 4.12$, $R^2 = 0.60$, $p < 0.0001$; *hatchling age*: $p = 0.0002$), and we therefore used the residuals of this regression in our analyses. We averaged these measures of investment recorded over multiple observations within each nest for our analyses. Since male effort may depend on the effort provided by his partner, we also considered proportional male investment. We found that proportional male care did not change with hatchling age for any investment variable (all $p > 0.26$), and we therefore averaged proportional effort recorded over multiple observations within each nest for our analyses. Our experimental manipulation did not appear to unduly affect male behavior, since there was no overall difference between control and cross-fostered nests in terms of male provisioning ($F_{1,25} = 1.55$, $R^2 = 0.06$, $p = 0.22$, $d = 0.48$, $CI_{0.95} = -0.31$ to $1.28$), male

threatening at the nest ($F_{1,25} = 0.74$, $R^2 = 0.03$, $p = 0.40$, $d = $ -0.33, $CI_{0.95} = $ -1.12 to 0.46),

male long call rate ($F_{1,25} = 3.17$, $R^2 = 0.11$. $p = 0.09$, $d = $ -.69. $CI_{0.95} = $ -1.50 to 0.12), or

male brooding length ($F_{1,25} = 2.79$, $R^2 = 0.10$, $p = 0.11$, $d = $ -0.65, $CI_{0.95} = $ -1.45 to 0.16).


*Statistical Analyses*

We used transformations to normalize data where necessary. We used generalized

linear models with nest identity as a random factor to assess the relationship between

colorimetric variables and laying order or offspring quality. We used simple correlations

to assess the relationship between our measures of female quality and average clutch

colouration. Similarly, we used correlations to determine the association between chroma

variables and paternal investment in control nests. For treatment nests, we used multiple

regression analyses with original and cross-fostered chromas as predictor variables and

measures of paternal investment as dependent variables. Some sample sizes vary because

we were unable to obtain all measurements for all individuals or eggs included in this

study.

We present standardized measures of effect size, and the confidence intervals (CI)

around those measures, where possible, to facilitate the interpretation of non-significant

results in our study (Nakawaga and Cuthill 2007). Standardized effect sizes estimate the

degree to which the null hypothesis is likely to be false (Cohen 1988; Nakagawa and

Foster 2004). Presentation of confidence intervals around the effect size is particularly

useful for the interpretation of non-significant results (Colegrave and Ruxton 2003;

Nakagawa and Foster 2004). Small effect sizes with corresponding CIs that encompass

zero provide support for the null hypothesis, indicating no real effect or a trivial effect if

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the null hypothesis is false. Standardized effect sizes can also be used to compare studies despite variation in sample sizes, and are useful for meta-analyses (Nakagawa 2004; Nakagawa and Cuthill 2007) and preferable to reporting retrospective power analyses (Colegrave and Ruxton 2003; Nakagawa and Foster 2004).

**Results**

*Biliverdin as a limiting factor*

If blue-green egg pigmentation is limiting in this species, we expected to see a decline in blue-green egg chroma with laying order. When controlling for nest identity, we found a relationship between the level of blue-green egg chroma and position in the laying order: the $2^{nd}$ egg had higher blue-green chroma than the other eggs (Figure 2; *whole model:* $F_{62,84} = 5.62$, $R^2 = 0.81$, $p < 0.0001$; *nest:* $p < 0.0001$; *laying order:* $p = 0.003$). In a similar model, laying order did not predict red chroma (*laying order:* $p = 0.08$).

*Egg colouration as a signal of female quality*

If blue-green egg colouration evolved as a signal of female quality, an important assumption of the sexual signaling hypothesis, it should correlate with female quality. We used female body condition index as a measure of female quality and H:L ratio as a measure of female immune stress. We found no association between female body condition index and either colorimetric variable (*blue-green chroma; r = -0.19, n = 24, p = 0.38,* $CI_{0.95} = -0.55$ to 0.23; *red chroma; r = 0.04, n = 24, p = 0.84,* $CI_{0.95} = -0.37$ to 0.44). Similarly, we found no relationship between female H:L ratio and either

colorimetric variable  (*blue-green chroma; r* = 0.03, *n* = 22,  *p* = 0.88, $CI_{0.95}$ = -0.39 to

0.44; *red chroma; r* = 0.05, *n* = 22,  *p* = 0.82, $CI_{0.95}$ = -0.36 to 0.45).

*Egg colouration as a signal of offspring quality*

According to our third prediction, blue-green egg colouration should indirectly

signal offspring quality, as investing in offspring of higher quality is the presumed benefit

of increased male investment in more chromatic clutches. Neither blue-green chroma nor

red chroma were significant predictors of fresh egg mass when controlling for nest ID

(Table 1). In a similar model, red chroma, but not blue-green chroma, was a significant

predictor of initial chick mass, such that larger chicks hatched from eggs that had higher

red chroma (Table 1).

*Paternal investment*

According to our fourth prediction, males should invest more in clutches with

more chromatic blue-green eggs. We addressed this prediction using both correlational

and experimental data. In a group of unmanipulated (control) nests, we found that neither

blue-green nor red chroma were correlated with male investment in long call rate, feeding

rate, neighbor threatening rate, and brooding length (all *p* > 0.58 and 0.36, respectively).

However, in control nests, male response to egg colouration could be confounded by

other variables (see Materials and Methods). Therefore, we used an experimental

manipulation to assess male parental care in relation to cross-fostered eggs. In

generalized linear models and regression analyses, neither original nor cross-fostered egg

colour significantly predicted measures of paternal investment (Table 2; Figure 3). We

also assessed proportional male investment, relative to the total investment provided by both parents, in relation to egg colouration in control and cross-fostered nests. We found that in control nests, only proportional male feeding rate was significantly correlated with blue-green egg chroma ($r = 0.56$, $n = 15$, $CI_{0.95} = 0.01$ to $0.85$, $p = 0.03$; all other variables $p > 0.14$ for blue-green chroma, and all $p > 0.60$ for red chroma). Using similar models, we found no measure of proportional male investment related to either original or cross-fostered blue-green or red chroma (all $p > 0.13$ and $p > 0.33$, respectively) in cross-fostered nests.

**Discussion**

In this study, we evaluated whether the sexual signaling hypothesis might explain egg colour variation in ring-billed gulls. We tested four assumptions and predictions of this hypothesis: that blue-green egg chroma would decrease over the laying period, that female health and condition would be positively correlated with the blue-green chroma of her eggs, that more chromatic blue-green eggs would be larger and would produce larger chicks, and that males would preferentially invest in clutches with more chromatic blue-green eggs. We found little support for these predictions, and therefore conclude that the sexual signaling hypothesis is unlikely to explain variation in blue-green egg colouration in ring-billed gulls.

A key assumption of the sexual signaling hypothesis is that blue-green egg pigmentation honestly reveals female quality and should therefore be limiting to females, such that only high-quality females can afford the cost of biliverdin deposition (Moreno and Osorno 2003). If biliverdin is limiting, we expected a negative relationship between

blue-green egg chroma and position in the laying order. Position in the laying order did influence blue-green chroma; however, the direction of the effect was rather ambiguous, with the second egg being more chromatic. We expected a negative relationship because of the comparatively high oxidative and energetic costs of egg laying in gulls (Ricklefs 1974; Monaghan et al. 1998), and because levels of a potent antioxidant decrease across the laying period in a congener (Royle et al. 2001). Under these stressful conditions, antioxidant limitation could be manifested as a decreased ability to deposit the pigment as the laying sequence progresses (Moreno and Osorno 2003). Alternatively, one could argue that the sexual signaling hypothesis should favor homogeneous pigment deposition across the clutch. Although egg colouration was more similar within clutches than between clutches, our analysis shows that pigment deposition was not homogeneous across laying order. Three other studies have documented laying order effects on blue-green egg colouration, including an increase in blue-green chroma (Siefferman et al. 2006), a decrease in egg brightness (Moreno et al. 2005), and a non-linear decrease in blue-green egg chroma (Krist and Grim 2007). Taken together, these studies suggest that there is no generalized relationship between laying order and blue-green egg pigmentation across species. Interestingly, one recent study found little difference between biliverdin levels in serum and excreta for hens laying blue-shelled and brown-shelled eggs; however, biliverdin levels differed significantly in the shell gland for these same females, suggesting that the biliverdin used in eggshell pigmentation is synthesized directly in the shell gland, and that it may be largely independent of circulating levels of biliverdin (Zhao et al. 2006). Physiological studies assessing whether biliverdin is

limiting to female birds during egg-laying would provide a stronger test of this prediction.

Another assumption of the sexual signaling hypothesis is that blue-green egg chroma signals female quality, and more specifically, female antioxidant capacity (Moreno and Osorno 2003). In this study, there was no significant association between the blue-green chroma of a female's eggs and her body condition index. We also found no association between female H:L ratio and average clutch blue-green eggshell colouration. It would be prudent to consider other measures of quality before ruling out a link between female condition and egg colour in ring-billed gulls. Several studies have supported an association between female quality and egg colour. For example, blue-green egg colouration was found to correlate with age, condition, or immunocompetence in a number of species (Moreno et al. 2005; Morales et al. 2006; Siefferman et al. 2006; Krist and Grim 2007), and two experimental studies have shown that manipulating female condition affects egg colour (Moreno et al. 2006a; Soler et al. 2008). Despite negative results presented here and elsewhere (Moreno et al. 2004; Moreno et al. 2005; Cassey et al. 2008), this is currently the most well-supported assumption of the sexual signaling hypothesis. Nevertheless, experimental manipulations of female antioxidant capacity or oxidative stress, and its resulting effect on egg pigmentation, would present stronger direct tests of this assumption. Moreover, it is important to recognize that other proposed functions of egg colour could yield positive associations between female quality and colour, even if the colour does not function as a signal directed at males (Bakken et al. 1978; Gosler 2005; Highham and Gosler 2006; Martinez-de la Puente et al. 2007).

Under the sexual signaling hypothesis, males should invest more in clutches laid by females of higher quality, as revealed by their egg colouration, because higher quality females should produce higher quality offspring (Moreno and Osorno 2003). We did not find evidence that blue-green chroma significantly predicted egg mass or nestling mass in ring-billed gulls. Nevertheless, an association between offspring quality and eggshell colouration in itself is not sufficient to broadly support the sexual signaling hypothesis, since these pigments may directly benefit the developing embryo without necessarily serving as a signal (Cassey et al. 2008). Studies of the relationship between offspring quality and blue-green egg colour in other species have yielded mixed results, and it is difficult to draw general conclusions since different authors tend to use different quality and egg colour measures. For example, Krist and Grim (2007) found a relationship between blue-green egg chroma and nestling tarsus length, but not mass or T cell mediated immunity. Moreno et al. (2005) found that nestlings had higher immunoglobulin levels, controlling for ectoparasites, when they hatched from eggs that were shifted away from blue-green colouration. Morales et al. (2006) found that blue-green egg chroma was positively associated with egg immunoglobulin levels. Most recently, Soler et al. (2008) found that nestlings supplemented with food showed a negative relationship between T-cell mediated immunity and blue-green egg chroma, whereas unsupplemented nestlings exhibited a positive relationship between the same two variables. Neither Siefferman et al. (2006) nor Lopez-Rull et al. (2007) found a relationship between egg colouration and egg characteristics. These findings suggest that this prediction of the sexual signaling hypothesis might also benefit from further experimental testing.

Our study also tested the prediction that males should provide a disproportionate amount of care to clutches with more chromatic blue-green colouration. We tested this key prediction using correlational data and a cross-fostering experiment. Males did not provide greater parental investment to clutches with more chromatic blue-green eggs in control clutches or experimentally cross-fostered clutches. In addition, male investment did not correlate with original egg colouration. When assessing proportional male investment, we found a positive relationship between blue-green chroma and male feeding rate in control clutches, but not in experimental clutches. No other proportional male investment variables were correlated with either colour variable. Our data suggest that male ring-billed gulls did not preferentially invest in more chromatic blue-green clutches. In pied flycatchers, *Ficedula hypoleuca*, males provided more provisioning to clutches with greater average blue-green clutch colouration (Moreno et al. 2004). A subsequent cross-fostering experiment in this species revealed that males did not adjust provisioning rate in response to average clutch colour, but rather adjusted proportional provisioning rate in response to the standard deviation of egg chroma and maximum egg chroma within a clutch (Moreno et al. 2006b). In the only study where egg colouration was experimentally manipulated, male spotless starlings, *Sturnus unicolor*, provided more care to artificial eggs painted a dark blue-green than to artificial eggs painted pale blue-green (Soler et al. 2008). By contrast, a recent experimental study found that males did not provide higher provisioning to more chromatic clutches in collared flycatchers, *Ficedula albicolis* (Krist and Grim 2007). Another spotless starling study did not support this prediction and showed that males instead used feather ornaments to assess female

quality and provided less care to clutches with more chromatic blue-green eggs (Lopez-Rull et al. 2007).

Using a combination of correlational and experimental data, we found that blue-green egg colouration did not decrease with laying order, did not correlate with female or offspring quality, and did not influence parental investment by males. Taken together, our findings suggest that the sexual signaling hypothesis is unlikely to explain variation in blue-green egg pigmentation in ring-billed gulls. Some of the analyses in our study were based on small sample sizes; however, most of the relationships did not suggest trends in the predicted direction and had low effect sizes with confidence intervals overlapping zero. Although further testing may be required before this hypothesis can be convincingly ruled out in ring-billed gulls, we suggest that other selective factors, such as egg recognition (Victoria 1972; Soler and Møller 1996; Lahti 2005) and crypsis (Lack 1958; Sánchez et al. 2004; Šálek and Cepáková 2006) are likely to play a more important role in explaining egg colour variation in this species. In addition, the ring-billed gull is single-brooded with 62% of pairs remaining together for two consecutive breeding seasons (Ryder 1993), which would suggest strong selection for a pre-mating, not post-mating, signal of quality. Indeed, blue-green egg colouration may have evolved in different avian lineages for different reasons (Kilner 2006). Since the sexual signaling hypothesis continues to receive mixed support in various species, future studies should continue to consider multiple hypotheses for the evolution of egg colouration in birds.

**Acknowledgements**

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**Table 2.1** - Univariate comparisons between egg colouration and indicators of offspring quality (fresh egg mass and hatchling mass), controlling for nest ID, in ring-billed gulls. We present approximate r values as a measure of effect size for fixed factors in these models.

| | $F$ | $R^2$ | $\beta$ | $df$ | $p$ | $r_{approx.}$ |
|---|---|---|---|---|---|---|
| Fresh egg mass | | | | | | |
| whole model | 2.58 | 0.59 | | 80,145 | <0.0001 | |
| nest ID | 2.60 | | | 79,225 | <0.0001 | |
| blue-green chroma | 3.12 | | 0.19 | 1,225 | 0.08 | 0.10 |
| | | | | | | |
| whole model | 2.52 | 0.58 | | 80,145 | <0.0001 | |
| nest ID | 2.55 | | | 79,225 | <0.0001 | |
| red chroma | 0.94 | | 0.09 | 1,225 | 0.33 | 0.05 |
| | | | | | | |
| Hatchling mass | | | | | | |
| whole model | 2.30 | 0.71 | | 20,19 | 0.04 | |
| nest ID | 2.35 | | | 19,39 | 0.04 | |
| blue-green chroma | 0.11 | | 0.12 | 1,39 | 0.74 | 0.06 |
| | | | | | | |
| whole model | 3.24 | 0.77 | | 20,19 | 0.007 | |
| nest ID | 3.40 | | | 19,39 | 0.005 | |
| red chroma | 5.68 | | 0.99 | 1,39 | 0.03 | 0.32 |

**Table 2.2** - Relation between male ring-billed gull parental investment and the colouration of eggs originally laid in their nests (original eggs; OR) and the colouration of eggs swapped into their nests shortly after laying (cross-fostered eggs; CF). We present partial $r$ values as a measure of effect size, and the 95% confidence interval around those effect sizes.

| Male investment | | $F$ | $R^2$ | β | df | p | partial r | CI$_{0.95}$ (lower, upper) |
|---|---|---|---|---|---|---|---|---|
| Long calls | whole model | 2.23 | 0.36 | | 2,8 | 0.17 | | |
| | CF blue-green chroma | 0.45 | | -0.20 | 1,10 | 0.52 | -0.21 | (-0.76, 0.52) |
| | OR blue-green chroma | 4.53 | | -0.62 | 1,10 | 0.07 | -0.56 | (-0.89, 0.16) |
| | whole model | 0.007 | 0.002 | | 2,8 | 0.99 | | |
| | CF red chroma | 0.006 | | 0.03 | 1,10 | 0.94 | 0.02 | (-0.64, 0.67) |
| | OR red chroma | 0.005 | | 0.03 | 1,10 | 0.95 | 0.02 | (-0.64, 0.67) |
| Feeding | whole model | 1.83 | 0.31 | | 2,8 | 0.22 | | |
| | CF blue-green chroma | 1.15 | | 0.33 | 1,10 | 0.31 | 0.32 | (-0.43, 0.81) |
| | OR blue-green chroma | 3.28 | | 0.55 | 1,10 | 0.11 | 0.49 | (-0.24, 0.87) |
| | whole model | 0.86 | 0.17 | | 2,8 | 0.46 | | |
| | CF red chroma | 1.71 | | 0.43 | 1,10 | 0.23 | 0.38 | (-0.37, 0.83) |
| | OR red chroma | 0.18 | | -0.14 | 1,10 | 0.68 | -0.13 | (-0.73, 0.57) |
| Threatening | whole model | 0.70 | 0.15 | | 2,8 | 0.53 | | |
| | CF blue-green chroma | 1.38 | | -0.40 | 1,10 | 0.27 | -0.35 | (-0.82, 0.40) |
| | OR blue-green chroma | 0.18 | | -0.14 | 1,10 | 0.69 | -0.13 | (-0.73, 0.58) |
| | whole model | 0.21 | 0.05 | | 2,8 | 0.81 | | |
| | CF red chroma | 0.03 | | 0.06 | 1,10 | 0.87 | 0.05 | (-0.63, 0.69) |
| | OR red chroma | 0.33 | | 0.20 | 1,10 | 0.58 | 0.18 | (-0.54, 0.75) |
| Brooding | whole model | 0.62 | 0.13 | | 2,8 | 0.56 | | |
| | CF blue-green chroma | 0.03 | | -0.06 | 1,10 | 0.87 | -0.05 | (-0.69, 0.63) |
| | OR blue-green chroma | 1.23 | | -0.38 | 1,10 | 0.30 | -0.33 | (-0.81, 0.42) |
| | whole model | 0.61 | 0.13 | | 2,8 | 0.58 | | |
| | CF red chroma | 1.13 | | -0.36 | 1,10 | 0.32 | -0.32 | (-0.81, 0.43) |
| | OR red chroma | 0.30 | | 0.19 | 1,10 | 0.60 | 0.17 | (-0.55, 0.74) |

Data are from multiple regression analyses. Investment variables are rates controlling for the number of chicks in the nest.
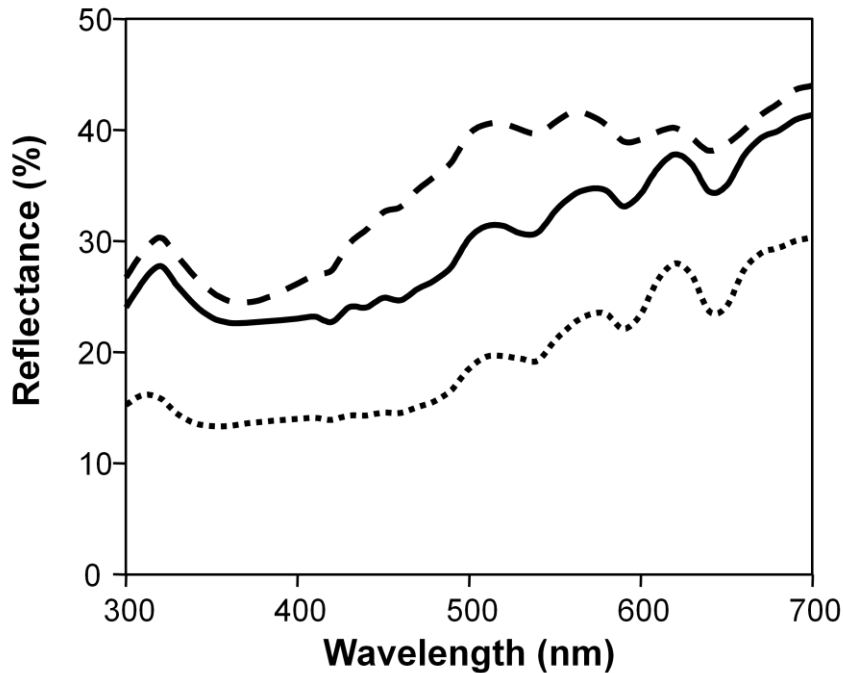
**Figure 2. 1**

Reflectance spectra of ring-billed gull eggs revealing extensive variation in egg colour.

Shown are the mean across all eggs sampled at Windermere Basin, Hamilton, Ontario,

Canada in 2007 (N = 267; solid line), and for a visual reference the mean of a blue-green

egg (dashed line), and a brownish egg (dotted line). The shapes of these spectra result

from the combination of blue-green biliverdin and brown porphyrin pigmentation (Ding

and Xu 2002; Falchuk et al. 2002; Scalise and Durantini 2004).
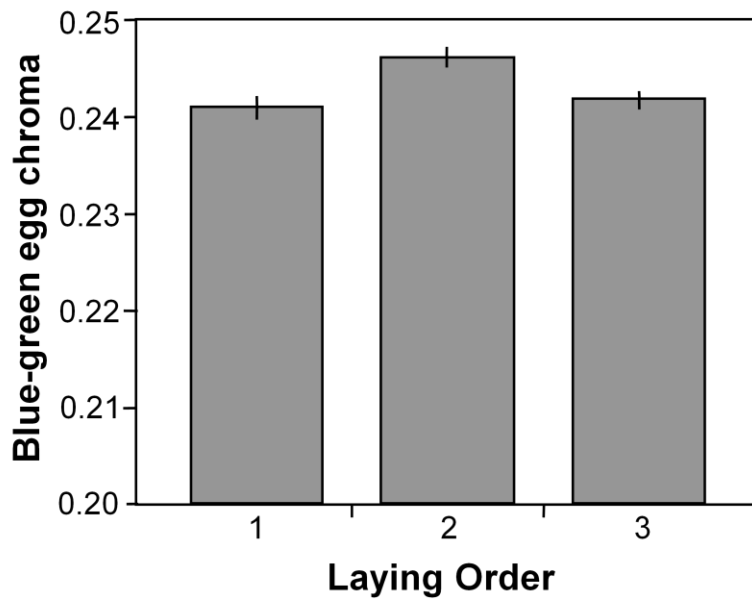
**Figure 2. 2**

Blue-green chroma in relation to laying order in ring-billed gulls. Data are least squares

means from an analysis controlling for nest identity.
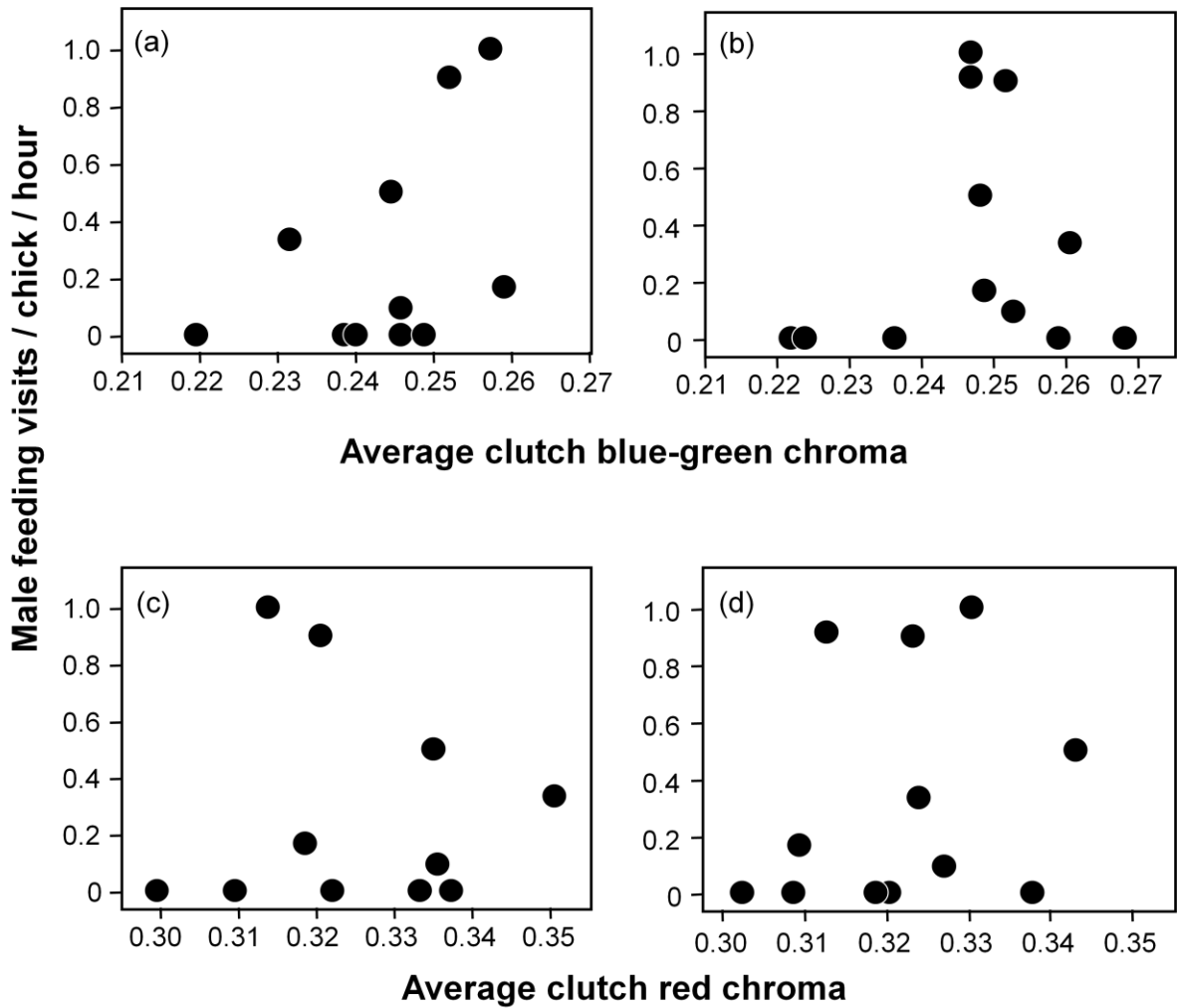
**Figure 2. 3**

The relationship between egg colouration and male feeding rates in ring-billed gulls. Data show male feeding rates in relation to the original blue-green (a) and red chroma (c) laid by his mate and the blue-green (b) and red chroma (d) we subsequently cross-fostered into his nest. Univariate data are shown; see Table 2 for multivariate analyses.

# CHAPTER 3: DOES ENVIRONMENTAL CONTAMINATION INFLUENCE EGG COLOURATION? A LONG-TERM STUDY IN HERRING GULLS[2]

**Chapter summary**

**1.** Although considerable progress has been made in reducing concentrations of persistent organic compounds in the environment, these contaminants are still found in many taxa. Here, we investigate the relationship between environmental contamination and egg colouration in herring gulls *Larus argtentatus*, using eggs from a long-term monitoring program.

**2**. The Herring Gull Monitoring Programme has documented changes in contaminant levels across the Great Lakes for nearly 40 years by monitoring contaminant levels in herring gulls and their eggs. We measured the colour of these eggs using reflectance spectrometry, and evaluated the influence of contaminants on egg colour using generalized linear mixed models. We also employed receptor-noise limited human visual models to determine whether humans would be able to visually distinguish differences in colour between eggs on the scale at which their colour is influenced by environmental contaminants.

**3**. Several contaminants were related to herring gull egg colouration; however, not all contaminants influenced colour in the same way. Blue-green chroma was positively related to concentration of both *trans*-nonAchlor and dioxin levels, whereas it was negatively related to PCB 1260 concentration. Brown chroma was positively related with *trans*-nonAchlor concentration only. We suggest that these patterns can be best described through each contaminant's distinct influence on the haem biosynthesis pathway, which may in turn influence the deposition of eggshell pigments.

**4.** *Synthesis and applications*. Our findings reveal associations between blue-green chroma and both PCB 1260 and dioxin concentration. This is an important first step in

using egg colouration as a proxy for assessing contaminant loads in a colonially breeding waterbird. Although the effect of colony was controlled for, the influence of some of these contaminants on colour is operating on a level that is visually detectable by field researchers, whereas others would need to be quantified using spectrometry. In either instance, assessing egg colouration may provide a rapid, inexpensive, and non-destructive means of estimating contaminant levels in the environment, which is essential for monitoring areas or species of concern as well as assessing potential human health risks.

**Key-words** – biliverdin, bioindicator, egg colour, environmental contaminant, herring gull, PCB, porphyrin

**Introduction**

Assessing habitat quality is imperative for monitoring and managing sensitive areas and wildlife. Bird eggs may provide an efficient means of assessing the environmental quality of avian breeding habitats, particularly with respect to industrial processes and agricultural pesticides. Many persistant organic compounds  associated with these activities are known to bioaccumulate in animal tissues as they are transferred from low trophic levels to higher ones. The influence of the bio-accumulation of these contaminants on avian reproduction became readily apparent during the late 1960s (Ratcliff 1967; Hickey and Anderson 1968), particularly through eggshell thinning induced by exposure to dichlorodiphenyldichloroethylene (DDE), a metabolite of the persistent insecticide dichlorodiphenyltrichloroethane (DDT) (Gilbertson 1974). Within the Great Lakes, the levels of persistent organic particulates have decreased dramatically

over the last half-century (Hebert et al. 1999); however, significant quantities of organic compounds are still prevalent in colonially nesting waterbirds (Antoniadou et al. 2007; Champoux et al. 2010; Lavoie et al. 2010). Recent models also suggest that cycles of contamination may be linked to oscillating currents and global patterns of climate change (Bustnes et al. 2010). Such studies reveal the importance of continued monitoring to track long-term patterns and evaluate potential risks to plants, animals, and humans from environmental contamination.

Our objective was to determine whether eggshell colouration could serve as a non-destructive bioindicator of environmental stress, using herring gulls *Larus argentatus* as an indicator species. Herring gulls have been the focus of a long-term monitoring program across several colonies along the shores of the Great Lakes in Canada and the United States. The objective of the Great Lakes Herring Gull Monitoring Programme has been to examine the concentrations and effects of environmental contaminants in herring gulls and their eggs (Hebert et al. 1999); the program has documented the levels of various organochlorines and metal contaminants in this species for 39 years. Key contributions of the program thus far include documenting reproductive dysfunction in herring gulls in relation to contaminant levels (Gilbertson 1974, 2001), discovering the presence of mirex and photomirex in herring gulls (1,2,3,4,5,5,6,7,8,9,10,10-dodecachloropentacyclo[5.3.0.0$^{2,6}$.0$^{3,9}$.0$^{4,8}$]decane and 1,2,3,4,5,5,6,7,9,10,10-undecachloropentacyclo[5.3.0.0$^{2,6}$.0$^{3,9}$.0$^{4,8}$]decane, respectively; Hallett et al. 1976), documenting the decline and stability of polychlorinated biphenyls (PCB) in the Great Lakes (Stow 1995), tracking temporal and spatial patterns in egg contaminants (Pekarik and Weseloh 1998; Weseloh et al. 2006), and spurring early work in the use of a

biochemical as an indicator of contaminant exposure (Ellenton et al. 1985). The eggs used in this long-term monitoring project have been stored in a national archive and are available for continued research projects. We measured the colouration of these eggshells using reflectance spectrometry to examine the relationship between egg colouration and levels of environmental contaminants.

Organisms that provide insights about changes in the health or quality of an ecosystem are known as bioindicators, and, more specifically, environmental indicators (McGeoch 1998). The herring gull is one such species. Early research on Great Lakes herring gulls documented decreases in hatchability in relation to DDT levels (Keith 1966). Fortunately, the levels of most legacy contaminants in Great lakes herring gull eggs have declined significantly since DDT was banned commercially in 1974 (Pekarik and Weseloh 1998; Jermyn-Gee et al. 2005). This dramatic temporal variation in contaminant load, as well as the herring gull's susceptibility to organochlorines (Neimi et al. 1986; Breton et al. 2008), makes this system ideal for examining the possible influence of contaminants on egg colouration. Moreover, herring gulls are colonial nesters, facilitating the collection of large quantities of data (Fox et al. 2007). In addition, herring gulls in the Great Lakes form a closed, non-migratory population (Weseloh 1984; Gilbertson 2001) so that contaminant levels found in their eggs are acquired from within the Great Lakes. Their ova also develop rapidly over the breeding season, increasing to approximately 1,472% of their pre-breeding size at time of peak laying (Harris 1964), and as with most birds the greatest change in ovum mass occurs within a week of laying (Romanoff and Romanoff 1949). Thus, the majority of the ovum mass accumulates at the

breeding site, suggesting that most of the contaminants found in herring gull eggs are derived from the breeding grounds.

A number of factors support the possible utility of avian pigments, and particularly avian egg pigments, as bioindicators of environmental stress. Proximity to urbanization (Horak et al. 2000) and exposure to PCBs (McCarthy and Secord 2000; Bortolotti et al. 2003a; Bortolotti et al. 2003b) are known to influence avian plumage and soft part colouration. Egg colouration in birds may be similarly influenced by environmental quality as it has been linked with female body condition (Morales et al. 2006; Soler et al. 2008) and health (Moreno et al. 2005; Martínez-de la Puente et al. 2007), yet this possibility has received surprisingly limited attention.

Despite dramatic variation in avian egg colouration within and among species (Collias 1993; Kilner 2006), only two related pigments are primarily responsible for this variation: biliverdin (blue-green in colour) and porphyrin (brown in colour) (Kennedy and Vevers 1976), both of which are derived from haem biosynthesis (Sorby 1875; Moore 1998; Ponka 1999; McGraw 2006). Although not all bird eggs contain both pigments, herring gull eggshells contain both biliverdin and porphyrin (Kennedy and Vevers 1976), which should allow for substantial variation in colour. The concentration of one of these pigments, porphyrin, has been recommended as a bioindicator in fecal samples (Akins et al. 1993; Casini et al. 2003). However, since both porphyrin and biliverdin occur along the same biochemical pathway, arguments for the use of porphyrin may also be relevant for biliverdin (Mateo et al. 2004; Jagannath et al. 2008).

The relationship between environmental contaminants and eggshell pigmentation was recently examined in the Eurasian sparrowhawk *Accipiter nisus* (Jagannath et al.

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2008). In a sample of eggs collected across the United Kingdom in a single year, Jagannath et al. (2008) found that blue hue was positively correlated with DDE concentration, while blue-green chroma, a measure of biliverdin content (Moreno et al. 2006), actually decreased with DDE concentration. A separate experimental study showed that contamination by lead caused a 53-fold increase in protoporphyrin and a 66-fold increase in biliverdin in fecal samples of Mallards *Anas platyrhynchos* (Mateo et al. 2004). These findings are consistent with the observation that organochlorines, halogenated hydrocarbons, and heavy metals influence the haem biosynthesis pathway (Kennedy et al. 1998; Casini et al. 2003; Mateo et al. 2003b; Mateo et al. 2004).

In this study, we investigated the relationship between egg colouration and environmental contaminants measured through the Great Lakes Herring Gull Monitoring Programme. We also used a receptor-noise-limited visual model to evaluate the practicality of human assessment of contaminant-induced egg colour variation in the field. By taking this approach we will be able to assess how large differences in egg chroma need to be to be detected in the field, and use this information to determine if effects on egg colour production are large enough to be detectable to a human observer. Based on previous research (Jagannath et al. 2008), we predicted that DDE concentration in eggs would be negatively related to blue-green chroma. Because little is known about the relationship between egg colouration and environmental stress, we used exploratory analyses to investigate possible relationships between egg colouration and other contaminants, with a particular emphasis on those that may be porphyinogenic compounds (compounds that increase porphyrin production).

**Materials and methods**

*Long-term dataset*

The National Wildlife Research Centre Specimen Bank in Ottawa, Ontario, Canada,

houses the eggshells for eggs sampled through the Great Lakes Herring Gull Monitoring

Programme (1971 – 2010). To our knowledge, this is the longest running annual

contaminants program on an indicator species, and has been conducted in a region that

has experienced a dramatic change in environmental quality with respect to numerous

forms of environmental contaminants (Heinz et al. 1985; Hebert et al. 1999; Jermyn-Gee

et al. 2005). The data collection protocol for this project has been relatively consistent

across all sampling years (Fox et al. 2007). Briefly, 15 colonies (Fig. 1) were visited

during early incubation once per year and 13 eggs, one per completed clutch, were

collected from each location and stored at 4 °C (Fox et al. 2007).

The protocol for organochlorine extraction has been described in detail (Pekarik

and Weseloh 1998). Briefly, within 2 weeks of collection, egg contents were placed in

hexane-rinsed jars, mixed with anhydrous sodium sulphate, and stored at -20 °C. Gas

chromatography was used to assess the level of contaminants in these aliquots. First, the

lipid content was eluted from the column and assessed with a gravimetric analysis. Lipid

soluble organochlorines were then separated and fractionated on Florisil. Another portion

of the homogenized aliquot was analyzed to determine organochlorine concentration

from the lipids. The first fraction contained DDE, mirex, photo-mirex, and a range of

PCB Arochlors, whereas the second contained DDT, dichlorodiphenyldichloroethane,

alpha-hexachlorocyclohexane, oxy-chlordane, and beta-hexachlorocyclohexane. More

details on the extraction methods, minor alterations to protocol, and extraction of other

contaminants can be found in published technical accounts (Bishop et al. 1992; Pekarik and Weseloh 1998; Pekarik et al. 1998; Jermyn-Gee et al. 2005).

Prior to 1986, contaminant levels were assessed for each egg individually. To reduce analytical costs, egg samples collected after 1986 were pooled together by colony and each pool was used to estimate average colony-level contaminant loads (Pekarik and Weseloh 1998). Previous research has shown that these methods result in comparable data (Turle et al. 1986). For our analyses, we used individual egg contaminant levels wherever possible and the pooled values for eggs that lacked individual data.

*Egg colour assessment*

We measured the colouration of 686 herring gull eggs from the National Wildlife Resource Specimen Bank at the National Wildlife Research Centre that had corresponding contaminant information from 4 years: 1977, 1985, 1989, and 1997. We measured egg colouration using a reflectance spectrometer (USB4000, Ocean Optics, Dunedin, Florida, USA) with a portable, full spectrum light source (PX-2 pulsed xenon, Ocean Optics, Florida, USA). All reflectance measurements were calculated relative to a Spectralon reflectance standard (WS-1-SL, Ocean Optics, Florida, USA). We measured each egg twice, once on the blunt end and once on the pointed end. Care was taken to avoid pigmented spots and only measure the uniform eggshell ground colour. We then averaged these measurements to obtain one spectrum per egg (Fig. 2). We visually inspected each spectrum prior to analyses to ensure that no aberrant readings were present in our dataset. The avian visual spectrum includes ultraviolet (UV) wavelengths (300 - 400 nm; Cuthill 2006), and birds are known to use UV colouration for detecting

and/or recognizing their own eggs (Cherry and Bennett 2001; Avilés et al. 2006). Because humans cannot perceive UV wavelengths, the consideration of UV wavelengths may reveal information that is not otherwise detectable by humans (Jacobs 1981; Kelber et al. 2003). We therefore included this region of the spectrum when calculating our colorimetric variables. To approximate biliverdin content, we calculated blue-green chroma as a proportion of reflectance in the blue-green region (450–550 nm) relative to that of the entire avian visible spectrum (300–700nm). To approximate porphyrin content, we calculated brown chroma as a proportion of reflectance in the brown region (600–700 nm) relative to that of the avian visible spectrum (Hanley and Doucet 2009). We calculated ultraviolet chroma as a proportion of reflectance in ultraviolet region (300–400 nm) relative to that of the avian visible spectrum.

*Possible egg fading*

Eggshell colouration may fade when eggs have been stored for a long period of time (Walters 2006), although some studies have found no evidence of egg fading (Soler et al. 2005; Jagannath et al. 2008). If eggs do fade with time, older eggs should have lower chroma values than recently collected eggs because chroma, a measure of biliverdin content (Moreno et al. 2006), should be highest in fresh eggs, before eggshell pigments have been subject to oxidation and degradation. Such fading has been documented in feathers in museum collections (McNett and Marchetti 2005; but see Armenta, Dunn & Whittingham 2008; Doucet & Hill 2009 ). We tested for possible eggshell fading by correlating collection date with our three colorimetric variables. Blue-green chroma ($r = -0.07$, $n = 686$, $P = 0.06$) was not related to collection date. Brown chroma ($r = -0.20$, $n = $

686, $P < 0.0001$) was negatively correlated with collection date, which is in the opposite

direction to that predicted by fading. To assess the possibility that substantial egg fading

might have occurred more rapidly, we compared the chroma values of 13 eggs collected

from Port Colbourne, Ontario, which we measured on the day they were collected in

2007, to the chroma values of eggs in our long-term dataset. There were no significant

differences between these fresh or stored eggs (*blue-green chroma*: $t_{707} = -0.54$, $P = 0.58$;

*brown chroma*: $t_{707} = -0.94$, $P = 0.35$). This lack of substantial fading may not be

surprising; herring gull eggs are exposed infrequently after laying due to long attentive

periods by the parents (Drent 1970; Pierotti and Good 1994), and the shells in our long-

term dataset were stored in sealed containers away from light shortly after being

collected.

*Testing discriminability using visual modeling*

We sought to determine whether variation in egg colouration associated with contaminant

level would be visually discernable in the field. The perception of visual signals depends

on the visual abilities of the viewer, the colouration of the object being viewed, the colour

of the viewing background, and the characteristics of the light illuminating the object

(Endler 1990; Kelber et al. 2003). To assess the detectability of differences in egg colour,

we employed a receptor-noise-limited opponent model that incorporated human spectral

sensitivity, a daylight irradiance spectrum, and our measurements of egg reflectance

(Schnapf et al. 1987; Endler 1993; Vorobyev and Osorio 1998). This receptor-noise-

limited opponent model accounts for chromatic differences in colour only (not

differences in brightness), and has been shown to provide good estimates of detectability for human subjects in bright light conditions (Vorobyev and Osorio 1998).

Colour vision can be represented by an $n$ –dimensional colour space, where $n$ refers to the number of cone types in the viewer (Vorobyev 2003) and the axes are maximum quantum catches for each cone type. Humans are trichromatic, with three cone-type photopigments that are maximally sensitive at 420 nm, 530 nm, and 560 nm (Jacobs 1981; Kelber et al. 2003). Quantum catch is calculated as by integration across the defined visible spectrum

$$Q_i = \frac{\int_\lambda R_i(\lambda)S(\lambda)I(\lambda)d(\lambda)}{\int_\lambda R_i(\lambda)} \qquad \text{eqn 1}$$

where $R_i$ represents the spectral sensitivity of cone type $i$, $S$ represents the spectral reflectance of the object, and $I$ represents the normalised irradiance spectrum. A viewer's ability to discriminate between two colours can be thought of as the distance between the two colours within this colour space. However, the visual sensation of stimuli is subject to noise occurring at the receptors as well as the subsequent stages of neural processing (Wyszceki and Stiles 1982; Vorobyev 2003). Therefore, we calculated receptor noise with a flexible function accounting for the inherent noise to signal ratio for all cone types relative to the proportion of receptor types in the eye (Vorobyev et al. 1998; Vorobyev 2003; Cheney and Marshall 2009) as

$$e_i = \sqrt{\frac{(1/(\log(T \frac{(Q_{i,a} + Q_{i,b})}{2}))^2 + w_i^2)}{n_i}} \qquad \text{eqn 2}$$

where T is a scaling factor for luminance level (set to 10,000), $w_i$ is the Weber fraction

accounting for differences in response sizes based on magnitude of stimuli (Wyszceki

and Stiles 1982) (set to 0.02 for all cone types), and $n_i$ is a constant representing the

relative number of receptor cells accounting for receptor type density. Here, we used

1:16:32 to represent the relative proportion of receptor types for the short, medium, and

long cone types found in humans, respectively (Wyszceki and Stiles 1982).

Thus, when accounting for receptor noise, the distance between colours within

human colour space, or discriminability, can be calculated as

$$(\Delta S)^2 = \frac{e_1^2 (\Delta f_3 - \Delta f_2)^2 + e_2^2 (\Delta f_3 - \Delta f_1)^2 + e_3^2 (\Delta f_1 - \Delta f_2)^2}{(e_1 e_2)^2 + (e_1 e_3)^2 + (e_2 e_3)^2} \qquad \text{eqn 3}$$

where $\Delta f_i$ is the difference in quantum catch between two stimuli (Vorobyev and Osorio

1998). Discriminability estimates calculated in this way represent units of just noticeable

differences, where a value of one or greater represents a difference in colour between two

objects that would be detectable by humans. We calculated quantum catch and

detectability using the program SPEC (Hadfield 2004) in R (Ihaka and Gentleman 1996)

between all pairwise comparisons of eggs in our dataset.

*Statistical analyses*

All data exhibited normal kurtosis and skewness values (all < 2). We used generalized

linear mixed models to determine the relationship between egg colouration and

contaminant levels. In each model, we included a colorimetric variable as the dependent

variable, and year of collection, a contaminant level, and colony (random effect) as

predictors.  We ran separate models for each colorimetric variable and each contaminant,

including DDE, PCB-1260, 2,3,7,8,-tetrachlorodienzo-p-dioxin (dioxin),

hexachlorobenzene (HCB), and *trans*-nonAchlor. We did not evaluate contaminants that

were not consistently detected within eggs (i.e., ordinarily at trace or undetectable levels).

**Results**

*Do contaminant levels explain variation in egg colouration?*

Variation in DDE levels did not explain variation in the colouration of herring gull eggs

for blue-green chroma (*whole model*: $r^2 = 0.07$, $F_{17,587} = 2.73$, P = 0.0002; *colony*: $F_{15,17} =$

2.85, P = 0.0003; *year*: $\beta = -0.19$, $F_{1,587} = 11.42$, P = 0.001; *DDE*: $\beta = -0.15$, $F_{1,587} = 3.51$,

$P = 0.06$), brown chroma (*whole model*: $r^2 = 0.13$, $F_{17,587} = 4.98$, P < 0.0001; *colony*: $F_{15,17}$

$= 3.32$, P < 0.0001; *year*: $\beta = -0.17$, $F_{1,587} = 9.48$, P = 0.002; *DDE*: $\beta = 0.13$, $F_{1,587} = 3.04$,

$P = 0.08$), ultraviolet chroma:  (*whole model*: $r^2 = 0.24$, $F_{17,587} = 11.04$, P < 0.0001;

*colony*: $F_{15,17} = 7.41$, P < 0.0001; *year*: $\beta = 0.36$, $F_{1,587} = 49.59$, P < 0.0001; *DDE*: $\beta = -$

0.01, $F_{1,587} = 0.02$, $P = 0.89$). Concentration of PCB 1260 predicted variation in both

blue-green chroma (*whole model: $r^2 = 0.12$, $F_{16,479} = 4.12$, $P < 0.0001$; colony*: $F_{14,16} =$

3.59, P < 0.0001; *year*: $\beta = -0.47$, $F_{1,495} = 28.41$, P < 0.0001; *PCB 1260*: $\beta = -0.35$, $F_{1,495}$

$= 7.59$, $P = 0.006$) and ultraviolet chroma (*whole model: $r^2 = 0.39$, $F_{16,479} = 19.38$, $P <$

0.0001; *colony*: $F_{14,16} = 13.79$, P < 0.0001; *year*: $\beta = 0.82$, $F_{1,495} = 126.25$, P < 0.0001;

*PCB 1260:* $\beta = 0.50$, $F_{1,495} = 22.47$, $P < 0.0001$).  However, PCB 1260 did not

significantly predict brown chroma (*whole model: $r^2 = 0.13$, $F_{16,479} = 4.67$, $P < 0.0001$;

*colony*: $F_{14,16} = 3.43$, P < 0.0001; *year*: $\beta = -0.37$, $F_{1,587} = 18.01$, P < 0.0001; *PCB 1260:* $\beta$

$= -0.18$, $F_{1,495} = 2.12$, $P = 0.15$).

Dioxin levels were also related to blue-green and ultraviolet chroma but in the opposite direction. Higher concentrations of dioxins were related to higher amounts of blue-green pigmentation (*whole model:* $r^2 = 0.08$, $F_{16,600} = 3.43$, $P < 0.0001$; *colony*: $F_{14,16} = 2.81$, P = 0.0004; *year*: $\beta = 0.15$, $F_{1,616} = 2.56$, P = 0.11; *dioxin:* $\beta = 0.38$, $F_{1,616} = 9.26$, $P = 0.002$), while ultraviolet chroma was inversely related to dioxin concentration (*whole model:* $r^2 = 0.31$, $F_{16,600} = 17.02$, $P < 0.0001$; *colony*: $F_{14,16} = 11.01$, P < 0.0001; *year*: $\beta = -0.01$, $F_{1,616} = 0.04$, P = 0.85; *dioxin:* $\beta = -0.59$, $F_{1,616} = 30.52$, $P < 0.0001$).

*Trans*-nonAchlor concentration was positively related to blue-green chroma (*whole model:* $r^2 = 0.09$, $F_{16,569} = 3.40$, $P < 0.0001$; *colony*: $F_{14,16} = 2.88$, P = 0.0003; *year*: $\beta = -0.09$, $F_{1,616} = 4.66$, P = 0.03; *trans-nonAchlor* $\beta = 0.15$, $F_{1,585} = 11.45$, $P = 0.0008$) and brown chroma (*whole model:* $r^2 = 0.14$, $F_{16,569} = 5.77$, $P < 0.0001$; *colony*: $F_{14,16} = 3.52$, P < 0.0001; *year*: $\beta = -0.19$, $F_{1,616} = 21.06$, P < 0.0001; *trans-nonAchlor* $\beta = 0.14$, $F_{1,585} = 10.42$, $P = 0.001$), while negatively related to ultraviolet chroma (*whole model:* $r^2 = 0.33$, $F_{16,569} = 17.34$, $P < 0.0001$; *colony*: $F_{14,16} = 9.68$, P < 0.0001; *year*: $\beta = 0.29$, $F_{1,616} = 61.20$, P < 0.0001; *trans-nonAchlor:* $\beta = -0.31$, $F_{1,585} = 65.33$, $P < 0.0001$). HCB concentration did not significantly predict any colorimetric variable (all $P > 0.09$).

*Are differences in chroma associated with contamination levels visually detectable in the field?*

The mean ± SD difference in brown chroma for pairs of eggs that would be discernable by humans (those with discriminability estimates over one) was 0.04 ± 0.00004, and the mean difference in blue-green chroma that would be discernable was 0.02 ± 0.00003. We used the unstandardized regression coefficients (representing units of change in the

predicted value of the dependent variable per unit change in a predictor variable) from

contaminants found to be significant predictors of egg colour in the mixed models to

predict concentration of contaminants that would correspond to a visually detectable

difference in egg chroma. The unstandardized beta values from our model for PCB 1260

suggest that a detectable change in blue-green chroma would reflect a 0.018 µg g$^{-1}$ wet wt

change in PCB 1260 concentration. Similarly, detectable differences in blue-green

chroma would correspond to a change of 54.6 µg g$^{-1}$ wet wt of dioxin (Table 1).

However, the concentration of *trans*-nonAchlor corresponding to detectable differences

in chroma fall far beyond any amount found in herring gull eggs (Table 1). Therefore, our

models predict that the range of contaminant concentrations found in herring gull eggs

would produce variation in colour detectable by humans.


**Discussion**

Based on analysis from a long-term dataset, our findings reveal significant associations

between persistent organic contaminants and egg colouration in herring gulls.

Interestingly, PCB 1260 and dioxin appear to have contrasting influences on blue-green

egg colouration. The only other study to examine the relationship between contaminant

load and egg colouration found a positive association between DDE levels and blue-green

chroma in Eurasian sparrowhawks (Jagannath et al. 2008). Contrary to our expectations

based on that study, we found no relationship between blue-green chroma and

concentration of DDE. However, the range in concentration of DDE in our dataset was

considerably lower (0.18 – 57.70 µg g$^{-1}$ wet wt) than the levels found in sparrowhawk

eggs (10 – 300 µg g$^{-1}$ wet wt), and it is possible that more variation would be necessary to

detect this pattern. In addition, herring gull eggs are considerably less blue-green than

sparrowhawk eggs, suggesting a lower level of biliverdin pigmentation. A marginal effect

of DDE may therefore be masked by larger quantities of porphyrin levels within herring

gull eggs. Another possibility is that we did not detect these patterns because DDE does

not induce the production of porphyrins, but is correlated to the presence of PCBs which

are known to be porphyinogenic (Kennedy et al. 1998). This suggests that the connection

between colouration and DDE may be the result of other contaminants that are associated

with DDE concentration.

      In our study, egg colouration in herring gulls was related to PCB 1260, dioxin,

and *trans*-nonAchlor concentrations. These contaminants have been found to accumulate

in the livers of adult herring gulls (Fox et al. 2007), and are known to interact with haem-

biosynthesis either directly or indirectly (Kennedy et al. 1998; Casini et al. 2003).

However, although PCBs are known to be related to higher concentrations of highly

carboxylated porphyrins in adult herring gull livers (Kennedy et al. 1998), a causal

relationship among the other contaminants is less certain. Dioxin levels have been found

to induce porphyrin production in some studies, while several other studies have found

marginal or no effect (Casini et al. 2003). Concentrations of *trans*-nonAchlor are also

known to relate to increases in porphyrin concentration; however, these patterns are not

believed to be a direct consequence of the contaminant on pigment, but rather a result of

the contaminant being related to other more influential contaminants (Kennedy et al.

1998).

      To understand the possible influence of environmental contaminants on egg

colour, it is necessary to understand how porphyrin and biliverdin are produced.  These

pigments are derived from haem-biosynthesis, which occurs endogenously through enzymatic interactions leading to the formation of various porphyrins (Moore 1998; McGraw 2006). Further enzymatic reactions along this pathway can convert these non-metallic porphyrins to haem through the addition of an $Fe^+$ ion (Ponka 1999; McGraw 2006), which can be oxidized to biliverdin (McDonagh 2001).

Although PCBs are known to have a direct effect on porphyrin concentration, they may also have an indirect effect on biliverdin. PCB contamination has been found to induce uroporphyrin production, thereby reducing protoporphyrin and haem production (Sano et al. 1985). Since haem is necessary for creating biliverdin, this process results in an elimination of biliverdin. Herring gull eggs have relatively high levels of porphyrin, and increases in this pigment may not cause changes that are as noticeable as the elimination of biliverdin, which is present in smaller quantities. This may explain why we did not find a relationship between PCB concentration and the brown pigment, porphyrin, despite the significant relationship between PCB 1260 and blue-green chroma.

It is possible to influence other stages in the haem biosynthesis pathway in addition to influencing porphyrin production. For instance, upstream degradation of haem through induced haem oxygenase activity has been proposed as a mechanism to explain increases in biliverdin associated with dioxin toxicity (Niittynen et al. 2002). We found that blue-green chroma increased with concentrations of both dioxin and *trans*-nonAchlor, while brown chroma also increased with concentration of *trans*-nonAchlor. Unlike the pattern related to PCB 1260, these mechanisms implicate changes in both pigments. Such differences in the effect of contaminants on egg colouration are feasible. For example, some species exposed to lead poisoning develop biliverdinuria, the excess

production of biliverdin (Mateo et al. 2003a; Mateo et al. 2004), whereas others develop

hemoglobinuria, the excess production of hemoglobin (Styles and Phalen 1998; Pollock

2006). In addition, various contaminants are known to influence haem biosynthesis in

different ways (Casini et al. 2003), and there are numerous forms of porphyria (Moore

1998), which may explain the different effects of different contaminants on egg

colouration.

The effect of environmental contaminants on egg colouration, although

detectable, is relatively low and there are many other environmental (Gosler et al. 2005;

Avilés et al. 2007) and genetic factors (e.g., Punnett 1933; Hardiman et al. 1975)

influencing avian eggshell pigmentation. Moreover, our models evaluated the effect of

contaminants independently, even though the contaminants we studied, as well as other

contaminants we did not quantify, may correlate with one another.  A more in-depth,

controlled experiment on the effect of contaminants on endogenous pigment deposition in

bird eggs is necessary to draw any definitive conclusions. In addition, future research

should examine the specific influence of these contaminants on the avian shell gland, the

site of egg pigment synthesis (Zhao et al. 2006), because contaminants are known to have

tissue-specific effects (Maines 1976; Leonzio et al. 1996).

Although the colouration of eggs is related to contaminant load, we are not

suggesting that these results qualify its immediate use as a bioindicator. While large

variation in herring gull egg colouration was useful for an initial test of the potential

utility of using egg colour as a bioindicator, large inter-clutch variation in egg colour may

make assessing relative colony-site contaminant loads challenging. Therefore, since egg

colour likely varies between colonies for reasons other than the influence of

contaminants, it will be critical to determine the normal amount of variation in colour and examine the influence of contaminant load on this variation within target colonies with preliminary research. Then in future years, use these baseline data to estimate the contaminant load based on variation in egg colour. In addition, while we demonstrate that egg colouration is related to a suite of contaminant levels, and outline the mechanistic link behind such relationships, future investigations into the value of this relationship as a monitoring tool would benefit from utilizing species with less variable pigment regimes (all porphyrin or all biliverdin), particularly those species that are subject to behavioural monitoring.

Our findings provide a critical first step towards a new avenue of conservation action and also increase our understanding of pigment deposition. Egg colouration would be most useful as a bioindicator if it could be assessed visually in the field. Our visual models suggest that visually detectable differences in herring gull blue-green egg chroma may be a useful measure of PCB 1260 concentration, and to some extent dioxin concentration. To assess levels of other contaminants, spectrophotometric methods would be necessary. Although we encourage future research evaluating the utility of human-perceived egg colour as a proxy for PCB contamination, the use of spectrometers is preferable because these devices are quantitative and more sensitive than the human visual system. Moreover, there are numerous handheld, battery operated spectrophotometers that would be convenient for use in the field. Although many of these hand-held devices do not capture the full ultraviolet range, our models suggest that variation within the human visual range may contain useful information.

The evaluation of contaminant levels in biota is important for the conservation of our natural resources and for monitoring long-term health risks to humans. Long-term monitoring programs provide a means to examine the progress of environmental remediation and for forecasting potential health risks. We have shown that the colour of herring gull eggs may be a useful bioindicator of PCB, dioxin, and *trans*-nonAchlor concentration. Because these are the only two pigments classes controlling egg colouration in birds (Gorchein et al. 2009), it is possible that these patterns are somewhat conserved across all birds. Therefore, egg colour may provide a simple, inexpensive, and non-destructive indicator of contaminant concentration. Moreover, there are numerous long-term monitoring programs on colonial and semi-colonial birds worldwide that may facilitate the global application of using avian egg colouration as a bioindicator of environment contamination.

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**Table 3.1**

Comparison between detectable changes in egg colouration and associated changes in contaminant levels in herring gull eggs for contaminants found to significantly predict variation in egg colour (see Results). Unstandardized beta scores refer to the units change in colour associated with a one-unit change in contaminant in our models. Mean differences in chroma detectable between eggs, as well as the maximum and minimum levels of contaminants found in our dataset, are included to provide context for the values associated with a detectable difference in colour.

| Chroma | Contaminant | $b \pm SE$ | detectable chroma | Concentration ($\mu g\ g^{-1}$ wet wt) | | |
| --- | --- | --- | --- | --- | --- | --- |
| | | | | detectable | min | max |
| Blue-green | PCB 1260 | $-0.005 \pm 0.002$ | 0.02 | 0.02 | 1.95 | 151.00 |
| | *trans*-nonAchlor | $0.002 \pm 0.0005$ | 0.02 | 220.26e2 | 0.01 | 0.16 |
| | Dioxin | $0.005 \pm 0.002$ | 0.02 | 54.60 | 3.16 | 91.00 |
| Brown | *trans*-nonAchlor | $0.002 \pm 0.0007$ | 0.04 | 485.17e6 | 0.01 | 0.16 |
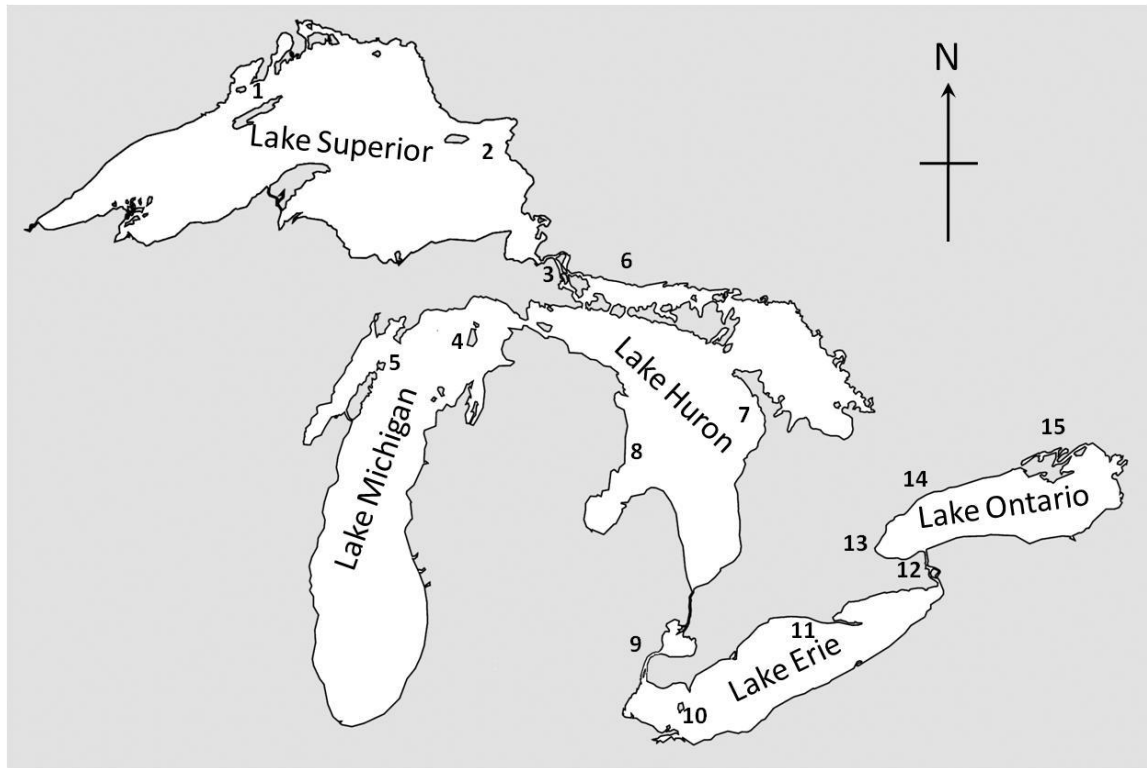
**Figure 3. 1**

A map of the Great Lakes showing the locations of herring gull colonies sampled in this study: 1) Granite Island, 2) Agawa Rocks, 3) Pumpkin Point, 4) Gull Island, 5) Big Sister Island, 6) Double Island, 7) Chantry Island, 8) Channel-shelter Island, 9) Fighting Island, 10) Middle Island, 11) Port Colborne, 12) Niagara River, 13) Hamilton Harbour, 14) Toronto Harbour, 15) Snake Island.
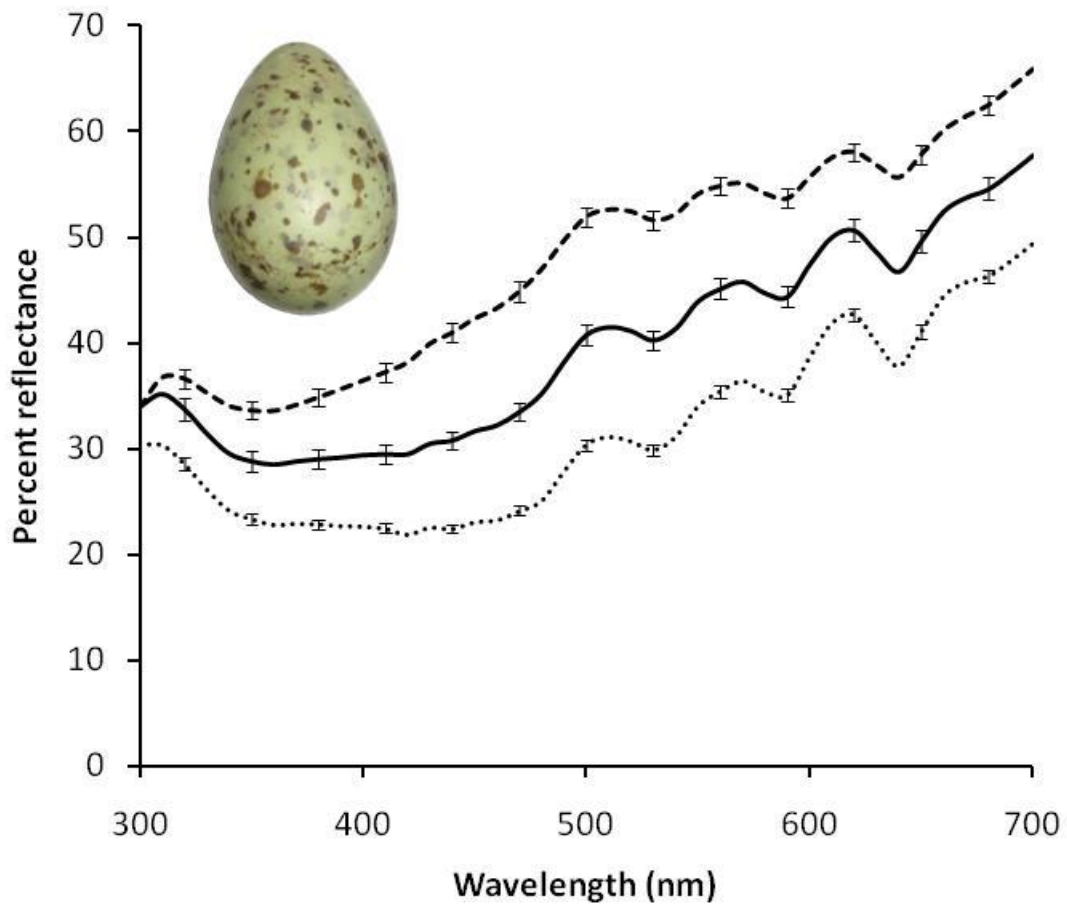
**Figure 3. 2**

The average spectral reflectance of herring gull eggs exhibiting mean blue-green chroma (±95% CI) , solid line, the average spectral reflectance of eggs within the upper 10%  of blue-green chroma, dashed line, and the average spectral reflectance of eggs within the lower 10% of the range of blue-green chroma in our study. Vertical bars represent standard error.

# CHAPTER 4 - PARENTS, PREDATORS, PARASITES, AND THE EVOLUTION OF COLOUR IN EGGS[3]

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[3] This manuscript is the product of joint research

**Summary**

The colour on the surface of birds' eggs varies dramatically between species, but the selective pressures driving this variation remain poorly understood. In this study, we used a large comparative dataset of 636 bird species to test several hypotheses proposed to explain the evolution of egg colouration. We tested the hypothesis that predation pressure might select for cryptic eggs by examining the relationship between predation rate and egg colouration. We found that predation rates were significantly positively related to eggshell brightness, suggesting that predation pressure may influence egg colouration in birds. Conspicuous eggs have been hypothesized to function as aposematic signals if conspicuous colours advertise unpalatability. In our dataset, only ultraviolet chroma was negatively related to egg predation rate, providing little support for the aposematism hypothesis. The blackmail hypothesis suggests that females lay colourful eggs to coerce males into providing additional care during incubation to keep colourful eggs covered. Therefore, colours that are conspicuous against the nest background should be found in situations with high risk of visual detection from predators or brood parasites. In support of this hypothesis, proportional blue-green chroma was positively related to parasitism risk, and parents provided higher nest attendance to eggs with higher proportional blue-green chroma or higher ultraviolet chroma. The sexual signalling hypothesis, which suggests that blue-green colour indicates female quality, was not supported by our findings. Likewise, our findings did not support the hypothesis that preferences for particular colours led to the diversification of egg colour in birds. We found some support for the hypothesis that brood parasitism may select for high inter-clutch variation in egg colour to facilitate egg recognition. In our dataset, parasitism risk was negatively related

to inter-clutch repeatability of blue-green chroma. Inter-clutch variability in egg colouration did not differ between solitary and colonial species, suggesting that a different mechanism for egg recognition may operate in colonial nesters. Our study highlights the diversity of selection pressures acting on the evolution of egg colour in birds.

Keywords: blackmail hypothesis, egg colour, egg recognition, evolution, parasitism, predation, sexual signalling hypothesis

**Introduction**

Avian egg colouration varies dramatically between species (Kilner 2006; Cassey et al. 2010b), and this variation has fuelled interest in egg colour from an evolutionary (Møller and Petrie 1991; Soler and Møller 1996; Soler et al. 2005; Cassey et al. 2010b), behavioural (Tinbergen 1962; Moreno et al. 2006; Siefferman 2006; Hanley et al. 2008), and ecological standpoint (Götmark 1992, 1993; Blanco and Bertellotti 2002; Magige et al. 2008). Unpigmented eggs are white in colouration, whereas the remaining diversity of egg colour variation results from the deposition of brown proto-porphyrin pigments, blue-green biliverdin pigments, or some combination of these two pigments (Kennedy and Vevers 1976). Predation pressure has long been hypothesized to be an important selective factor in egg colour evolution (Wallace 1889), and many species' eggs are pigmented in such a way that they appear to match the colouration or patterning of their nest material (Solís and de Lope 1995; Šálek and Cepáková 2006; Mayer et al. 2009). A number of studies have also shown that egg colouration influences predation (Verbeek 1990; Yahner and Mahan 1996; Blanco and Bertellotti 2002; Castilla et al. 2007). However, despite this important influence of predation pressure, many species appear to lay seemingly conspicuous eggs (Lack 1958). Eggshell conspicuousness may be favoured in some situations, such as in dark nest cavities (Chapter 5). Nevertheless, many other instances of egg conspicuousness continue to intrigue biologists.

The widespread existence of conspicuous eggs has spurred a number of attempts to explain egg colour evolution, with many hypotheses focussing specifically on explaining the presence conspicuous eggs from both non-signalling (McAldowie 1886; Gosler et al. 2005; Lahti 2008; Ishikawa et al. 2010) and signalling perspectives

(Swynnerton 1916; Swynnerton 1918; Cott 1948; Moreno and Osorno 2003; Hanley et al.

2010). Variation in egg colour and patterning has also been found to facilitate egg

recognition in colonial nesters (Gaston et al. 1993), and to be involved in an arms race

between brood parasites and host species (Øien et al. 1995; Langmore et al. 2009;

Stoddard and Stevens 2010). Considering these numerous and varied selection pressures

on egg colour and patterning, it is unlikely that egg colour has evolved for a single reason

(Reynolds et al. 2009). Evolutionarily distinct lineages should experience some

independent selection pressures (Kilner 2006; Cassey et al. 2010b), and any single

species will often face multiple counteracting pressures, which in some cases may result

in trade-offs between opposing selection factors (Magige et al. 2008; Mayer et al. 2009).

Here, we use a large comparative dataset to investigate multiple hypotheses for

the evolution of egg colouration in birds. We focus specifically on seven hypotheses

relating to the visual information that is provided by variation in egg colour rather than

other possible functions of avian egg pigments, which we explain in greater detail below.

(1) The crypsis hypothesis suggests that egg colouration serves to minimize egg detection

by predators (Wallace 1889). (2) The aposematism hypothesis suggests that conspicuous

egg colours signal distastefulness to predators (Swynnerton 1916; Cott 1948). (3) The

blackmail hypothesis suggests that conspicuous egg colours have evolved to coerce males

into providing greater parental care (Hanley et al. 2010). (4) The sensory bias hypothesis

suggests that egg colour has evolved based on species-specific inherent preferences for

egg colours (Abercrombie 1931; Lack 1958; Weeks 1973; Schwartz and Lentino 1984).

(5) The sexual signalling hypothesis suggests that blue-green chroma signals female

quality to mates (Moreno and Osorno 2003). (6) The parasitic recognition hypothesis

suggests that egg colour has evolved to enhance recognition of brood parasitic eggs (Swynnerton 1918; Victoria 1972). Finally, (7) the colonial recognition hypothesis suggests that egg colouration has evolved to enhance recognition of eggs within dense breeding colonies (Noble and Lehrman 1940; Baerends and Vanrhijn 1975; Gaston et al. 1993). Because these hypotheses relate to the visual information provided by eggs, our analyses focus on species that use open nests rather than closed nests such as domed nests, cavities, and burrows, because light levels in closed nests are thought to be too low to allow for detection of variation in colouration (Cassey 2009; Holveck et al. 2010).

One classic explanation for the diversity of colour in avian eggs suggests that colour serves to camouflage the eggs (Wallace 1889; Lack 1958; Oniki 1985). Brown egg colouration has been shown to reduce predation pressure in numerous species with varying nesting strategies (Götmark 1992; Solís and de Lope 1995; Yahner and Mahan 1996; Castilla et al. 2007; Westmoreland 2008). Although blue-green or white eggs seem more conspicuous than brown or spotted eggs, it has been suggested that they may be cryptic in nests exposed to particularly blue-green light or in which eggs may be viewed from below through sparse nest materials (Wallace 1889; Lack 1958; Oniki 1985). However, numerous predation studies suggest that it is unlikely that blue-green and white eggs are cryptic to natural nest predators (Westmoreland and Best 1986; Blanco and Bertellotti 2002; Magige et al. 2008), perhaps because these colours produce high visual contrast against the brownish colour of many avian nests. We therefore predicted that browner eggs should experience lower predation rates whereas eggs that are brighter, more blue-green, or more reflective in the UV should experience higher predation rates. These predictions focus on the assumption that visually orienting nest predators that use

colour to find eggs will more easily detect colours that do not match nesting materials (Hunt et al. 2003; Jourdie et al. 2004). Birds, mammals, and reptiles all are important nest predators, but their colour vision differs dramatically (de Cock Buning 1983; Jacobs 1992; Jacobs 1993; Hart 2001; Kelber et al. 2003; Weatherhead and Blouin-Demers 2004). We therefore do not attempt to use specific visual models for each predator type but instead focus on colorimetric variables that describe spectral shape over a broad range (300 – 700 nm).

Another early hypothesis concerning conspicuous eggs suggested that these eggs may be unpalatable, with colour serving as an aposematic signal to nest predators (Swynnerton 1916; Cott 1948). Although this idea received support from subsequent tests (Cott 1952; Cott 1953), issues regarding the statistical approach called these findings into question (Lack 1958). There are numerous examples of aposematic colouration in both vertebrate and invertebrate body colouration (Mallet and Joron 1999), and feather toxicity has been discovered in at least two bird species (Dumbacher et al. 1992; Dumbacher et al. 2000), suggesting that avian egg toxicity or unpalatability could similarly evolve. This hypothesis has not yet been examined with a comparative approach. If eggshell colouration serves as an aposematic signal to deter predators, we expect blue-green chroma, ultraviolet (UV) chroma, and eggshell brightness to be negatively associated with predation levels.

We recently proposed a new hypothesis, termed the blackmail hypothesis, suggesting that conspicuous egg colouration may reinforce paternal investment to mitigate predation and parasitism risks (Hanley et al. 2010). According to this hypothesis, females can exploit males by producing conspicuous eggs, such that the male's optimal

strategy is to assist in keeping these eggs concealed by assisting with incubation or by provisioning to the incubating female. If conspicuous egg colours serve to coerce males into providing paternal care, species with brighter eggs, more blue-green eggs, or eggs with higher UV chroma should initiate incubation earlier, should exhibit higher nest attendance during the incubation stage, and should be more likely to exhibit male-only or bi-parental incubation. An underlying assumption of this hypothesis is that conspicuous eggs should experience greater risk of predation or brood parasitism, and we therefore expect these colorimetric variables to be positively associated with the risk of predation or parasitism.

Several authors have suggested an inherent link between egg colour and the attentiveness of the parents (Abercrombie 1931; Lack 1958; Weeks 1973; Schwartz and Lentino 1984), particularly in the context of seemingly conspicuous eggs. We term this hypothesis the sensory bias hypothesis. Although this idea was never fully developed as a hypothesis, we propose that a connection between parental attentiveness and egg colouration could be mediated via a hormonal response when parents are presented with a preferred colourful stimulus. Such a pattern could evolve through a sensory bias mechanism (Endler and Basolo 1998; Ryan 1998). This hypothesis has never been tested, but it has been deemed unlikely on multiple occasions (Lack 1958; Weeks 1973; Schwartz and Lentino 1984; Brennan 2009). Nevertheless, intra-specific behavioural experiments suggest that parental nesting behaviours can be motivated by coloured stimuli in a species-specific manner (Baerends and Kruijt 1973; Baerends and Vanrhijn 1975). Strong preferences for specific egg colours should reduce inter-clutch variability within a species through stabilizing selection for "preferred" colours. If egg colouration

serves to exploit the sensory system of the parents, there should be a positive relationship between the inter-clutch repeatability of egg colour and incubation attendance as well as the timing of incubation initiation. Because there should be no restrictions on which colour is preferred within a particular species, we will test these predictions across several colorimetric variables.

Another recent hypothesis, known as the sexual signalling hypothesis, proposes that egg colour may indicate the quality of the laying female to her partner (Moreno and Osorno 2003). According to this hypothesis, since the blue-green pigment biliverdin has antioxidant properties (Kaur et al. 2003), high-quality females should be able to deposit more egg pigments during the oxidatively stressful laying period (Monaghan et al. 1998). Males could then use this information to evaluate relative mate quality and contribute paternal investment accordingly. This hypothesis has been the subject of numerous investigations and has received mixed support (*reviewed in:* Reynolds et al. 2009; Cherry and Gosler 2010). If blue-green egg colour indicates female quality, blue-green egg colouration should be enhanced in species with some degree of paternal care, where males provide assistance during the incubation period, the nestling period, or throughout the entire breeding season. In addition, in species where parents feed offspring, relative male provisioning should be greatest for species with higher blue-green chroma.

Heterospecific brood parasitism may influence the evolution of egg colouration by selecting for parents that make correct egg rejection decisions either through discordance (identification of a dissimilar egg) or through true egg recognition (Underwood and Sealy 2002; Kilner 2006). One strategy to counteract heterospecific brood parasitism is to produce a clutch of eggs that looks distinct from clutches laid by

other conspecifics (Swynnerton 1918; Victoria 1972). When individuals lay distinctive eggs, the variation between clutches is high, resulting in a lower likelihood that a brood parasite can produce an accurate match. Indeed, numerous studies have supported the prediction that inter-clutch variability is related to parasitic egg rejection behaviour (Øien et al. 1995; Soler and Møller 1996; Moskat et al. 2002; Stokke et al. 2002; Avilés and Møller 2003; Avilés et al. 2006; Kilner 2006) and host suitability (Stokke et al. 2002). In addition, inter-clutch variability decreases when there is no longer a risk of inter-specific parasitism (Lahti 2005). The majority of investigations relating to this hypothesis have considered variation in eggshell patterning and appearance rather than ground colour (see reviews, Underwood and Sealy 2002; Kilner 2006; Cherry and Gosler 2010). Interestingly, recent studies suggest that in hosts exposed to inter-specific brood parasitism, eggshell ground colouration may be more important in regulating egg rejection behaviour than egg spot density (Moskat et al. 2008; Avilés et al. 2010; but for species exposed to high conspecific brood parasitism, see Lopez-de-Hierro and Moreno-Rueda 2010). If inter-clutch variability evolves in response to risk of brood parasitism, inter-clutch repeatability of blue-green, brown, and ultraviolet chroma should decrease with risk of parasitism (i.e., within a species experiencing a high risk of parasitism there should be greater differences in colour between clutches).

A similar recognition function of egg colouration may be expected within open-nesting species that breed in dense nesting colonies. In colonial nesters, egg colour may facilitate a rapid return to the correct clutch and retrieval of displaced eggs (Noble and Lehrman 1940; Baerends and Vanrhijn 1975; Gaston et al. 1993). In certain species, the clutch can even be a more important orientation signal for colonial birds than either the

nest or nest site (Kirkman 1937; Noble and Lehrman 1940; Baerends et al. 1970). If inter-clutch variation in egg colour facilitates recognition within a colony, inter-clutch repeatability in blue-green, brown, and ultraviolet chroma should be lower in colonially nesting birds, particularly in species that nest in the open, where eggs may serve as a useful signal for visual recognition and egg retrieval.

**Methods**

*Egg reflectance*

We measured egg colouration of 5,604 eggs from 636 species ($3.06 \pm 0.07$ clutches per species, $8.81 \pm 0.27$ eggs per species) representing 26 of 27 avian orders (excluding Pterocliformes). We obtained these reflectance measurements from preserved specimens at four natural history collections: the American Museum of Natural History, the Field Museum, the University of Michigan Museum of Zoology, and the National Museum at Tring. There were minor differences in how we obtained the spectra between the museums. We measured the eggs from National Museum at Tring (31% of eggs sampled) at a coincident normal measurement angle using an Ocean Optics USB2000 Miniature Fiber Optic Spectrophotometer with illumination by a DT mini lamp (Cassey et al. 2010b). We measured reflectance spectra from the other collections at 45 degree coincident oblique measurement geometry using an Ocean Optics USB 4000 and a PX-2 pulsed xenon light source (Ocean Optics, Dunedin, FL). We compared both sets of spectra to a Spectralon white standard (WS-1) and summarized reflectance spectra into 5nm bin classes using a script written in SAS v9.2 for the eggs sampled at National Museum at Tring and using CLR for all other eggs (Montgomerie 2008).

Three species that were measured at three separate collections revealed that eggs sampled with different measurement geometries only differed significantly in brightness in one species (*Guira guira* $F_{2,36} = 40.3$, $P < 0.0001$), and this species has a variable white lattice pattern across the entire shell surface and exceptional variation in blue-green chroma, such that all collections were significantly different from one another, even if they were measured using the same measurement geometry ($F_{2,36} = 106.85$, $P < 0.0001$). Therefore, we pooled the data from all museums because variation across collections was not as important as variation between species.

For each egg, we recorded six spectra across the entire shell surface such that two measurements were collected from each of three distinct regions of the egg: blunt end, equator, and pointed end. Since this study focused specifically on the evolution of ground colouration, rather than spotting, we avoided measuring spots wherever possible. Our measurements should have adequately captured general ground colouration because any spotting we could not avoid measuring should have been fine enough to create a uniform impression across the entire shell surface and effectively become part of the perceived ground colour. Although this perception would depend on viewing distance and visual acuity, it is likely that most birds would perceive fine spotting as a relatively uniform surface because most birds have poorer acuity than the average human viewer (Columbiformes, Blough 1971; Hodos 1993; Passeriformes, Fife et al. 1975). There are exceptions, however, such as raptors that possess greater acuity than humans (Jones et al. 2007). The high acuity in this group should not overly influence our interpretation because in most raptor eggs, the spots are spaced widely enough that we could avoid spots when measuring ground colouration.

We visually inspected each spectrum (N = 33,624) to screen for any aberrant recordings and excluded these prior to analysis. We then averaged the remaining readings to obtain a single reflectance spectrum per egg (N = 5,604). We used these spectra to calculate four standard colorimetric variables: ultraviolet chroma (the sum of reflectance between 300 – 400 nm as a proportion of the sum of reflectance between 300 - 700nm), blue-green chroma (the sum of reflectance between 450 – 550 nm as a proportion of the sum of reflectance between 300 -700nm), brown chroma (the sum of reflectance between 600 – 700 nm as a proportion of the sum of reflectance between 300 -700nm), and brightness (average reflectance across entire visible spectrum 300 – 700 nm). To test hypotheses relating to egg recognition and sensory bias, we calculated inter-clutch repeatability for these colorimetric variables (Lessells and Boag 1987).

While chroma values may be adequate for intraspecific examinations of colour, they do not perform as well in interspecific studies for comparisons of spectra that differ in shape, particularly when comparing spectra characterized by peaks and plateaus. For example, the blue-green chroma value calculated for a white egg can be similar to that of a blue-green egg (Figure 1). This can occur when the total amount of reflectance above the blue-green spectrum between 550 -700 nm is equivalent to the total amount of reflectance below the blue-green spectrum between 300 – 450 nm. To obtain a measure of chroma that we could compare across species, we calculated the proportion of blue-green to brown chroma, a measure we call proportional blue-green chroma. This metric should allow us to assess the relative contribution of pigments. A high proportional blue-green chroma should correspond to eggs containing more biliverdin relative to porphyrin,

whereas a low proportional blue-green chroma should correspond to egg containing more

porphyrin relative to biliverdin.

*Influence of egg fading*

Our data were obtained from eggs collected over more than 100 years (1825 – 2006);

however, most eggs in our dataset were collected within the interquartile range of 1896 –

1924. It is possible that these eggs have faded since they were collected; therefore, we

correlated average clutch colorimetric values by date of collection to assess the effect of

fading on this dataset. We accompany these measures of effect with their 95% confidence

intervals, which represent an estimate of precision for the effect statistic (Nakagawa and

Cuthill 2007). Brightness and ultraviolet chroma were significantly correlated with

collection date (brightness: $r = 0.07$, $CI_{0.95} = 0.02$ to $0.12$, $N = 1618$, $P = 0.005$; ultraviolet

chroma: $r = 0.08$, $CI_{0.95} = 0.04$ to $0.13$, $N = 1618$, $P = 0.001$), however our other

colorimetric variables were not significantly correlated with collection date (blue-green

chroma: $r = -0.04$, $CI_{0.95} = -0.09$ to $0.008$, $N = 1618$, $P = 0.10$; brown chroma: $r = -0.03$,

$CI_{0.95} = -0.08$ to $0.02$, $N = 1618$, $P = 0.24$; proportional blue-green chroma: $r = 0.02$,

$CI_{0.95} = -0.03$ to $0.07$, $N = 1618$, $P = 0.34$). Despite the fact that some of our colorimetric

variables were correlated with collection year, the size of the correlation coefficients and

their confidence limits indicate that the effect of collection date in our dataset is small

(Cohen 1988). Furthermore, recent research on a subset of these eggs found no effect of

collection date (Cassey et al. 2010a), which seems to be a general trend in studies

utilizing egg collections (Soler et al. 2005; Jagannath et al. 2008). Although this does not

mean that the eggs we measured did not undergo any fading, it shows that this effect was only marginal in our dataset collected over a range of over 100 years.

*Natural history data*

We compiled information on the life histories of the species in our dataset from 564 peer reviewed articles and species accounts (details provided in Appendix I). We preferentially selected average values from studies with large sample sizes, and avoided reports that were poorly documented (e.g., values mentioned in a single study with no information about how those data were collected). Specifically, we recorded information on nest type (ground, open cup, cliff, dome, cavity, burrow, or mound), degree of sociality (social or solitary), incubation period (in days), the egg in the laying sequence when incubation begins (egg #), clutch size (number of eggs), incubation attendance (percentage of time spent on the nest), form of parental care (male only, female only, bi-parental), incubation roles (male only, female only, bi-parental), parasitism risk (percent of population parasitized), predation risk (percentage of eggs depredated, avoiding any records that were associated with the introduction of invasive species), male and female provisioning rate (feeding trips per hour), and developmental category (super-precocial, precocial, semi-precocial, semi-altricial, altricial; *sensu* Stark 1993). We divided the egg number in the laying sequence where incubation begins by the clutch size as an estimate of incubation initiation relative to clutch completion (where low values represent species that begin incubating early in the laying sequence). In addition, we also calculated relative male provisioning rate as male provisioning rate divided by female provisioning rate.

*Phylogenetic reconstruction & comparative analyses*

We used Mesquite (version 2.6) to reconstruct a phylogeny based on the species represented in our dataset. For this purpose, we combined data from numerous published sources, including recent hypotheses for relationships among all birds (Ericson et al. 2006; Hackett et al. 2008) and among passerines (Jønsson and Fjeldså 2006). We utilized current molecular phylogenies, and in a few cases we used data from sources that combined molecular and morphological phylogenies (Appendix II). As our data originated from multiple sources, branch lengths could not be preserved from the source trees, and we therefore used ultrametricized branch lengths which sets the distance from the root to all tips as equal (Lapointe and Legendre 1991).

We used the 'nlme,' and 'ape' packages in R, v 2.7.1 (Ihaka and Gentleman 1996) to run phylogenetic least squares (PGLS) analyses, which can be applied to phylogenies with polytomies (Pagel 1997). For our PGLS analyses we used the maximum likelihood value of Pagel's $\lambda$ (Pagel 1997, 1999), which transforms a phylogeny to make the data best fit a Brownian motion model of evolution (Freckleton et al. 2002). We used Box-Cox transformations on non-normal continuous variables to improve normality (for specific transformation details see Appendix III). In addition, we used multiple assessments to evaluate model fit (Freckleton 2009), including the distribution of normalised residuals as well as quantile-quantile plots. When comparing variation in traits across groups, we applied a Tukey's HSD post-hoc test. These were calculated based on fitted value for the group levels and mean square error from the PGLS analysis, rather than from a separate non-phylogenetic ANOVA model. To assist in interpretation

of the relationships, we present partial correlation coefficients from the PGLS analyses bounded by their lower and upper confidence limits (Nakagawa and Cuthill 2007).

**Results**

*Crypsis and aposematism hypotheses*

Contrary to our expectations, there was no significant relationship between predation rate and egg proportional blue-green chroma (r = - 0.17, $CI_{0.95}$ = - 0.38 to 0.08, N = 65, P = 0.19) or brightness (r = - 0.09, $CI_{0.95}$ = -0.32 to 0.15, N = 65, P = 0.46). However, predation rates were negatively related to ultraviolet chroma (r = -0.26, $CI_{0.95}$ = -0.46 to - 0.02, N = 65, P = 0.04), suggesting that eggs with high UV chroma experienced lower predation. This finding is contrary to our expectation that UV chroma would decrease crypsis, but is consistent with our predictions for the aposematism hypothesis. We also examined these patterns within species nesting in open cups above the ground. In this group, eggshell brightness was positively related to predation rate (r = 0.89, $CI_{0.95}$ = 0.80 to 0.93, N = 33, P < 0.0001), suggesting that brighter eggs are at higher risk of nest predation. There was no significant relationship between predation rate and proportional blue-green chroma (r = 0.10, $CI_{0.95}$ = -0.25 to 0.41, N =33, P = 0.59) or UV chroma, (r = 0.22, $CI_{0.95}$ = - 0.14 to 0.50, N = 33, P = 0.23).

*Blackmail hypothesis*

The timing of incubation initiation was unrelated to egg brightness (r = 0.03, $CI_{0.95}$ = - 0.10 to 0.16, N = 223, P = 0.66), proportional blue-green chroma (r = 0.04, $CI_{0.95}$ = -0.09 to 0.17, N =223, P = 0.59), or ultraviolet chroma (r = -0.01, $CI_{0.95}$ = -0.14 to 0.12, N =

223, P = 0.85). In analyses focussing on nest attendance, we controlled for the length of the incubation period because higher nest attendance is known to be associated with shorter incubation periods (Martin et al. 2007). In support of our predictions, nest attendance was positively related to proportional blue-green chroma (r = 0.33, $CI_{0.95}$ = 0.12 to 0.51, N = 76, P = 0.003) and UV chroma (r = 0.39, $CI_{0.95}$ = 0.18 to 0.55, N = 0.76, P < 0.0001), but was not related to brightness (r = 0.12, $CI_{0.95}$ = -0.11 to 0.33, N =76, P = 0.30). Contrary to our predictions, however, proportional blue-green chroma was lower in species with male-only incubation than in species with either biparental or female-only incubation ($F_{2,339}$ = 3.19, P = 0.04; Figure 2), while neither brightness ($F_{2,339}$ = 2.10, P = 0.13) nor UV chroma ($F_{2,339}$ = 0.66, P = 0.52) were related to incubation roles. An assumption of the blackmail hypothesis is that more colourful eggs should be at higher risk of predation or brood parasitism. Accordingly, risk of brood parasitism was positively related to proportional blue-green chroma (r = 0.33, $CI_{0.95}$ = 0.11 to 0.51, N =76, P = 0.005), but was unrelated to brightness (r = 0.12, $CI_{0.95}$ = - 0.11 to 0.34, N = 76, P = 0.34) or UV chrom (r = -0.02, $CI_{0.95}$ = -0.25 to 0.20, N =76, P = 0.83). In addition, predation rate was positively related to brightness but negatively related to UV chroma (see results for *Crypsis and aposematism hypotheses* above).

*Sensory bias hypothesis*

The timing of incubation initiation was unrelated to inter-clutch variation in egg brightness (r = -0.05, $CI_{0.95}$ = - 0.19 to 0.09, N = 191, P = 0.51), proportional blue-green chroma (r = 0.02, $CI_{0.95}$ = - 0.12 to 0.16, N = 191, P = 0.82), or UV chroma (r = 0.01, $CI_{0.95}$ = -0.13 to 0.15, N = 191 P = 0.85). Contrary to our predictions, inter-clutch

repeatability of ultraviolet chroma was negatively related to incubation attendance when controlling for incubation period (r = -0.26, $CI_{0.95}$ = -0.46 to -0.02, N = 67, P = 0.03) and was unrelated to eggshell brightness (r = 0.23, $CI_{0.95}$ = -0.02 to 0.43, N = 67, P = 0.07) or proportional blue-green chroma (r = 0.06, $CI_{0.95}$ = -0.29 to 0.18, N = 67, P = 0.63).

*Sexual signalling hypothesis*

Contrary to our predictions, among species with open nests, blue-green chroma was lower in species with male-only care than in species with bi-parental or female-only care ($F_{2,278}$ = 3.91, P = 0.02; Figure 3). There were no differences between these groups in terms of proportional blue-green chroma ($F_{2,278}$ = 1.99, P = 0.14). In species with open nests, excluding female-only care and precocial species, there was no relationship between blue-green chroma (r = 0.17, $CI_{0.95}$ = -0.16 to 0.45, N = 37, P = 0.30) or proportional blue-green chroma and relative male provisioning (r = -0.02, $CI_{0.95}$ = - 0.33 to 0.30, N =37, P = 0.91).

*Parasitism recognition hypothesis*

We found that parasitism risk was significantly negatively related to inter-clutch repeatability of blue-green chroma (r = -0.24, $CI_{0.95}$ = -0.44 to 0.003, N = 68, P = 0.05), but not brown (r = -0.07, $CI_{0.95}$ = -0.30 to 0.17, N = 68, P = 0.56), or ultraviolet chroma (r = 0.07, $CI_{0.95}$ = - 0.17 to 0.30, N =68, P = 0.55). We also assessed this relationship again with the addition of dome-nesting species, as visual egg recognition signals have previously been established in some dome nesting species that suffer from high rates of brood parasitism (Davies 2000). As with the previous analyses, parastisim risk was

negatively related to inter-clutch repeatability of blue-green chroma (r = -0.24, $CI_{0.95}$ = -0.43 to -0.01, N = 76, P = 0.04), but not brown (r = - 0.13, $CI_{0.95}$ = -0.34 to 0.10, N = 76, P = 0.26) or ultraviolet chroma (r = -0.03, $CI_{0.95}$ = - 0.25 to 0.19, N = 76, P = 0.77).

*Coloniality recognition hypothesis*

Contrary to our predictions, inter-clutch repeatability of blue-green ($F_{1,261}$= 2.10, P = 0.15), brown ($F_{1,261}$= 0.05, P = 0.82), and ultraviolet chroma ($F_{1,261}$= 0.29, P = 0.59) did not differ between colonial and solitary species.

**Discussion**

In this study, we used a large comparative dataset to investigate multiple hypotheses for the evolution of egg colour. Our findings supported the predictions of some hypotheses but not others. In support of the crypsis hypothesis, we found that egg brightness was positively correlated with predation rate, suggesting that brighter eggs might be more conspicuous to predators. We also found that eggs with high UV chroma experienced lower predation rates, which contradicts the crypsis hypothesis but supports the aposematism hypothesis. The risk of parasitism was higher in species with eggs exhibiting high proportional blue-green chroma, supporting a key assumption of the blackmail hypothesis that risk should be associated with conspicuous egg colours. Our findings also supported the prediction that nest attendance should increase with proportional blue-green and ultraviolet chroma. Our findings contradicted predictions of the sensory bias and sexual signalling hypotheses, and provided no support for the coloniality recognition hypothesis. However, we did find that variability in blue-green

chroma between clutches increased with risk of parasitism, supporting the hypothesis that

brood parasitism could drive selection for high inter-clutch variability in egg colour. Our

findings highlight the diversity of selective factors that can influence the evolution of

avian egg colour, including predation risk, parental behaviour, and egg recognition.

Nest predation has an important selective influence on egg colour evolution

(Haskell 1996). Numerous studies illustrate the relative importance of eggshell spotting

in reducing predation rates (Sánchez et al. 2004; Šálek and Cepáková 2006;

Westmoreland 2008), and provide evidence that egg predation is related to the predator's

visual system (Blanco and Bertellotti 2002). The relationship between ground colouration

and predation pressure is not as clear. Some studies have found no influence of eggshell

ground colouration on predation levels (Götmark 1992; Weidinger 2001; Avilés et al.

2006; Brennan 2010). Others have proposed that immaculate white eggs could resemble

transparent holes in the forest canopy when viewed from beneath loosely constructed

nests, and in that way appear cryptic (Oniki 1985). Our data suggest the opposite pattern,

as predation rates were higher for brighter eggs. This is in agreement with previous

research which has shown that when placed outside of the nest, blue and white eggs are

more likely to be depredated than brown eggs (Götmark 1992). Moreover, an

observational study investigating egg predation rates (within the nest) in a species

exhibiting blue and white egg colour polymorphism found no difference in predation

rates between white and blue eggs (Kim et al. 1995). Relationships between predation

pressure and egg colour are generally rationalized by focusing on nests and nest activity

(Skutch 1976; Götmark 1992). This has led some to suggest that egg crypsis may be

relaxed in conspicuous nests (Götmark 1993). It may be more parsimonious to conclude

that brown eggs are generally cryptic, while both immaculate white and blue-green eggs are not, especially when these eggs are laid within a brown nesting substrate. In addition, nest predation studies have found that open cup nests generally experience higher predation levels than closed nests (Martin 1995). Therefore, it is not surprising that we found egg brightness to be positively related to predation levels in open cup nests, because egg brightness would be a detectable cue to any visually orienting predator (Kelber et al. 2003). Nevertheless, the predation levels reported in experimental and observational studies should be viewed cautiously since it is likely possible that predators are attracted by foreign odours (of the researcher), not egg colour (Kilner 2006).

Our findings supported a single prediction of the aposematism hypothesis: that ultraviolet egg chroma should be negatively related to predation rate. In the absence of other supporting relationships, however, it is difficult to interpret this as evidence for aposematism, particularly since many nest predators may not have the ability to detect UV wavelengths (Guilford and Harvey 1998; Bowmaker and Hunt 2006). It is also unlikely that this pattern is a result of UV-chromatic eggs providing a better match to the nest material, because nest material is not highly reflective in the UV region and usually increases the contrast of UV reflective objects in the nest (Hunt et al. 2003; Jourdie et al. 2004). Instead, a negative relationship between UV chroma and predation risk may be more reasonably explained by our finding that nest attendance behaviour is higher for eggs with higher UV chroma. Thus, predation would be lower for these eggs because they are obstructed from view.

We found two lines of support for the blackmail hypothesis: proportional blue-green chroma was significantly positively related to parasitism risk, and eggs with higher

proportional blue-green and ultraviolet chroma had higher nest attendance. The blackmail

hypothesis proposes that the risks associated with predation and parasitism may force

males to invest more to help keep conspicuous eggs covered (Hanley et al. 2010). Our

data appear to support the blackmail hypothesis only within the context of brood

parasitism because parents have high attendance at nests containing eggs with a relatively

higher proportion of blue-green chroma, and these eggs experienced higher parasitism

pressure. The blackmail hypothesis also suggests that this risk should result in parents

covering eggs earlier, rather than waiting until clutch completion. However, our data

suggest that eggshell ground colour is selectively neutral in regards to incubation

initiation. In addition, contrary to our predictions, we found that proportional blue-green

chroma was actually lowest in species exhibiting male-only incubation. An alternative

explanation for the relationship between proportional blue-green chroma and nest

attendance involves potential anti-microbial properties of egg pigments.  Recent research

has revealed that proto-porphyrin possesses a photodependent mechanism for protecting

the shell from infection by gram positive bacteria, whereas biliverdin does not possess

this mechanism (Ishikawa et al. 2010). Previous research has also shown that incubation

attendance significantly reduces microbial infection rates (Cook et al. 2005a; Cook et al.

2005b). This may explain why eggs with high proportional blue-green chroma (and hence

low brown chroma) experience higher incubation attendance. However, such an

explanation would also favour an early onset of incubation for these eggs (Cook et al.

2003), which we did not find.

Inter-clutch repeatability in UV chroma was negatively related to nest attendance,

such that nest attendance decreased when inter-clutch repeatability was high. This finding

is in direct contrast to the predictions of the sensory bias hypothesis. Sensory bias mechanisms for preferences in colour have been discovered in a wide range of taxa (Gerhardt 1994; Rodd et al. 2002; Raine and Chittka 2007), including birds (Møller and Erritzøe 2010). It is possible that we did not detect any patterns in this study because we considered the question too broadly. If preferences for specific egg colours explain the evolution of conspicuous eggs, future investigations may benefit from examining this question in a group with high egg colour diversification rates, where extant species vary greatly in terms of eggshell colour as well performing field investigations to establish species-specific egg colour preferences.

A recent comparative investigation of the sexual signalling hypothesis revealed an association between blue-green egg colouration and the length of the nestling period (Soler et al. 2005), which the authors interpreted as an indication that paternal effort was higher for birds with blue-green eggs. However, numerous other factors are known to influence the length of the nestling period, and degree of male paternal effort, relative to the female, varies across species with different mating systems (Kendeigh 1952; Bosque and Bosque 1995). Whereas this previous study focused on the nestling period to assess paternal effort, we investigated whether different forms of parental care may be related to differences in egg colour. Under the sexual signalling hypothesis, species with bi-parental care should have greater blue-green chroma than species with either male- or female-only care (Moreno and Osorno 2003). Contrary to this idea, we found that species with male-only care had significantly lower blue-green chroma than other forms of parental care, with no differences between female- or bi-parental care. In addition, relative male provisioning was unrelated to either blue-green chroma or proportional chroma. There

has been mixed experimental support for the sexual signalling hypothesis (Reynolds et al. 2009), and our lack of support in this broad comparative analysis suggests that future comparative investigations of this hypothesis should focus on specific lineages where such a mechanism is likely.

Our findings also provide support for an association between heterospecific brood parasitism and eggshell ground colouration. Recognizing parasitic eggs as a defence mechanism is an evolutionary viable strategy, although host anti-parasitic behaviours may be limited by both the visibility of eggs within the nest and the host's ability to eject the eggs or otherwise modify the nesting attempt (Davies 2000; Langmore et al. 2005; Antonov et al. 2009). If parasitic eggs remain in the nest, some early-hatching young parasites may eject their host's eggs, which is an advantageous strategy for the parasitic young, despite the physical costs associated with egg ejection (Grim et al. 2009). The distinctiveness of a clutch may allow a female to recognize a foreign parasitic egg, as well as confound parasites to find an appropriate match (Swynnerton 1918; Victoria 1972; Davies and Brooke 1989). Intra-clutch variation appears to be less related to parasitism pressure than inter-clutch variation (Stokke et al. 2002), which may occur because distinctiveness is not merely defined by low variation within a clutch. In this study, we found that inter-clutch repeatability in ground colouration was related to parasitism risk. Our findings provide support for an influence of inter-clutch variation in ground colour, aside from any effect of speckling, on the evolutionary arms race between hosts and brood parasites. Our findings also contribute to a growing body of research revealing similar associations between inter-clutch variation and parasitism risk (Øien et al. 1995; Soler and Møller 1996; Avilés et al. 2004; Avilés et al. 2010; Yang et al. 2010)

and suggest that the overall colour of a bird's egg may be useful for recognition of parasitic eggs. The relative importance of speckling and colour in egg recognition appears to differ between species that lay speckled versus immaculate eggs (Rothstein 1982; Lopez-de-Hierro and Moreno-Rueda 2010), yet at the inter-specific level, egg ground colour alone was an important enough factor to be uncovered in our analyses.

A similar argument could apply to colonially-nesting species that may need to recognize their own eggs in dense breeding colonies. However, we found no support for the idea that inter-clutch repeatability of egg colour was related to coloniality. Our findings may illustrate an interesting difference between egg recognition under parasitism pressure versus egg recognition in colonial breeding. Recent research suggests that in the context of conspecific brood parasitism (i.e., egg dumping), eggshell ground colouration is not as important as egg speckling (Øien et al. 1995; Siefferman 2006; Lopez-de-Hierro and Moreno-Rueda 2010). Within a dense colony, variation between clutches in terms of egg appearance is hypothesized to enhance recognition of one's own egg from that of nearby conspecifics. Furthermore, previous research has revealed that egg spotting patterns are important in egg recognition of colonially nesting birds (Gaston et al. 1993). The support for a link between inter-clutch repeatability of egg colour in the context of brood parasitism, but a lack of an association in the context of coloniality, may indicate an underlying difference in how egg recognition has evolved in these two situations. Perhaps variation in eggshell spotting is more important for egg recognition in colonially-nesting birds, or perhaps recognition of one's own eggs, rather than differentiation from those of conspecifics, is more important in this context. If eggshell patterning is a more important cue than eggshell ground colour, differences in costs between the two

strategies may be responsible. As pigment deposition is thought to be costly (Morales et al. 2008), altering the placement of pigments (speckling) in response to parasitic pressure should be less costly than altering the relative contribution of the pigments themselves (ground colouration).

Although our omission of eggshell spotting may have limited our ability to detect patterns associated with the hypotheses we tested, our focus on ground colouration may provide additional insight into the evolution of avian eggshell colouration. The omission of eggshell spotting likely adds a significant amount of noise to our dataset because some of the species we measured had immaculate colouration, whereas some were heavily spotted. For example, although spotting has been shown to have a powerful effect on predation rates (Montevecchi 1976; Castilla et al. 2007), our data revealed an association between predation rates and eggshell ground colouration aside from any influence of spotting. We encourage future investigations to examine the combined effects of eggshell ground colouration and eggshell spotting, and tests of hypotheses focusing on egg conspicuousness would benefit from including quantitative variation in eggshell spotting, eggshell ground colouration, nest material colour and patterning, and light environments.

In this paper we use a large comparative dataset to test multiple hypotheses related to the evolution of egg colouration. It is important to emphasize that these hypotheses are not mutually exclusive, and the predictions we tested are not exclusive to these hypotheses. For example, greater male nest attendance in species with greater blue-green egg colouration may indicate support for the blackmail hypothesis or may simply suggest that males always cover colourful eggs without implicating any sexual conflict. Furthermore, the interpretation of non-significant results in a broad comparative analysis

may be complicated when species with differing life-history strategies are included in a single analysis; in this way, lineage-specific patterns could be masked.For example, we found no support for the hypothesis that eggshell colour is used in egg recognition in colonially nesting birds. However, such a mechanism may be adaptive for a small set of colonial birds, while the majority use other recognition cues. A broad comparative approach may overlook this variation, and in such cases lineage- or species-specific studies would be appropriate follow-up tests. By contrast, significant results found in broad comparative analyses indicate patterns that are strong enough to be detected despite taxonomically diverse datasets.

Our findings make an important contribution to our understanding of the diversity of selection pressures that influence egg colouration. We found several consistent patterns between eggshell ground colouration and life history traits, revealing that egg colouration may provide visual information in the form of cues or signals to parents, parasites, and predators. Variation in egg colouration has likely evolved for numerous and complex reasons, and the fact that we were able to support some of these hypotheses in such a large and diverse group of birds highlights the importance of these selective pressures. In such large comparative analyses, multiple, competing influences on egg colouration, and large differences in life history traits between distantly related species, may mask some important patterns. We encourage the contribution of future comparative studies that examine egg colour evolution in groups of closely related species, which may help to control for some of these confounding influences on egg colour evolution.

**Acknowledgments**

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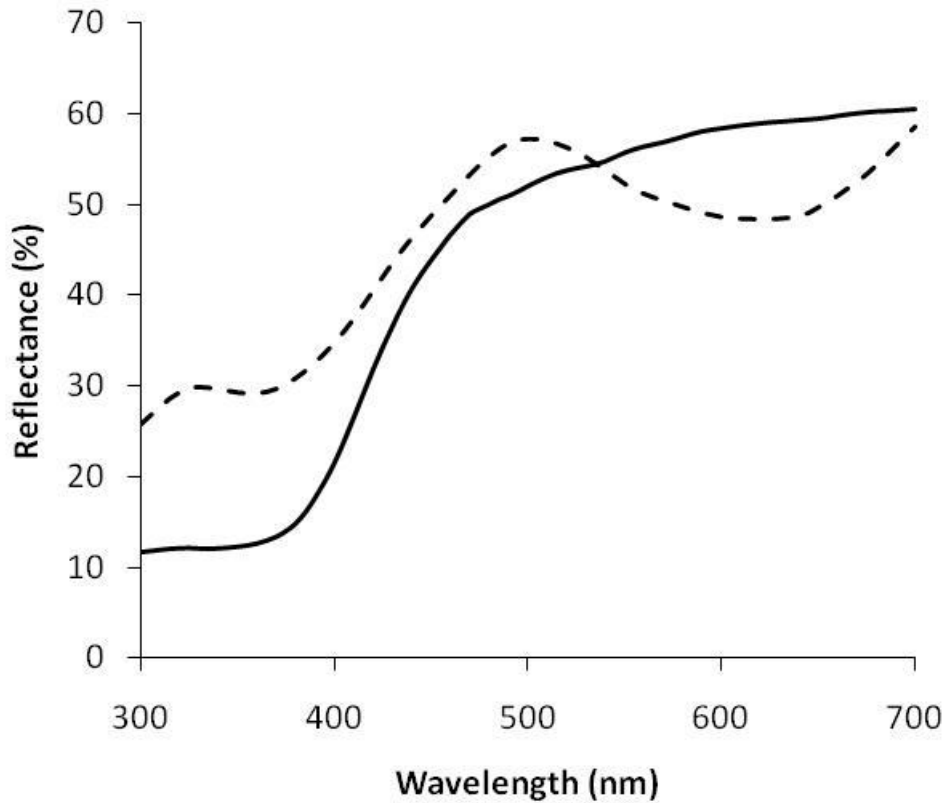
**Figure 4. 1**

Average reflectance spectra of naturally blue-green eggs from *Turdus philomelos* depicted by the
dashed line (N = 23), and naturally white eggs of *Struthio camelus* depicted by the solid line (N =
3), smoothened with a locally weighted polynomial regression using the lowess package in R.
Despite appearing quite different n colouration, these species have an identical value for blue-
green chroma (0.31). Proportional blue-green chroma values (blue-green chroma/ brown chroma)
were different: *T. philemons* (1.06) and *S. camelus* (0.86).

**Figure 4. 2**

Data represent the back transformed predicted values from a phylogenetic generalized least squares analysis predicting proportional blue-green chroma by forms of incubation behaviour (male-only, bi-parental, and female-only; *for details on back transformation see Appendix III*). SE bars used here depict the standard error of the raw data, however small letters above the bars represent Tukey's honest significant differences between the group means calculated from the predicted values and MSE of the PGLS analysis.
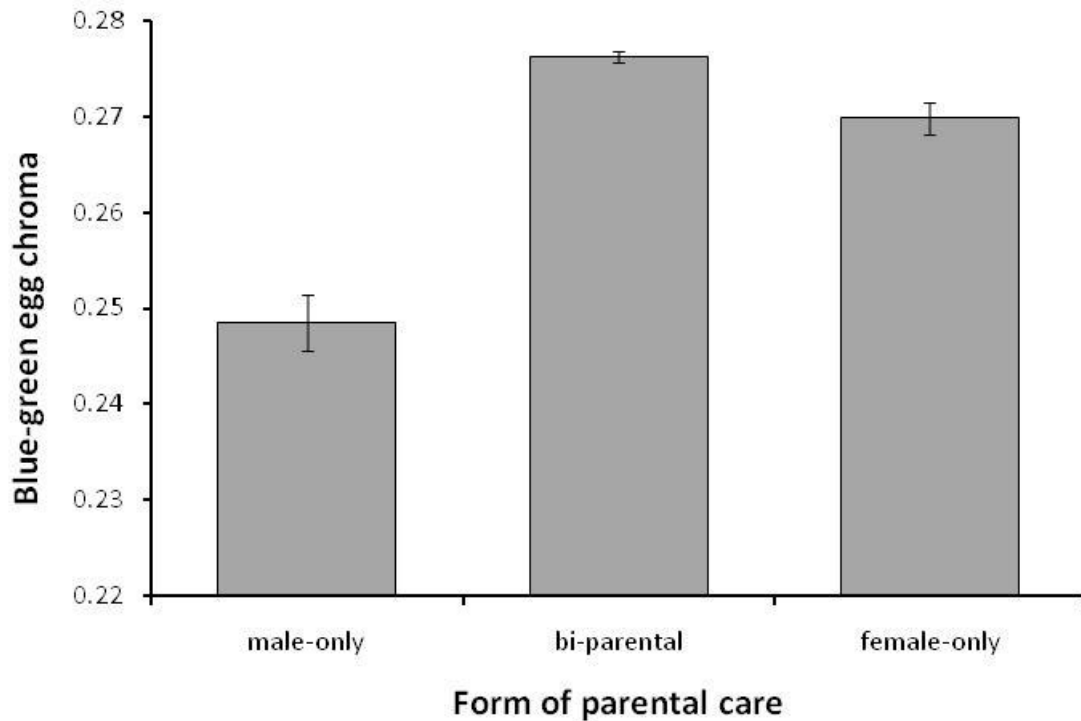
**Figure 4. 3**

Data represent the back transformed predicted values from a phylogenetic generalized least squares analysis predicting blue-green chroma by forms of parental care not necessarily restricted to incubation duties (male-only, bi-parental, and female-only; *for details on back transformation see Appendix III*). SE bars used here depict the standard error of the raw data, however small letters above the bars represent Tukey's honest significant differences between the group means calculated from the predicted values and MSE of the PGLS analysis

# CHAPTER 5 - A COMPARATIVE TEST OF VISIBILITY, ANTI-MICROBIAL, AND SOLAR RADIATION HYPOTHESES FOR THE EVOLUTION OF EGG COLOUR IN BIRDS[4]

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[4] This manuscript is the product of joint research

**Summary**

There exits dramatic variation in the colouration of birds' eggs. This variation has been the subject of much interest and investigation. In this study, we provide the largest comparative examination of selection pressures on avian eggshell colouration to date. We examine whether nest type and broad environmental factors, such has habitat type, appear to favour certain egg colours or pigmentation strategies. We find support for the hypothesis that eggshell brightness increases egg visibility in cavity and burrow nests by showing that brightness was significantly higher in dark nest types, and that hatching success was positively correlated with egg brightness only within dark nest types. We also provide evidence that this is not simply a result of decreased selection for pigmentation in dark nests. Though recent research suggests that some eggshell pigments may provide antimicrobial protection, we did not find comparative support for this hypothesis. We also investigate whether certain egg colours might reduce the effects of harmful solar radiation on developing embryos by examining the colour of eggs found in open nests across different habitat types. We provide suggestive evidence that eggs found in the tundra, a very open habitat type, have significantly darker and potentially more pigmented eggs. Our findings suggest that a diversity of environmental factors likely influence the evolution of egg colouration in birds. Future studies may benefit from re-examining these hypotheses through comparative analyses within groups of closely-related species, and through experimentation in the field and laboratory.

**Introduction**

Avian eggshell colouration represents one of the most diverse examples of natural colour variation in animals, yet the evolutionary mechanisms explaining this variation remain poorly understood. Avian egg colouration is particularly interesting because eggshell pigmentation is unique to birds, is present in even the most basal species, and exhibits dramatic interspecific variation (Kilner 2006; Cassey et al. 2010b). Despite considerable speculation about the origin and maintenance of the diversity of avian egg colours, few comparative examinations have investigated how life-history traits might have influenced the evolution of quantitative variation in the uniform colouration covering the shell surface, known as ground colouration (Soler et al. 2005; Avilés et al. 2006). Indeed, most research on avian egg colouration has focussed on intra-specific studies, with a recent emphasis on the possible signal function of variation in colour (reviewed in Reynolds et al. 2009). Although these studies have enhanced our understanding of the various selective pressures that can influence egg colouration, comparative investigations allow us to test multiple hypotheses for the evolution of egg colour across taxonomically diverse species. In this study, we use a broad comparative analysis to investigate multiple hypotheses relating to the influence of life-history variables such as nest type and nest environment on the evolution of egg colour. In particular, we test hypotheses relating to egg visibility, the risk of microbial invasion, and vulnerability to damaging solar radiation.

One of the most notable patterns observed in relation to avian egg colouration is that the eggs of cavity-nesting species are generally white or de-saturated (Lack 1958; Avilés et al. 2006; Kilner 2006). Increased egg brightness may be adaptive in nests with

low light levels if this makes them more visible to the parents, thereby facilitating incubation and egg rotation while reducing the risk of breakage (Abercrombie 1931; von Haartman 1957; Holyoak 1969); we term this the *egg visibility hypothesis*. For example, one experiment found that artificially darkened eggs were cracked more often by parents upon entering and exiting their nesting cavities (Holyoak 1969). Recent research employing avian visual modelling also suggests that in the cavity-nesting blue tit, *Cyanistes caeruleus*, egg brightness is a more important factor in eggshell discrimination than differences in colour (Holveck et al. 2010). Another recent study found that experimental eggs reflecting more ultraviolet (UV) light were retrieved from the nest perimeter significantly more often than eggs that did not reflect in the UV within the dimly lit nests of spotless starlings, *Sturnus unicolor* (Avilés et al. 2006), suggesting that some colour information is also useful for discrimination at low light levels.

Although previous research highlights the potential benefits of bright egg colouration in nests with low light levels, these data don't necessarily demonstrate that there is selection for bright eggs in dark nests. It is possible, for example, that within dim nest cavities, bright white eggs evolve via genetic drift from relaxed selection pressure on egg colouration (Oniki 1985). Alternatively, there could be selection against egg pigmentation in nests with poor visibility, especially if pigments are costly to deposit (Morales et al. 2008). If selection favours brighter eggs in dark environments, species nesting in enclosed nests should have brighter eggs than species nesting in open nests. In addition, brighter eggs should have greater hatching success in closed nests. We do not expect this relationship in fully lit nests where egg detection should not be limited by egg brightness. In addition, if UV chroma enhances egg visibility (Avilés et al. 2006), we

expect to find higher UV chroma in enclosed nests and higher hatching success in relation to UV chroma in these nests. If the evolution of bright eggs in cavity-nesting species results from genetic drift due to relaxed selection pressure, egg brightness should follow a Brownian motion model of evolution, which approximates a process of random genetic drift (Antonelli et al. 1977). This combination of predictions should allow us to distinguish between natural selection for brighter egg colour in dark nests and genetic drift resulting from relaxed selection.

Another intriguing possibility is that eggshell pigments are a functional component of the insoluble shell matrix that reduces microbial growth and invasion of the egg (Soler et al. 2005), which we term the *anti-microbial hypothesis*. Recent research has shown that open-cup nesting species experience greater bacterial growth on shell surfaces and greater penetration of microbes through the shell than cavity-nesting species (Godard et al. 2007). Open nests are exposed to rainfall, and the material in these nests may retain more moisture. Moreover, nest temperatures in cavities are regularly higher than 27°C, which is high enough to initiate the antibacterial enzymatic activity of the albumen (Beissinger et al. 2005). In open-cup nests, incubation reduces the severity of these bacterial infestations (Cook et al. 2005a), suggesting a possible role for incubation prior to the completion of egg laying (Cook et al. 2003, 2005b). These findings suggest that morphological (egg colouration) and behavioural (incubation) adaptations may represent two independent or synergistic mechanisms for protecting eggs from microbial invasion.

In support of the idea that avian egg pigments may help prevent microbial invasion, recent research has demonstrated that proto-porphyrin IX reduces the survival

of gram positive bacteria (Ishikawa et al. 2010). This pigment, which is responsible for

the production of brown colouration in eggshells, is a macrocyclic tetrapyrrole with

photo-dependent antimicrobial properties (Malik et al. 1988; Karmakar et al. 1995;

Papkovsky et al. 1995; Stojiljkovic et al. 2001; Bozja et al. 2003; Bozja et al. 2004).

These findings suggest that in addition to the beneficial camouflage properties that brown

pigmentation may confer to eggs laid in open nests (Götmark 1992; Haskell 1996;

Weidinger 2001; Svagelj et al. 2003), porphyrin pigments may also provide photo-

dependent anti-microbial protection. We therefore predict that brown chroma should be

lower in nests that have a lower risk of microbial invasion, which include cavity and

burrow nests, and higher in habitats where risk of microbial invasion is greatest. Since

cavity and burrow nests have a lower risk of microbial infection, we expect hatching

success to be positively related to brown egg chroma in high-risk nest types (controlling

for differences in colour due to nest type), but do not expect the same finding in the

lower-risk cavity and burrow nests. The blue-green pigment biliverdin has also been

suggested to provide anti-microbial defense (Soler et al. 2005), and we therefore

examined these predictions in relation to blue-green chroma as well.

It is possible that eggshell pigments reduce the harmful effects of solar radiation

on eggs laid within open nests (McAldowie 1886), hereafter referred to as the *solar

radiation hypothesis*. Such protection would be beneficial because heat and ultraviolet

radiation have detrimental effects on embryonic development (Webb 1987; Perotti and

Diegeuz 2006). Egg colouration may provide protection from radiation through the

reflectance or absorbance of harmful wavelengths. Blue-green, brown, and white eggs

have relatively high near-IR reflectance levels, suggesting that they can prevent

overheating of the embryo (Bakken et al. 1978). Brown egg pigmentation is also known to reduce UV transmission (Shafey et al. 2002), while biliverdin has an absorbance peak in the UV region which may reduce ultraviolet transmittance in blue-green eggs as well (Falchuk et al. 2002). These findings suggest that egg pigmentation may serve as protection against exposure to near-IR and UV radiation, and this idea has been examined in several taxa with varied results (Montevecchi 1976; Bakken et al. 1978; Westmoreland et al. 2007; Lahti 2008; Magige et al. 2008). A recent study focusing on natural egg colouration found no difference in internal egg temperature between differently coloured eggs (Westmoreland et al. 2007). Another natural experiment revealed that blue-green egg chroma increased in an introduced population of African village weavers (*Ploceus cucullatus*) that has been released from brood parasitism by the diedrik cuckoo (*Chrysococcyx caprius*) for more than 100 years (Lahti 2008). Lahti (2008) suggested that when freed from the pressures of brood parasitism, this trait evolved via natural selection for increased solar protection. Accordingly, Lahti (2008) expanded the solar radiation hypothesis by suggesting that blue-green pigmentation may preferentially block blue-green filtered ambient light (Lahti 2008), which could explain the common occurrence of blue-green eggs in open nests (Kilner 2006).

If egg colour serves to prevent excess light or heat from damaging developing embryos in open nests, eggs should be brighter in open nests within open habitats because brighter eggs have greater overall reflectance, and also have high near-IR reflectance (Bakken et al. 1978). If eggshell absorbance in the UV protects developing embryos by reducing UV transmittance, UV chroma should be lower in open habitats. In addition, if blue-green pigmentation acts as a solar filter, blue-green chroma should be

higher in the eggs of species inhabiting forests, where blue-green light is dominant (Endler 1993).

 We used a large comparative dataset to examine the influence of life history variables on the evolution of eggshell colouration in birds, with a particular emphasis on selection for egg visibility, antimicrobial protection, and protection from solar radiation. We used reflectance spectrometry to obtain quantitative measures of egg colouration from 636 bird species spanning all but one avian order (Pterocliformes), which represents the largest comparative dataset on egg colour evolution to date. Despite the fact that only two classes of pigments are responsible for producing variation in egg colour (Kennedy and Vevers 1976; Miksik et al. 1994; Miksik et al. 1996; Gorchein et al. 2009), we documented an astounding diversity of variation in colouration. Our broad investigation will provide the scale necessary to begin to understand this diversity of colour.

**Methods**

*Egg reflectance*

We quantified eggshell reflectance from preserved museum samples located at four natural history museums: the University of Michigan Museum of Zoology, the American Museum of Natural History, the Field Museum of Chicago, and the National History Museum at Tring. We measured the eggs of 636 species representing all orders except Pteroclidiformes (sandgrouse). We measured six spectra across the entire shell surface such that two measurements were taken from each pole and the equator. We specifically targeted eggshell ground colouration, rather than spotting, wherever possible. To our eyes, speckling that was too fine to be avoided by our measurement configuration

generally created the impression of a nearly uniform colouration. As the visual acuity of humans is greater than that of most vertebrates (Kirk and Kay 2004), we expect that potential visual receivers (avian parents, avian brood parasites, and vertebrate predators) would likewise perceive very fine speckling as nearly uniform colouration.

We used slightly different methods to obtain reflectance spectra from different museums. At the National History Museum at Tring (31% of eggs sampled), we measured eggs using an Ocean Optics USB 2000 spectrometer and a DT mini light source. We measured these eggs at a coincident normal measurement angle (Cassey et al. 2010b). We then summarized spectra across 5nm bins using a script written in SAS v9.2. At the three other museums, we measured egg reflectance spectra using an Ocean Optics USB 4000 spectrometer and a PX-2 pulsed xenon light source (Ocean Optics, Dunedin, FL). We used a 45 degree coincident oblique measurement geometry (Andersson and Prager 2006). We summarized spectra across 5nm bins using CLR (Montgomerie 2008). Percent reflectance was calculated relative to the same white standard (WS-1) at all collections. Previous research on a subset of these eggs found little evidence of fading (Cassey et al. 2010a), which is consistent with other studies (Soler et al. 2005; Jagannath et al. 2008). We visually inspected each spectrum (N = 33,624) and removed erroneous readings before obtaining an average for each egg (N = 5,604). These data were obtained across multiple clutches per species (3.06 ± 0.07 clutches per species, 8.81 ± 0.27 eggs per species). We found no evidence of eggshell fading, and found that museum and measuring technique were unlikely to influence our results (Chapter 4).

We calculated multiple colorimetric variables for each egg: ultraviolet chroma (the sum of reflectance between 300 – 400 nm as a proportion of the sum of reflectance

between 300 – 700 nm), blue-green chroma (the sum of reflectance between 450 – 550

nm as a proportion of the sum of reflectance between 300 - 700nm), brown chroma (the

sum of reflectance between 600 – 700 nm as a proportion of the sum of reflectance

between 300 - 700nm), and brightness (the mean reflectance between 300 – 700 nm).

Chroma values do not always describe interspecific differences in colour adequately

because there are often large interspecific differences in spectral shape, such as the

presence of peaks or plateaus. Because chroma measures reflectance in a specific region

of the spectrum relative to the rest of the spectrum, two spectra that differ markedly in

shape can have similar chroma values depending on the reflectance values within the

region of interest and the reflectance values outside the region of interest. For example, a

blue-green egg could have high blue-green chroma if it has high reflectance in the blue-

green region and moderate reflectance at short and long wavelengths. Similarly, a white

egg could have high blue-green chroma if it has high reflectance in the blue-green region,

low reflectance in the UV, and high reflectance at long wavelengths (see Chapter 4).

 Therefore, we calculated the proportion of blue-green to brown chroma, a measure we

call proportional blue-green chroma, to obtain a measure of chroma that would be

comparable between species. This metric should assess the relative contribution of

pigments, with high values indicating more biliverdin relative to porphyrin, and low

values indicating more porphyrin relative to biliverdin.


*Natural history data*

We collected natural history data for species in our dataset from 564 peer reviewed

articles and species accounts (details in Appendix I). In addition to selecting references

from peer reviewed sources, we also preferentially selected average values from studies

with large sample sizes that should be less prone to outliers. In addition, we avoided

reporting behaviours that were poorly documented in the reference (e.g., no information

about how those data were collected). Specifically, we collected information on nest type

(ground, open-cup, cliff, dome, cavity, burrow, mound, buried or mound), habitat type

(forest, field or savannah, shore, marsh, tundra, or rocky surface), the length of the

incubation period (in days), incubation attendance (the proportion of time the eggs are

covered by either parent), and hatching success (the percentage of eggs laid that hatch).

This measure of hatching success incorporates all eggs that were laid, and may include

eggs that did not hatch because they were infertile (Deeming 1995), experienced failed

embryonic development due to environmental stress (Ohlendorf et al. 1989;

Scheuhammer 1991), or were lost due to predation or accidental breakage by the parents.

Although egg hatchability provides valuable insight into success at the egg stage, it is

likely to be influenced by a number of factors which may not have been related to our

hypotheses and may have introduced noise in our analyses (Koenig 1982). For the anti-

microbial hypothesis, we subdivided habitat type into low- and high-risk groups. Because

previous research has determined cavity nests are at lower risk of microbial invasion than

open nests (Godard et al. 2007), we classified cavity and burrow nests in the low risk

group, and the other nest types as high-risk nests.


*Phylogenetic reconstruction and comparative analyses*

We used Mesquite (version 2.6) to reconstruct a phylogeny based on the species

represented in our dataset. For this purpose, we combined data from numerous molecular

phylogenies and in a few cases phylogenies that combined molecular and morphological information (Appendix II). We based basal relationships on recent hypotheses for relationships among birds (Ericson et al. 2006; Hackett et al. 2008). Because the source trees used different markers and techniques, branch lengths could not be preserved. We therefore used randomly ultrametricized branch lengths, which includes setting the distance between the root and all tips equal (Lapointe and Legendre 1991). This large composite phylogeny could then be truncated for individual tests to contain only species for which we were able to obtain the necessary life history data.

We ran our comparative analyses in R version 2.7.1 using the packages 'nlme' (Pinheiro et al. 2010) and 'ape' (Paradis et al. 2004). For regression analyses, we used the phylogenetic least squares (PGLS) approach (Pagel 1997). To improve fit to normality, we used Box-Cox transformations on variables that deviated significantly from normality (Appendix 3). We also evaluated model fit by assessing the distribution of normalised residuals and examining quantile-quantile plots (Freckleton 2009). For each analysis we used the maximum likelihood value of Pagel's $\lambda$ (Pagel 1997, 1999) obtained with a maximum likelihood procedure (Freckleton et al. 2002). For PGLS analyses that compared differences between levels of a categorical variable, we applied Tukey's HSD test. For these calculations we used the predicted group means and the mean square error from the PGLS analysis, rather than from a separate non-phylogenetic ANOVA model.

**Results**

*Egg visibility hypothesis*

There were significant differences in egg brightness between nest types when controlling for the influence of phylogeny ($F_{6,603} = 4.5$, $P < 0.001$; Figure 1). Post-hoc comparisons show that these differences are the result of eggs in cavity and burrow nests being significantly brighter than eggs in ground or open-cup nests, supporting the hypothesis that egg brightness may increase visibility in dark nests. Moreover, egg hatching success was significantly positively predicted by eggshell brightness within cavity and burrow nest types but not in other nesting strategies (Table 1, Figure 2). Ultraviolet chroma also differed between nest types ($F_{6,603} = 2.24$, $P = 0.04$); however, the ultraviolet chroma of cavity and burrow nesters was not significantly higher than other nest types (Figure 3). Likewise, there was no relationship between egg hatchability and ultraviolet chroma in all birds ($r = 0.04$, $CI_{0.95} = -0.12$ to $0.20$, $N = 152$, $P = 0.63$), open and dome nesting birds ($r = -0.04$, $CI_{0.95} = -0.21$ to $0.15$, $N = 116$, $P = 0.69$), or cavity and burrow nesting species ($r = 0.20$, $CI_{0.95} = -0.13$ to $0.48$, $N = 36$, $P = 0.24$).

*Anti-microbial hypothesis*

If pigments serve as a microbial defense system, there should be differences in brown chroma, blue-green chroma, or proportional blue-green chroma between nests at lower risk of microbial infection (closed nests) and nests at higher risk of microbial infection (open nests). We found no support for this prediction for any colorimetric variable (brown chroma: $F_{1,608} = 3.60$, $P = 0.06$; blue-green chroma: $F_{1,608} = 0.001$, $P = 0.98$; proportional blue-green chroma: $F_{1,608} = 0.54$, $P = 0.46$). We also examined the

relationship between these colorimetric variables and hatching success. Because cavity and burrow-nesting birds have brighter eggs (see *egg visibility hypothesis* above), we controlled for nest type in these analyses. Hatchability was positively related to blue-green chroma in all risk conditions, negatively related to brown chroma in all but the high risk group, and positively related to proportional blue-green chroma in all but the high risk group (Table 2).

*Solar radiation hypothesis*

If high eggshell reflectance provides protection from solar radiation, species using open nests in open habitats should have brighter eggs. By contrast, if eggshell pigments provide protection from solar radiation, species using open nests in open habitats should have darker eggs with lower UV chroma. We found that eggs laid in the tundra were darker than eggs laid in forests, fields, or shore habitats (brightness: $F_{5,389} = 3.81$, P = 0.002, Figure 4A). UV chroma also differed between habitat types (ultraviolet chroma: $F_{5, 389} = 2.41$, P = 0.04; Figure 4B). A post-hoc comparison test was unable to elucidate the significant relationships. If blue-green egg colouration provides protection from solar radiation in forests, species using open nests in forests should have higher blue-green or proportional blue-green chroma. However, eggs in open nesting species within forests did not have significantly different in blue-green ($F_{5,389} = 1.18$, P = 0.32) or proportional blue-green chroma ($F_{5,389} = 1.41$, P = 0.22) than other habitats.

**Discussion**

Our study examined broad evolutionary pressures on avian egg colouration, particularly as they apply to nest type and nesting habitat. We found strong evidence for selection for brighter eggs in nest cavities; species nesting within cavities had brighter eggs and egg brightness was positively related to egg hatching success. We also investigated the potential anti-microbial function of eggshell pigments, which had not yet been tested using a comparative framework. Our findings did not support this hypothesis for brown pigments, but do suggest that blue-green egg colouration may be related to risk of microbial invasion. We also examined how egg colour varied across habitat types to test the solar radiation hypothesis. Our results provide only mixed support for the solar radiation hypothesis. In particular, species nesting in the tundra, where eggs are exposed, produce darker eggs than species nesting in other environments. Our findings suggest that several of these selective pressures may influence the evolution of egg colouration in birds.

One longstanding observation regarding patterns of avian egg colour has been that birds nesting in cavities generally have white or unsaturated eggs (von Haartman 1957). Brighter eggs may enhance egg visibility in dark nests, which should select for increased egg brightness (von Haartman 1957). Previous comparative studies have shown that cavity nesting species have brighter eggs (Avilés et al. 2006; Kilner 2006) and an experimental study has shown that within cavities, artificially darkened eggs are at greater risk of breaking than white eggs (Holyoak 1969). In this study, we found that eggs are brighter in closed nests and that hatching success is positively associated with egg brightness within closed nests. Although hatching success may be influenced by factors that may be unrelated to egg visibility, there is unlikely to be greater predation on

dark eggs within cavities, and it is also unlikely that a connection exists between egg brightness and infertility. Our findings contribute to a growing body of literature highlighting the importance of bright signals in dark nest environments, including flange visibility in nestling house sparrows (*Passer domesticus*) and cliff swallows (*Petrochelidon pyrrhonota*), and the visibility of egg teeth in northern flickers (*Colaptes auratus*) (Dugas 2010; Wiebe 2010).

Contrary to our findings with brightness, we did not support the hypothesis that ultraviolet chroma increases egg visibility in dark nests. Our findings contrast with other studies showing that UV colouration enhances the visibility of eggs (Avilés et al. 2006; Avilés 2008) and nestling gapes in closed nests (Hunt et al. 2003). Ultraviolet wavelengths may temporarily enhance egg visibility as a result of a shift in retinal sensitivity toward shorter wavelengths when a bird first moves into a dark environment (Hart 2001), as a product of the bird's eye transitioning from photopic to scotopic vision. Although there is a gap in our knowledge regarding avian vision under mesopic illumination, where both rods and cones contribute to perceived chromaticity (Wyszceki and Stiles 1982; Hart 2001), ultraviolet wavelengths are unlikely to confer any sustained benefit to visibility when eggs are viewed in a scotopic nest environment where rods are the predominant photoreceptor used (Cassey 2009; Lind and Kelber 2009). It is therefore not surprising that we found different patterns between egg brightness and UV chroma.

Egg pigments may also serve as a line of defence against microbial invasion into the shell (Soler et al. 2005). We considered cavity and burrow nests as low risk nests since these nests generally have sufficiently high temperatures to activate lysozymes in the albumen, which serve as an anti-microbial defence (Beissinger et al. 2005; Godard et

al. 2007), and we considered other types of nests to be at higher risk of microbial invasion through increased exposure to the elements (Godard et al. 2007). We did not find differences in brown, blue-green, or proportional blue-green chroma between high and low risk nests. In addition, brown chroma was negatively related to hatching success in all birds and birds in low risk nests, and unrelated to hatching success in species that should experience a high risk of microbial invasion. Although our findings did not support an antimicrobial function of porphyrin pigmentation, our findings for biliverdin colouration are intriguing. Proportional blue-green chroma was related to hatching success across all birds and birds at low risk of microbial invasion, and blue-green chroma was related to hatching success in all risk categories. Our findings provide some support for the hypothesis that biliverdin may possess anti-microbial properties (Soler et al. 2005). If biliverdin does provide protection against microbes, the mechanism would necessarily differ from the photo-dependent mechanism proposed for porphyrin (Ishikawa et al. 2010), since biliverdin does not share these photo-dependent properties (Needham 1974). Our findings are still puzzling, however, because the relationship between blue-green colouration and hatching success was present in all risk categories. We encourage future experimentation on the influence of eggshell pigments in reducing microbial infection, and the mechanisms responsible for producing this protection from microbes. One alternative explanation for our findings is that parents may spend more time incubating eggs with proportionally greater blue-green chroma, as predicted by some hypotheses (Moreno and Osorno 2003; Hanley et al. 2010), which would reduce microbial infection (Cook et al. 2003) and enhance egg viability (Arnold et al. 1987).

If egg pigments serve as a form of solar protection, eggs should be darker and have lower UV chroma in open habitats. We found few overall differences in colour between eggs in different habitats, except that eggs laid in the tundra were darker than eggs laid in other habitats. In our dataset, tundra was the habitat type where eggs in open nests would be exposed to the greatest direct solar radiation. Our comparative data therefore provide some support for the solar radiation hypothesis by suggesting that pigment deposition increases within a habitat where eggs may be exposed to high light levels. However, previous research has shown that ground-nesting birds attempt to match egg pigmentation to their nesting substrate to lower predation risks (Solís and de Lope 1995; Šálek and Cepáková 2006; Mayer et al. 2009), and this may be especially important in the tundra where ground nesting is the predominant nesting strategy (Preston and Norris 1947). These different selective pressures could lead to reinforcement of mutually beneficial strategies or trade-offs between protection from solar radiation and protection from predators. Future studies may benefit from investigation the interaction between these selection pressures.

An extension of the solar radiation hypothesis proposed that blue-green colouration may provide protection from solar radiation in forested habitats with blue-green light (Lahti 2008). In our study, neither blue-green chroma nor proportional of blue-green chroma was higher in open nests within forests. Generally, our findings provide only modest support for the solar radiation hypothesis. It is important to note, however, that our analyses were based on broad classifications of habitat and exposure to solar radiation. Our data may not have been able to detect this type of pattern because ambient light irradiance varies greatly within habitat types on both large and small scales

(Endler 1993; Leal and Fleishman 2002; Altshuler 2003; Cervantes et al. 2005). Future research should determine the transmittance properties of the eggshells, which would provide a better idea of the characteristics of light and infra-red radiance that embryos are exposed to, while also determining the specific ambient light conditions the eggs are exposed to within their nests. This would provide a holistic approach to determining if eggshell pigments are adapted to protect the embryo from the harmful effects of solar radiation.

Our study has revealed several intriguing patterns of variation in egg colour in relation to nest type and nest environment. The recent resurgence of interest in avian egg colouration has been fuelled by the remarkable diversity of colour variation found across all birds; however, much of the recent literature has focused on intra-specific patterns of egg colour (Reynolds et al. 2009; Cherry and Gosler 2010). These studies have revealed many novel patterns, such as the relationship between male investment and eggshell colour (Moreno et al. 2006; Hanley et al. 2008; Soler et al. 2008), female quality and eggshell colouration (Morales et al. 2006; Martínez-de la Puente et al. 2007; Morales et al. 2008), as well as associations between egg colour and soil composition (Gosler et al. 2005), climate (Avilés et al. 2007), and environmental contamination (Jagannath et al. 2008). Our broader comparative approach has shown that the presence of bright eggs in cavities may be the result of selection for lighter eggs within these dim lit habitats. In addition, we present comparative evidence that blue-green pigmentation may be linked to hatching success across nest types, which provides partial support for the anti-microbial hypothesis. Our findings also provide limited support for the hypothesis that solar exposure favours greater pigment deposition. Interestingly, habitat alone was not a good

predictor of egg colour, which suggests that common selection pressures shared by species within multiple habitat types may be driving the evolution of egg colouration, and that associations between egg colour and habitat characteristics may need to focus on small scale microhabitat characterizations.

**Acknowledgments**

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**Table 5.1**

Results from univariate PGLS analyses assessing the relationship between egg brightness and hatching success across all birds in our dataset, in birds with open nests, and in birds with closed nests. The lower and upper limits of the 95% confidence interval of the effect size estimate are indicated by LCL and UCL, along with sample size and test significance. Hatching success was Box-Cox transformed to fit a normal distribution.

|              | r    | LCL   | UCL  | N   | P    |
|--------------|------|-------|------|-----|------|
| all birds    | 0.15 | -0.01 | 0.30 | 152 | 0.06 |
| open nests   | 0.10 | -0.08 | 0.27 | 116 | 0.29 |
| closed nests | 0.42 | 0.10  | 0.63 | 36  | 0.01 |

**Table 5.2**

Results from univariate PGLS analyses assessing the relationship between hatching success and three colorimetric variables, while controlling for the influence of nest type (to account for variation in colour attributable to nest type). We assessed these relationships in all birds, birds using nests with a low risk of microbial invasion and birds using nests with a high risk of microbial invasion. The lower and upper limits of the 95% confidence interval around the effect size measure are indicated by LCL and UCL, along with sample size and test significance. Hatching success and chroma values were Box-Cox transformed fit a normal distribution.

| Colour | risk of microbial infection | r | LCL | UCL | N | P |
|---|---|---|---|---|---|---|
| Blue-green chroma | all birds | 0.22 | 0.06 | 0.36 | 152 | 0.01 |
| | low risk | 0.35 | 0.02 | 0.58 | 36 | 0.04 |
| | high risk | 0.20 | 0.01 | 0.36 | 116 | 0.04 |
| Brown chroma | all birds | -0.21 | -0.35 | -0.05 | 152 | 0.01 |
| | low risk | -0.57 | -0.73 | -0.30 | 36 | 0.0003 |
| | high risk | -0.12 | -0.29 | 0.07 | 116 | 0.21 |
| Proportional blue-green chroma | all birds | 0.22 | 0.06 | 0.36 | 152 | 0.01 |
| | low risk | 0.45 | 0.14 | 0.66 | 36 | 0.01 |
| | high risk | 0.18 | -0.01 | 0.35 | 116 | 0.06 |
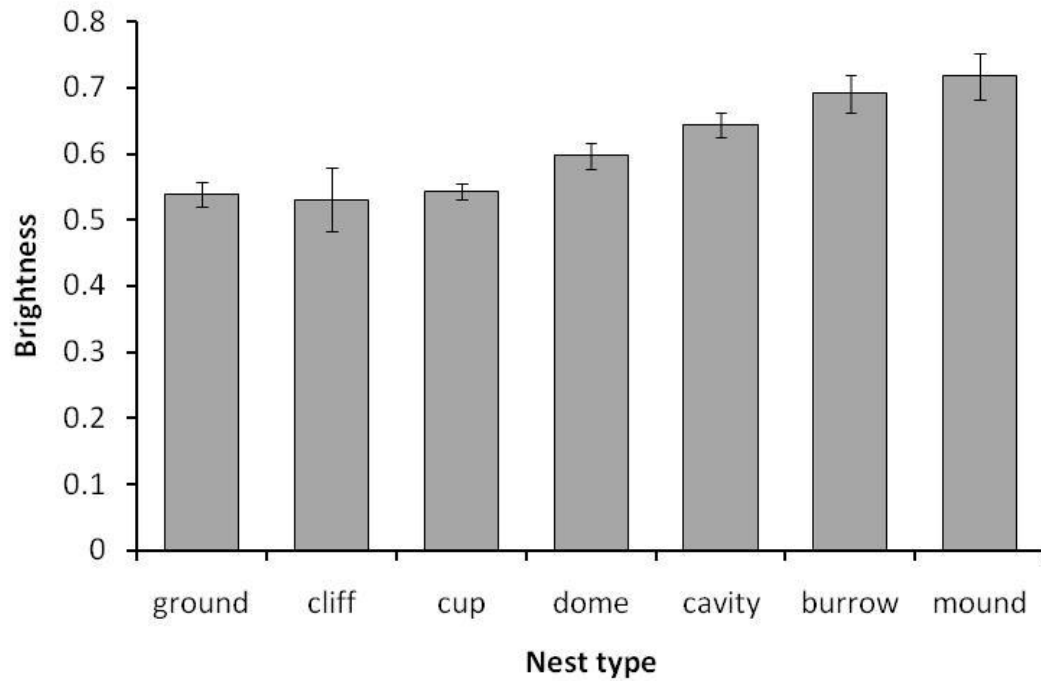
**Figure 5. 1**

Variation in egg brightness across nest types. Data are from a PGLS analysis controlling

for phylogenetic relatedness. The fitted values from a PGLS analysis with only nest type

as a predictor variable represent the phylogenetically corrected means for these groups

We provide the standard error for the raw values, as standard error is not obtainable for

the phylogenetically corrected group means.
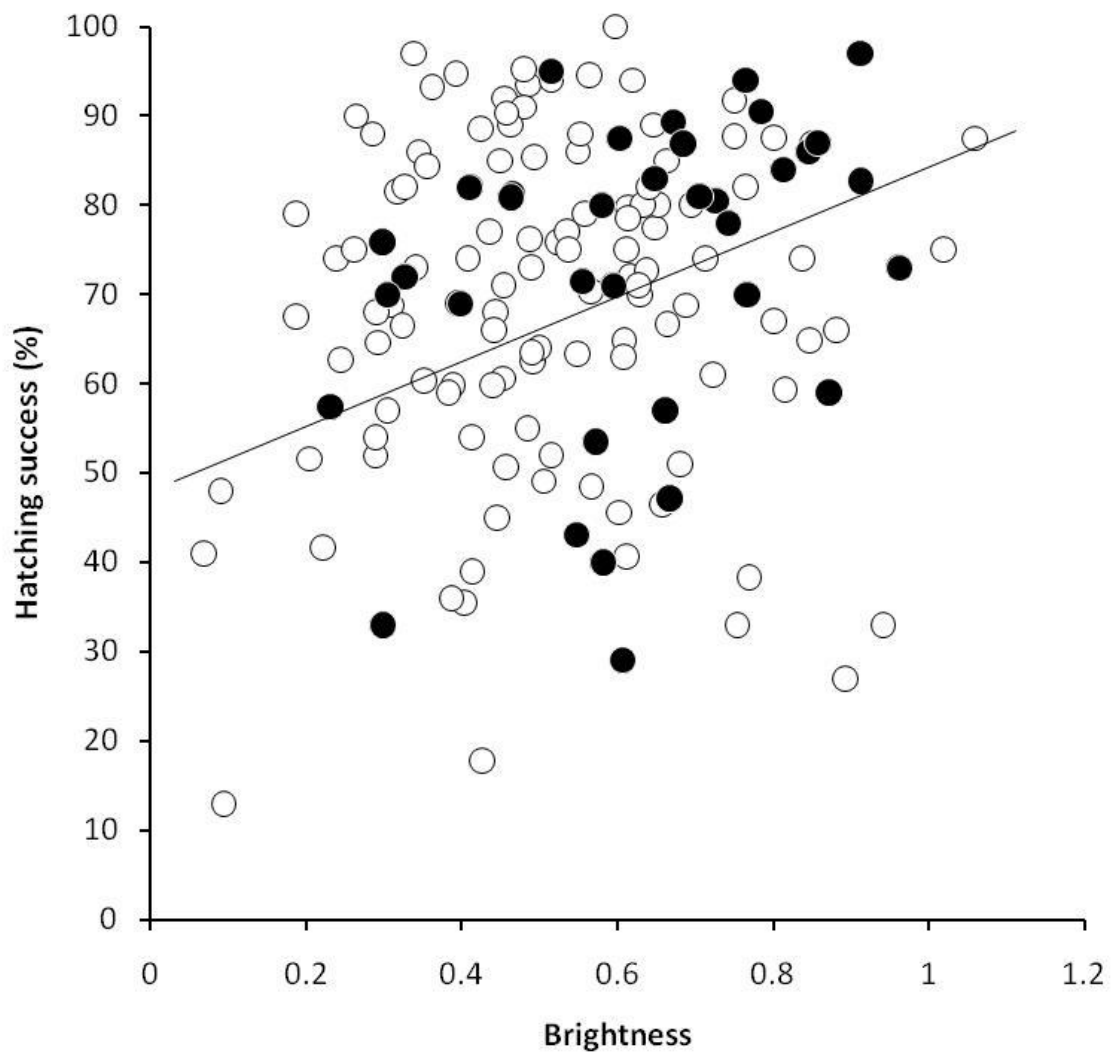
**Figure 5. 2**

Relationship between hatching success and eggshell brightness. Solid dots represent

closed nests, while open circles represent open nests. Points show raw data, whereas the

solid line represents the relationship established with a PGLS analysis between these

variables for closed nesting species.
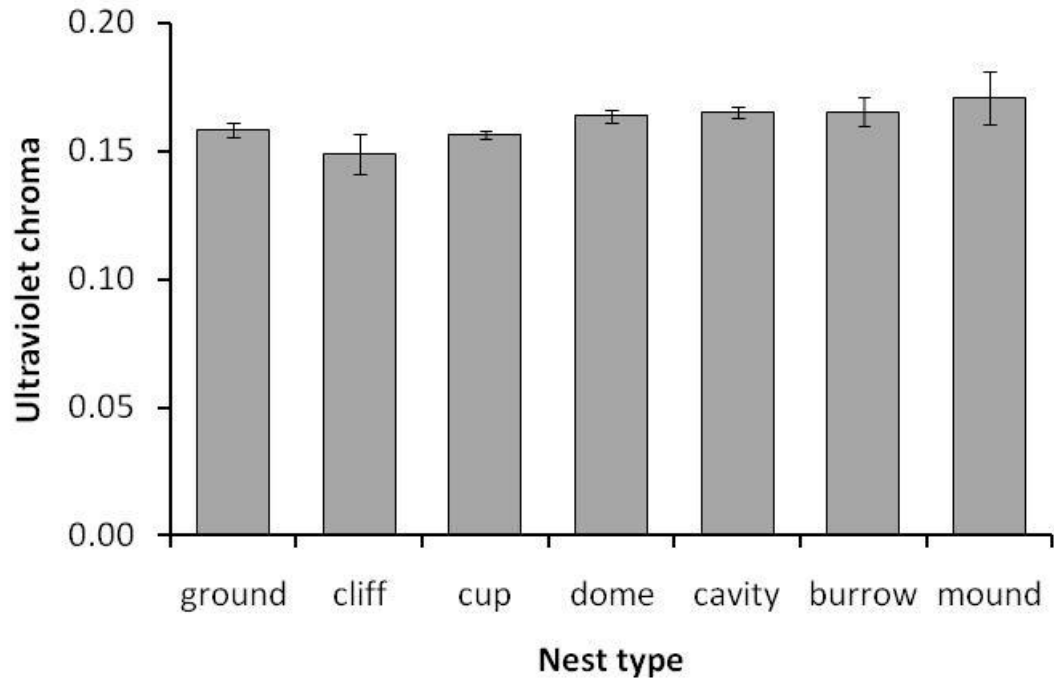
**Figure 5. 3**

Variation in egg ultraviolet chroma across nest types. Data are from a PGLS analysis controlling for phylogenetic relatedness. The fitted values from a PGLS analysis with only nest type as a predictor variable represent the phylogenetically corrected means for these groups. We provide the standard error for the raw values, as standard error is not obtainable for the phylogenetically corrected group means.
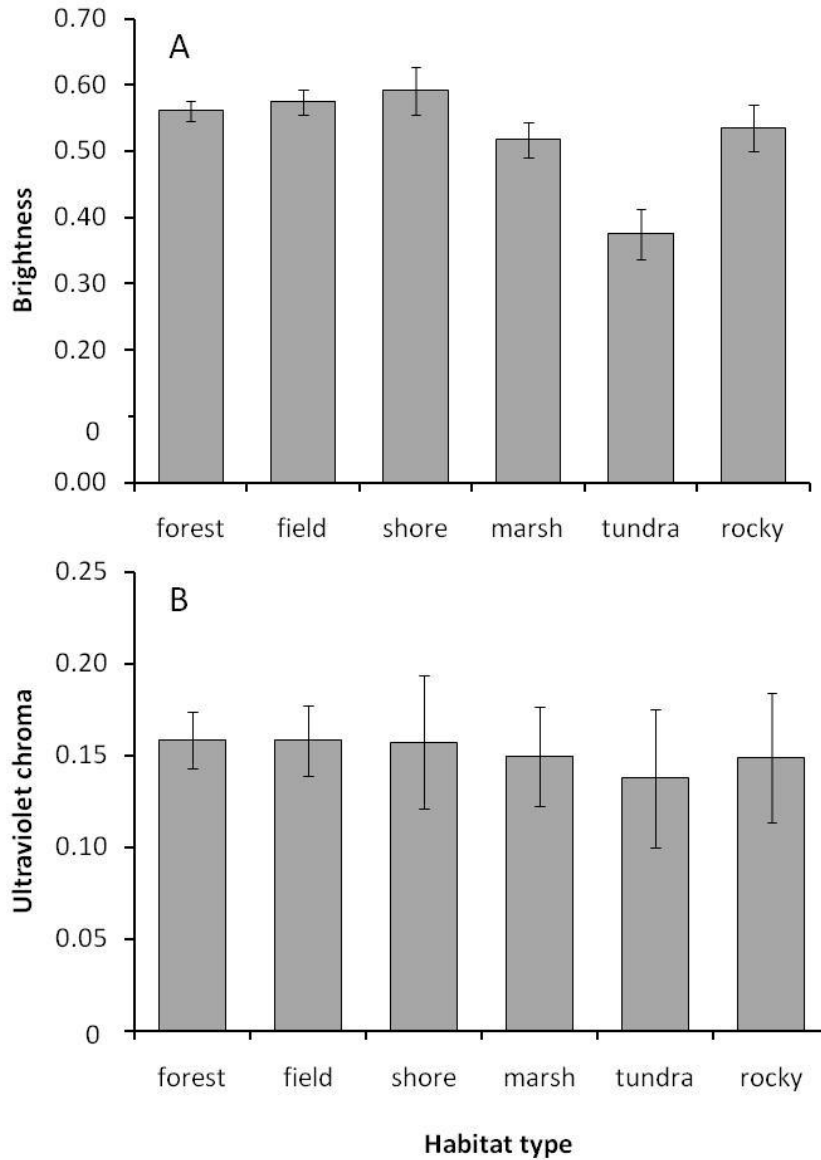
**Figure 5. 4**

Variation in egg brightness A) and ultraviolet chroma B) across habitat types. Data are

from a PGLS analysis controlling for phylogenetic relatedness. The fitted values from a

PGLS analysis with only habitat type as a predictor variable represent the

phylogenetically corrected means for these groups. We provide the standard error for the

raw values, as standard error is not obtainable for the phylogenetically corrected group

means.

# CHAPTER 6 - AVIAN EGGS PHOSPHORESCE[5]

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[5] This manuscript is the product of joint research

**Summary**

There has been a recent resurgence of interest in the evolution of egg colouration, and thus numerous studies have quantified eggshell colouration using reflectance spectrometry. In spite of this work, the fluorescent properties of avian eggshells have been poorly studied and, to our knowledge, eggshell phosphorescence remains undescribed. Here, we document that phosphorescence is widespread in avian eggs, occurring in 95% of the species we assessed. Based on known egg pigment composition, our data suggest that eggshell phosphorescence is associated with porphyrin pigmentation. By measuring eggs with irradiance including and excluding ultraviolet light, we found that phosphorescence is unlikely to influence egg reflectance measures. Future studies should assess the mechanisms and possible adaptive significance of avian egg phosphorescence.

Keywords: egg colouration, porphyrin, biliverdin, phosphorescence, fluorescence, pigmentation

**Introduction**

Naturalists have been curious about variation in avian eggshell colouration for more than a century (Wallace 1889; Kilner 2006; Cherry and Gosler 2010), and several hypotheses have been proposed to explain variation in egg colour (Kilner 2006; Cherry and Gosler 2010). Avian egg colouration is produced mainly by two pigments. Porphyrin produces brown hues and biliverdin produces blue-green hues, although colour isn't always a clear indication of pigment composition (Kennedy and Vevers 1976). Early research on eggshell colouration revealed that some avian eggs can also fluoresce (Derrien 1924; Schönwetter 1932). Fluorescence occurs when an object absorbs some wavelengths of light and re-emits this energy in the form of light at longer wavelengths (Nassau 1997). The emitted photons give fluorescent objects a glowing appearance when viewed under ultraviolet (UV) or near-UV radiation (e.g., blacklights). Fluorescence is common in abiotic structures such as emeralds and rubies (Nassau 1997), and is also found in biotic structures such as in arthropod hardparts (Lawrence 1954) and some bird feathers (McGraw and Nogare 2005). When exposed to ultraviolet light, avian eggs fluoresce in a species-specific manner, differing dramatically from their colour in normal lighting (Schönwetter 1932). This fluorescence is believed to be caused by porphyrin, due to its fluorescent properties *in vivo*, and has been used to infer porphyrin composition in eggs (e.g., With 1973). Aside from this diagnostic use, the fluorescent properties of avian eggshells have not been examined in detail.

While conducting a comparative study of avian egg colour, we noticed that many eggs also possess phosphorescent properties, a phenomenon which, to our knowledge, has not yet been described (see Video, Appendix 4). Phosphorescence is similar to

fluorescence in both mechanism and appearance. The principal difference is that phosphorescence occurs over a longer period of time after the excitation radiation has ceased, and thus the egg continues to glow for a few moments. In phosphorescence, an electron in an excited single state moves to a higher energy triplet state and must then undergo an unfavourable transition back to ground state, which takes longer than in fluorescence (Needham 1974). This transition from triplet to singlet state results in re-emission of light over longer periods of time after excitation has stopped (Goodwin 1953). In contrast, fluorescence emission occurs when an excited photon transitions to ground state directly from the excited singlet state.

Porphyrin pigments are macrocyclic tetrapyrroles that contain substitutions around the ring perimeter. This molecule is ideally planar, has a conjugated double bond system, and has complete delocalisation of electrons. These properties of porphyrin increase its resonance energy and extend its $\pi$ orbital which decreases in the energy required for electron transitions (Needham 1974). Biliverdin is an open-chain tetrapyrrole found in a cis configuration and is therefore not as planar as porphyrin and has a lower resonance. These differences in molecular structure explain why porphyrin fluoresces while biliverdin does not (Needham 1974).

Current interest in avian egg colour necessitates an examination of phosphorescent properties in eggshells. Here, we provide the first description of phosphorescence in avian eggshells and document its occurrence in relation to known eggshell pigments. To determine the possible influence of phosphorescence on egg reflectance measurements, we also assess the reflectance spectra of eggs using different light sources (including and excluding excitation wavelengths in the ultraviolet region). If

egg phosphorescence influences reflectance measurements, we expect phosphorescing eggs to exhibit higher reflectance at visible wavelengths. We discuss the impact of emission spectra on egg colour research as well as the possible adaptive significance of phosphorescence in avian eggs.

**Materials and Methods**

While measuring egg reflectance in a darkened room, we noticed that some eggs phosphoresced after being illuminated by our reflectance light source (PX2, Ocean Optics, USA). We compiled a list of species that we found to either possess or lack eggshell phosphorescence whenever visually detectable (N = 82 species; for a complete list see, Appendix 5, Table S1). We compared this information to known egg pigment composition (Kennedy and Vevers 1976) using a Fisher's Exact Test. To determine the whether phosphorescence influenced egg reflectance, we measured the eggshell reflectance of 97 pied-billed grebe (*Podilymbus podiceps*) eggs using different light sources with and without UV illumination, as this species appeared to phosphoresce longer than any other species we measured. Briefly, we measured grebe egg colouration using a USB-4000-UV-VIS reflectance spectrometer and four different light sources (Ocean Optics, USA): combined deuterium and halogen light source (HD), halogen alone (H), deuterium alone (D), and a pulsed xenon light source (PX2). The halogen light source, which emits very little light in the UV, was the only light source did not induce any visually detectable phosphorescence (for further details see supplementary material 2). All reflectance measurements were collected relative to a Spectralon white standard that reflects 99% of incoming light (WS-1, Ocean Optics, USA).

Because phosphorescence is detectable within the visual range and some of our
measurements excluded the UV range, we focused on the wavelength range between 400
– 700 nm to make all treatments comparable. We quantified blue-green chroma as the
proportion of reflectance between 450 – 550 nm relative to the total reflectance (between
400 – 700 nm), and brown chroma as the proportion of reflectance between 600 – 700 nm
relative to the total reflectance. We calculated brightness as the mean reflectance between
400 – 700 nm. We used generalized linear mixed effect models to determine whether
colour was predicted by light treatment using egg identity as a random factor, and used a
Tukey HSD test to examine differences across light sources.

**Results**

A diversity of species ranging from ratites to passerines exhibited egg
phosphorescence. In fact, 78 of 82 (95%) species assessed exhibited visually detectable
phosphorescence (Appendix 5). Although knowledge of pigment composition in avian
eggs is still limited, the eggs in our dataset that were known to possess porphyrin always
phosphoresced, whereas eggs known to lack porphyrin did not phosphoresce (Fisher's
Exact Test: $p = 0.03$).

Pied-billed grebe eggs measured with different light sources exhibited subtle
differences in spectral shape (Figure 1). These differences in spectral shape translated
into colorimetric differences (Table 1). Light source had a significant influence on egg
brightness, blue-green chroma, and brown chroma (Table 1, Figure 2). In particular,
brightness was significantly different between all light treatments except the HD and D.
In addition, chroma values measured with either the HD or D light sources differed from

those measured with either the H or PX2 light sources. Since the HD and D light sources

cover the same illumination range, the fact that they did not produce different reflectance

spectra suggests that differences between the spectra resulting from other light sources

were not a result of human error or an artefact of the experimental design. Importantly,

the light sources capable of inducing egg phosphorescence (D, HD, PX-2) did not

produce brighter reflectance spectra than the light source that did not induce

phosphorescence (H).

**Discussion**

In this study, we document that egg phosphorescence is prevalent in a broad

diversity of avian taxa. We also provide evidence that phosphorescence is associated with

known presence of porphyrin pigmentation whereas lack of phosphorescence is

associated with known absence of porphyrin. Indeed, there were only a few species

where phosphorescence was not observed and these eggs were always blue-green. The

fact that such a large proportion of eggs phosphoresce can likely be explained by the fact

that porphyrin pigments are very common, while eggs pigmented solely with biliverdin

are relatively rare (Kennedy and Vevers 1976). Interestingly, there appeared to be a

disconnect between apparent brown chroma and the degree to which eggs phosphoresced.

This may suggest the presence of different forms of porphyrin with different

phosphorescent properties. Indeed, we did not detect phosphorescence on large dark egg

spots whereas we did notice phosphorescence on lighter speckling, suggesting that there

may be differences in porphyrin composition between two common forms of eggshell

markings. Future research should characterize the mechanism of phosphorescence, its likely association with porphyrin pigmentation, and its possible use as a diagnostic tool.

We also found that pied-billed grebe eggs differed in brightness and chroma when measured with different light sources. The differences in spectral shape were minor in a species that exhibits striking phosphorescence, and our observations suggest that phosphorescence was not responsible for the differences in egg reflectance produced by different light sources. First, contrary to our expectation, the light sources that produced phosphorescence also produced darker egg reflectance measurements in the visible spectrum. Second, although the light sources produced visible phosphorescence, individual variation in egg colour was a more important source of variation for both chroma models. We therefore believe that phosphoresce is unlikely to have a negative impact on studies of egg colouration, as has been concluded in a similar study of feather fluorescence (but see Arnold et al. 2002; Pearn et al. 2003). Nevertheless, it would be cautious to use light sources that include UV irradiance and approximate natural lighting in studies of egg colouration. Researchers interested in comparisons between studies or meta-analyses should also be aware that different light sources may produce different colorimetric values, independent of UV irradiance.

Phosphorescent properties of eggs may also be adaptive by providing a functional mechanism for the hypothesis that egg pigments reduce the risk of microbial invasion (Soler et al. 2005). Recent research has shown that eggshell porphyrin pigmentation inhibits the growth of gram positive bacteria when photo-stimulated (Ishikawa et al. 2010). Interestingly, these defensive capabilities are directly related to the photons' transition from triplet to ground state (Papkovsky et al. 1995), which produces the light

we detect as phosphorescence. In addition to crypsis (Solís and de Lope 1995) and enhanced shell strength (Gosler et al. 2005), porphyrin pigmentation may contribute to the shell's natural defence system when deposited in eggs exposed to solar radiation. Future studies should determine the levels of photo-excitation necessary to elicit oxidative or reductive quenching within the shell matrix.

**Acknowledgements**

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**Table 6. 1 -** Whole model and effect tests from generalized linear mixed models constructed to predict variation in colorimetric variables of pied-billed grebe eggs. Model predictors included egg identity as a random factor and light source as a fixed factor (HD, H, D, or PX2; see Materials and Methods).

| | whole model | | | light source | | egg identity | |
|---|---|---|---|---|---|---|---|
| | $F_{99, 156}$ | $r^2$ | $p$ | $F_{3,255}$ | $p$ | $F_{96,255}$ | $p$ |
| brightness | 16.28 | 0.91 | $< 0.0001$ | 107.13 | $< 0.0001$ | 12.63 | $< 0.0001$ |
| blue-green chroma | 42.67 | 0.96 | $< 0.0001$ | 41.19 | $< 0.0001$ | 42.73 | $< 0.0001$ |
| brown chroma | 43.78 | 0.97 | $< 0.0001$ | 14.01 | $< 0.0001$ | 44.72 | $< 0.0001$ |
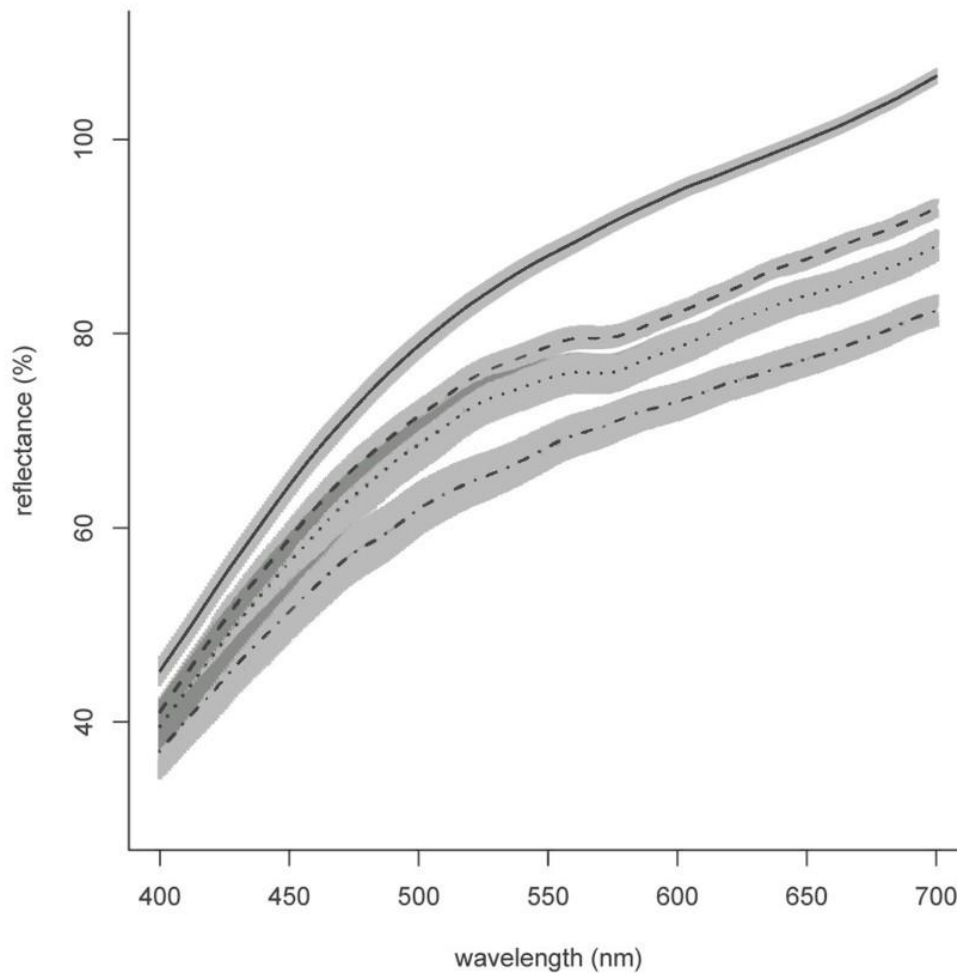
**Figure 6. 1**

Average reflectance spectra of 97 Pied-billed Grebe eggs as measured with four light

sources: halogen (H) = solid line, halogen+deuterium (HD) = dashed line, deuterium (D)

= dotted line, and pulsed xenon (PX2) = dashed and dotted line. The halogen light source

lacks the UV irradiance necessary for producing phosphorescence. The shaded areas

around each curve represent the standard error. These spectra have been smoothed with a

locally-weighted polynomial regression using the *lowess* function implemented in R

(Cleveland 1981).
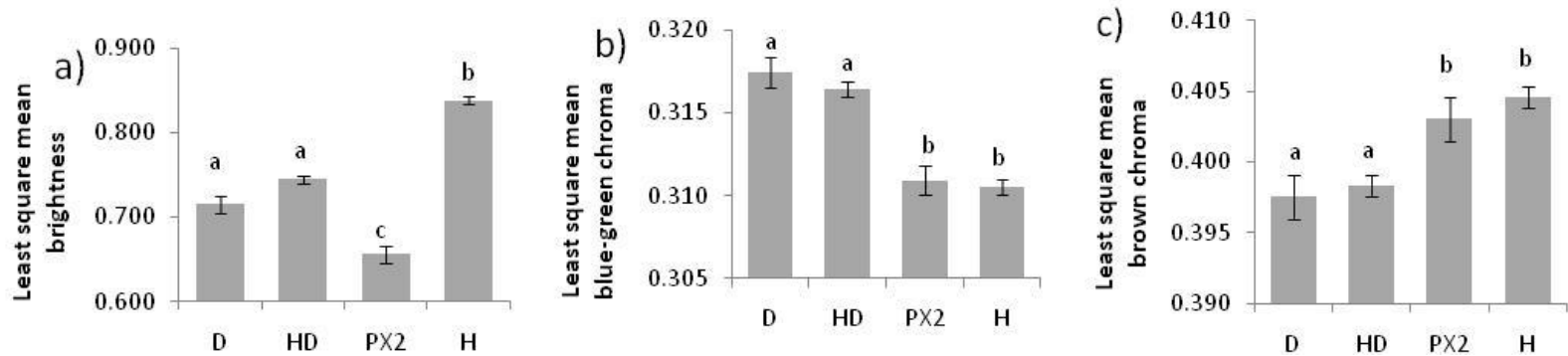
**Figure 6. 2**

Variation in a) brightness, b) blue-green chroma, and c) brown chroma of Pied-billed Grebe eggs measured using four different light sources: halogen (H), halogen+deuterium (HD), deuterium (D), and pulsed xenon (PX2). The halogen light source lacks the UV irradiance necessary for producing phosphorescence. Data are least square means (± SE) from linear mixed models controlling for egg identity, and significant differences ($\alpha = 0.05$) between light sources are indicated by letters above bars.

# Chapter 7 – General Discussion

**Dissertation summary and implications**

Variation in avian eggshell pigmentation has been of biological interest for more than 100 years, and provides a unique avenue to investigate selection pressures on a single trait. Unlike in other life stages, the egg is particularly susceptible to risks of predation and over-exposure, and is either directly, or indirectly in the case of the Megapodes (Elliott 1994; Jones et al. 1995), dependent upon its parents. There has been an abundance of research on egg colouration (reviewed in, Underwood and Sealy 2002; Kilner 2006; Cherry and Gosler 2010); however, there are still many unanswered questions. My dissertation addresses some of these unanswered questions by taking a broad approach, ranging from examining the luminescent properties of pigments, to investigating whether egg colour may be used as a signal at the species level, to assessing the long-term environmental impacts associated with egg colouration, and investigating the selection pressures associated with avian egg colour.

One recently proposed hypothesis (Moreno and Osorno 2003), known as the sexual signalling hypothesis, suggests that the blue-green pigment biliverdin serves as a signal of female quality. This can occur if biliverdin exhibits antioxidant properties within the laying female, and higher quality females can afford to deposit more biliverdin in their eggs. Males could evaluate egg colour as an indicator of female quality, and preferentially invest in clutches that are of presumed higher quality. There has been some support of this idea in terms of paternal provisioning increasing with greater blue-green chroma (Moreno et al. 2004; Moreno et al. 2006; Hanley et al. 2008; Soler et al. 2008), and also links between female quality and blue-green egg chroma (Siefferman et al. 2006; Hanley et al. 2008; Morales et al. 2008). However, there are some fundamental problems

with this hypothesis (Reynolds et al. 2009), and tests of the hypothesis. For instance, in

species used to examine this hypothesis, males rarely have the opportunity to evaluate

other females' eggs to determine the relative quality of their mate. In chapter 2, we

examined the predictions of this idea in a colonially nesting bird, the ring-billed gull

(*Larus delawarensis*). This species has ample opportunity to evaluate the colouration of

their clutch relative to thousands of other nests (Ryder 1993). We found no support for

this hypothesis despite testing multiple predictions. More specifically, biliverdin did not

appear to be limited across the laying period, two measures of female condition were

unrelated to egg colour, blue-green chroma was unrelated to either egg or chick mass, and

no measure of total or proportional male effort was correlated with blue-green egg colour.

Our findings suggest that the sexual signalling hypothesis does not explain variation in

blue-green colour in the ring-billed gull (Hanley and Doucet 2009). We encourage a

meta-analysis to examine the findings of this hypothesis, to determine the overall level of

support and potentially uncover patterns about why there is support in some avian groups

and not in others.

As other studies failed to support many of the predictions of the sexual signalling

hypothesis (Krist and Grim 2007; Lopez-Rull et al. 2007), some researchers began

investigating environmental influences on egg colour and found that many factors can

impact the coulour of birds' eggs, including soil calcium levels, temperature, and

environmental contaminants (Gosler et al. 2005; Avilés et al. 2007; Jagannath et al.

2008). In Chapter 3, I examined the relationship between egg colouration and

environmental contamination in a related gull species, the herring gull (*Larus

argentatus*). Specifically, I utilized data from the Herring Gull Monitoring Program,

which has monitored the levels of environmental contaminants in the Great Lakes since the early 1970's. I found that some persistent organic compounds influenced egg colour and found that a human observer may be able to detect the influence of some persistent organic compounds in the field; however, the use of spectroscopy would undoubtedly be a more reliable method. These findings contribute to our understanding of environmental influences on eggshell colour production, and provide a foundation for future investigations concerning the utility of using egg colour as a non-destructive bio-indicator. Follow up experimentation should examine the functional link between eggshell colouration and environmental contaminants. To evaluate the role of egg colour as a bio-indicator, researchers should focus more heavily within one population rather than spreading effort across multiple sites. In addition, field investigations should collect spectrometric data as well as human based assessments of colour in relation to a colour contact sheet and colour assessments from photographs. This will allow for a better assessment of the relative costs and benefits (time, funds, and accuracy) associated with each technique.

In Chapter 4, I evaluated the potential for egg colouration to function as a visual cue or signal. One idea suggests that conspicuous egg colouration may have evolved to coerce males into providing care (Hanley et al. 2010). This could occur either through males directly keeping eggs covered, or increasing behaviours such as incubation feeding that allow the female to keep the nest contents concealed. I found support for the notion that colour-induced parasitism risk may invoke a parental response to keep colourful eggs covered, effectively lowering the risk of detection. These patterns provide partial support

for the blackmail hypothesis, and suggest that conspicuous egg colour may evolve as a consequence of conflict between the sexes (Hanley et al. 2010).

We also investigated the relationship between eggshell ground colouration and pressure from brood parasites. We found that species experiencing high levels of parasitism have low inter-clutch repeatability of egg colour. Our findings suggest that high inter-clutch variability in ground colouration may enhance parents' ability to recognize foreign eggs and may make it more difficult for parasites to match host eggs. Thus, eggshell ground colouration may be involved in a brood parasitic arms race.  As an interesting point of comparison, within a single species that was introduced in a location without brood parasites, the clutches of the parasite-free population have had lower inter-clutch variation in eggshell ground colour, and the source population, still experiencing parasitism pressure, has higher inter-clutch variation (Lahti 2005).

I also found that egg brightness was positively related to predation levels in species that use open nests and that nest above the ground. Detecting relationships between traits and predation levels has long eluded biologists (Lahti 2009). Although there are numerous possible reasons for this, perhaps the most important one is that few researchers have approached the question from the perspective of the predator, or at least considered the how predators detect prey (Lahti 2009). The relationship we found between eggshell brightness and predation pressure is important because it indicates that eggs are not inconsequential relative to conspicuous nests, as some have suggested (Skutch 1976; Götmark 1993). One reason why this effect may have been detectable is that eggshell brightness should enhance the conspicuousness of eggs to predators independent of their colour vision abilities (Jacobs 1981; Kelber et al. 2003; Hanley et al.

2010). However, eggshell ground colouration only captures part of overall egg conspicuousness; egg speckles, nest material and location, and parental colouration all play an important role in keeping nest contents concealed. Future investigations should examine the conspicuousness of eggs within their nests, incorporating both eggshell ground colouration and patterning, as well as colouration and patterning of the nesting substrate. A few new analytical methods have become available for undertaking such an investigation (Stevens et al. 2007; Stoddard and Stevens 2010).

In Chapter 5, I found evidence for selection for increased eggshell brightness within nest cavities, and also found some supporting evidence that eggshell pigments may provide protection from solar radiation. These are among two of the oldest hypotheses for eggshell colouration (McAldowie 1886; Wallace 1889), and finding comparative support for these hypotheses suggests that future research should examine these ideas in more detail in lineages with high variation in nesting strategies and egg colour. Future investigations of the hypothesis that egg brightness has evolved to enhance visibility would benefit from focusing on cavity nesting species that do not have white eggs, and lineages that have evolved enclosed nesting multiple times, such as in Cisticolidae (Nguembock et al. 2007). Future investigations of the hypothesis that eggshell pigments protect the developing embryo from solar radiation would benefit form careful experimentation on the influence of light transmission on embryonic growth and development.

In Chapter 6, I provided the first documentation of eggshell phosphorescence. Our findings suggest that egg phosphorescence does not have detrimental impacts on egg reflectance measurements. We suggest that this property may be a useful diagnostic tool

for documenting the presence of proto-porphyrin within an egg, which may be useful

considering recent interest in proto-porphyrin-based quality signals (Martínez-de la

Puente et al. 2007; Sanz and García-Navas 2009). Such a diagnostic tool would be most

useful when a species of interest has unknown pigment composition and may have trace

levels of porphyrin, such as in the European Starling (*Sturnus vulgaris*) (Kennedy and

Vevers 1976; Miksik et al. 1996), which visually appear blue-green.

**Areas of future research**

One valuable avenue for future research would be to more carefully characterize

the pigments involved in avian eggshell colouration. Studies of the signalling potential of

egg colouration lack key information underlying the assumptions of signal-based

hypotheses. In particular, we need to establish whether eggshell colouration is in fact

produced by only proto-porphyrins and biliverdin. There has been debate about this

supposition, but the methods of extraction have not allowed for the isolation of particular

pigment layers (Sorby 1875; With 1973; Kennedy and Vevers 1976; Miksik et al. 1994;

Miksik et al. 1996; Gorchein et al. 2009). Analyses of pigment composition would

benefit from an approach that isolates areas of the eggshell that appear to have different

pigments (e.g., cuticular pigments, shell pigments, ground colour across the palisade

layer). Understanding which pigments are in avian eggs, and the properties of these

pigments, will allow us to refine existing hypothesis and to formulate new hypotheses for

the evolution of egg colouration.

While we have focussed on variation in ground pigmentation, and provided

valuable insights into the selection pressures acting on this trait, we have excluded

variation in spotting from our evaluation. Consideration of the ground colouration and spotting will be necessary for a truly holistic evaluation of egg colour. Therefore, we suggest that future researchers adopt this holistic approach in future comparative studies, and continue to investigate intraspecific variation in egg spot colouration and pattering (Martinez-de la Puente et al. 2007; Sanz and García-Navas 2009; Stoddard and Stevens 2010).

In addition, researchers should consider the diversity of egg colouration as broadly as possible, and not neglect the extant ranges and phylogenetic relationships between species. Although some favour non-adaptive explanations for the diversification of avian egg colouration, it is possible that we are looking for adaptations in the wrong place. Instead of looking at the benefits of biliverdin and porphyrin within the eggshell matrix, perhaps it would be advantageous to consider the benefits of these pigments to female physiology. We know that females with differently-coloured eggs can have similar levels of pigment within their shell gland, and that the shell gland is the likely site of production for these pigments (Zhao et al. 2006; Liu et al. 2010). It is possible that the antioxidant, antimicrobial, and protease inhibition properties of these pigments (McPhee et al. 1996; Stojiljkovic et al. 2001; Kaur et al. 2003) improve the reproductive health of the female, particularly at the site of the shell gland. Such a defensive mechanism may be important in areas where the prevalence of parasites and harmful microbes may be high. A comparison of breeding female parasite loads across a broad geographical range spanning temperate and tropical zones with the diversification rate and extant diversity of egg colouration may allow for an initial examination of this hypothesis. Such measures of parasite load should be obtainable directly from the cloaca as a measure of digestive or

reproductive microbes present at the time of breeding (Ruiz-Rodriguez et al. 2009). Life history variables such as clutch size and gregariousness have been found to be related to parasite resistance and immunity (Lee et al. 2008), and examining other potential mechanisms for the diversity of egg colouration may be worthwhile.

In this body of work, we have shown that egg pigmentation has a significant environmental component, and that numerous selective pressures are acting on this variation in colour. It seems that diversity in avian egg colouration may be rivalled by the diversity of selection pressures acting on these eggs. Our work strongly suggests that life history traits, as well as environmental conditions, have shaped the dramatic expression of colouration across birds' eggs.

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**APPENDIX 1 – NATURAL HISTORY REFERENCES**

| Species | Common name | References |
|---|---|---|
| *Aepyornis maximus* | Elephant Bird | [1] |
| *Struthio camelus* | Ostrich | [1-4] |
| *Pterocnemia pennata* | Lesser Rhea | [1, 5] |
| *Rhea americana* | Greater Rhea | [1, 5-7] |
| *Casuarius bennetti* | Dwarf Cassowary | [1] |
| *Casuarius casuarius* | Southern Cassowary | [1, 8] |
| *Casuarius unappendiculatus* | Northern Cassowary | [1] |
| *Dromaius novaehollandiae* | Emu | [1] |
| *Apteryx australis* | Brown Kiwi | [1, 8] |
| *Crypturellus soui* | Little Tinamou | [1] |
| *Crypturellus noctivagus* | Yellow-legged Tinamou | [1] |
| *Crypturellus undulatus* | Undulated Tinamou | [1] |
| *Crypturellus cinnamomeus* | Thicket Tinamou | [1] |
| *Crypturellus obsoletus* | Brown Tinamou | [1] |
| *Crypturellus parvirostris* | Small-billed Tinamou | [1] |
| *Crypturellus tataupa* | Tataupa Tinamou | [1] |
| *Eudromia elegans* | Elegant Crested-Tinamou | [1, 9, 10] |
| *Tinamus osgoodi* | Black Tinamou | [1] |
| *Tinamus major* | Great Tinamou | [1, 11] |
| *Tinamus solitarius* | Solitary Tinomou | [1, 7] |
| *Nothura boraquira* | White-bellied Nothura | [1] |
| *Nothura maculosa* | Spotted Nothura | [1] |
| *Rhynchotus rufescens* | Red-winged Tinamou | [1] |
| *Nothoprocta curvirostris* | Curve-billed Tinamou | [1] |
| *Nothoprocta cinerascens* | Brushland Tinamou | [1] |
| *Nothoprocta perdicaria* | Chilean Tinamou | [1, 9] |
| *Nothocercus bonapartei* | Highland Tinamou | [1, 12] |
| *Tinamotis pentlandi* | Puna Tinamou | [1, 9] |
| *Eudyptes chrysolophus* | Macaroni Penguin | [13, 14] |
| *Eudyptes chrysocome* | Rockhopper Penguin | [8, 14-16] |
| *Pygoscelis adeliae* | Adelie Penguin | [8, 14, 17-19] |
| *Pygoscelis papua* | Gentoo Penguin | [8, 14, 20] |
| *Spheniscus magellanicus* | Magellanic Penguin | [14, 21] |
| *Gavia adamsii* | Yellow-Billed Loon | [22] |
| *Gavia arctica* | Arctic Loon | [23-26] |
| *Gavia pacifica* | Pacific Loon | [23, 27] |
| *Gavia immer* | Common Loon | [28-31] |
| *Gavia stellata* | Red-throated Loon | [23, 32-34] |
| *Podiceps auritus* | Horned Grebe | [35-39] |
| *Podiceps cristatus* | Great Crested Grebe | [35, 39-41] |
| *Podiceps grisegena* | Red-necked Grebe | [35, 39, 42] |

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| *Podilymbus podiceps* | Pied-billed Grebe | [35, 39, 43] |
| *Tachybaptus pelzelnii* | Madagascar Grebe | [35, 39] |
| *Tachybaptus dominicus* | Least Grebe | [35, 39, 44] |
| *Tachybaptus novaehollandiae* | Australasian Grebe | [8, 35, 39] |
| *Tachybaptus ruficollis* | Little Grebe | [35, 39, 40] |
| *Phoebastria albatrus* | Short-tailed Albatross | [45, 46] |
| *Diomedea exulans* | Wandering Albatross | [8, 46] |
| *Phoebastria immutabilis* | Laysan Albatross | [46, 47] |
| *Phoebastria nigripes* | Black-footed Albatross | [46, 48] |
| *Thalassarche chlororhynchos* | Atlantic yellow-nose Albatross | [8, 46, 49-51] |
| *Thalassarche chrysostoma* | Grey-headed Albatross | [8, 9, 46, 52] |
| *Phoebetria palpebrata* | Light-mantled Albatross | [46, 51, 53, 54] |
| *Bulweria bulwerii* | Bulwer's Petrel | [55-57] |
| *Fulmarus glacialis* | Northern Fulmar | [56, 58, 59] |
| *Daption capense* | Cape Petrel | [8, 56, 60, 61] |
| *Macronectes giganteus* | Southern Giant Petrel | [8, 56] |
| *Pachyptila vittata* | Broad-billed Prion | [8, 56] |
| *Pterodroma macroptera* | Great-winged Petrel | [56] |
| *Pterodroma neglecta* | Kermadec petrel | [8, 9, 56, 62] |
| *Puffinus lherminieri* | Audubon's Shearwater | [51, 56, 63, 64] |
| *Puffinus puffinus* | Manx Shearwater | [56, 65] |
| *Puffinus yelkouan* | Yelkouan Shearwater | [45, 56] |
| *Hydrobates pelagicus* | European Storm-Petrel | [45, 66] |
| *Oceanites oceanicus* | Wilson's Storm-Petrel | [8, 66] |
| *Oceanodroma castro* | Band-rumped Storm-Petrel | [66, 67] |
| *Oceanodroma furcata* | Fork-Tailed Storm-Petrel | [66, 68] |
| *Oceanodroma homochroa* | Ashy Storm-Petrel | [66, 69] |
| *Oceanodroma melania* | Black Storm-Petrel | [66, 70] |
| *Oceanodroma monorhis* | Swinhoe's Storm-Petrel | [66] |
| *Oceanodroma tethys* | Wedge-rumped Storm-Petrel | [9, 66] |
| *Pelagodroma marina* | White-faced Storm-Petrel | [8, 66] |
| *Pelecanoides garnotii* | Peruvian Diving-petrel | [9, 71] |
| *Pelecanoides georgicus* | South Georgia Diving-petrel | [8, 71] |
| *Pelecanoides urinatrix* | Common Diving-petrel | [8, 71] |
| *Phaethon aethereus* | Red-billed Tropicbird | [4, 72-74] |
| *Phaethon lepturus* | White-tailed Tropicbird | [4, 72, 74-78] |

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| *Phaethon rubricauda* | Red-Tailed Tropicbird | [72, 76, 79-81] |
| *Pelecanus erythrorhynchos* | American White Pelican | [82, 83] |
| *Pelecanus occidentalis* | Brown Pelican | [83, 84] |
| *Morus serrator* | Australian Gannet | [8, 85] |
| *Morus bassanus* | Northern Gannet | [85-87] |
| *Sula dactylatra* | Masked Booby | [73, 85, 88-90] |
| *Sula leucogaster* | Brown Booby | [85, 87, 90, 91] |
| *Sula nebouxii* | Blue-footed Booby | [85] |
| *Sula sula* | Red-footed Booby | [80, 85, 92, 93] |
| *Sula variegata* | Peruvian Booby | [9, 85] |
| *Phalacrocorax pygmeus* | Pygmy Cormorant | [45, 94, 95] |
| *Phalacrocorax africanus* | Long-tailed Cormorant | [94-97] |
| *Phalacrocorax aristotelis* | European Shag | [45, 73, 94, 95, 97] |
| *Phalacrocorax bougainvillii* | Guanay Cormorant | [9, 94, 95] |
| *Phalacrocorax magellanicus* | Rock Shag | [9, 94, 95] |
| *Phalacrocorax pelagicus* | Pelagic Cormorant | [94, 95, 98] |
| *Phalacrocorax urile* | Red-faced Cormorant | [94, 99] |
| *Anhinga anhinga* | Anhinga | [100, 101] |
| *Anhinga melanogaster* | Darter | [96, 101-103] |
| *Fregata magnificens* | Magnificant Frigatebird | [104, 105] |
| *Fregata minor* | Great Frigatebird | [105, 106] |
| *Fregata ariel* | Lesser Frigatebird | [105] |
| *Ardea herodias* | Great Blue Heron | [89, 107-109] |
| *Ardea picata* | Pied Heron | [8, 107] |
| *Butorides striata* | African green Heron | [8, 103, 107, 110] |
| *Gorsachius melanolophus* | Malayan Night Heron | [8, 103, 107, 111] |
| *Ixobrychus exilis* | Least Bittern | [107, 112, 113] |
| *Mycteria americana* | Wood Stork | [114, 115] |
| *Platalea ajaja* | Roseate Spoonbill | [116, 117] |
| *Platalea regia* | Royal Spoonbill | [8, 117, 118] |
| *Eudocimus albus* | White Ibis | [89, 117, 119] |
| *Eudocimus ruber* | Scarlet Ibis | [89, 117, |

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| *Plegadis falcinellus* | Glossy Ibis | [8, 117, 121, 122] |
| *Plegadis chihi* | White-Faced Ibis | [117, 118, 123, 124] |
| *Phoenicopterus ruber* | Greater Flamingo | [125] |
| *Anhima cornuta* | Horned Screamer | [126, 127] |
| *Chauna chavaria* | Northern Screamer | [126, 128] |
| *Anas platyrhynchos* | Mallard | [129-131] |
| *Anas strepera* | Gadwall | [130-132] |
| *Anas flavirostris* | Speckled Teal | [8, 9, 131] |
| *Aythya americana* | Redhead | [73, 133, 134] |
| *Mergus serrator* | Red-Breasted Merganser | [131, 135] |
| *Somateria mollissima* | Common Eider | [33, 131, 136] |
| *Branta ruficollis* | Red-breasted Goose | [45, 131] |
| *Cathartes aura* | Turkey Vulture | [137, 138] |
| *Coragyps atratus* | Black Vulture | [137, 139, 140] |
| *Gyps rueppellii* | Ruppells Vulture | [45, 96, 141, 142] |
| *Gyps coprotheres* | Cape Griffon Vulture | [141, 142] |
| *Pandion haliaetus* | Osprey | [141, 143] |
| *Haliaeetus vocifer* | African Fish Eagle | [141, 142] |
| *Aquila clanga* | Greater Spotted Eagle | [45, 142] |
| *Buteo albonotatus* | Zone-tailed Hawk | [142, 144] |
| *Melierax canorus* | Pale-chanting Goshawk | [141, 142, 145] |
| *Polyboroides typus* | African Harrier-Hawk | [141, 142] |
| *Circus cyaneus* | Northern Harrier | [142, 146] |
| *Ictinia mississippiensis* | Mississippi Kite | [142, 147] |
| *Sagittarius serpentarius* | Secretarybird | [148-150] |
| *Caracara cheriway* | Crested Caracara | [151, 152] |
| *Phalcobaenus australis* | Striated Caracara | [151] |
| *Falco columbarius* | Merlin | [151, 153] |
| *Falco peregrinus* | Peregrine Falcon | [154, 155] |
| *Falco concolor* | Sooty Falcon | [45, 151, 154] |
| *Nystalus maculatus* | Spot-bellied Puffbird | [156] |
| *Monasa nigrifrons* | Black-fronted Nunbird | [156] |
| *Megapodius nicobariensis* | Nicobar Scrubfowl | [103, 111, 157] |

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| *Megapodius reinwardt* | Orange-footed Scrubfowl | [8, 157, 158] |
| *Megapodius freycinet* | Dusky Scrubfowl | [157] |
| *Megapodius cumingii* | Philippine Scrubfowl | [103, 157, 159] |
| *Megapodius pritchardii* | Polynesian Scrubfowl | [157] |
| *Macrocephalon maleo* | Maleo | [157, 160, 161] |
| *Aepypodius arfakianus* | Wattled Brush-turkey | [157] |
| *Megapodius eremita* | Melanesian Scrubfowl | [157] |
| *Ortalis vetula* | Plain Chachalaca | [162-164] |
| *Meleagris ocellata* | Ocellated Turkey | [165] |
| *Meleagris gallopavo* | Wild Turkey | [4, 165, 166] |
| *Bonasa umbellus* | Ruffed Grouse | [139, 167, 168] |
| *Lagopus muta* | Rock Ptarmigan | [139, 167, 169] |
| *Tympanuchus cupido* | Greater Prairie-Chicken | [139, 167, 170] |
| *Tympanuchus phasianellus* | Sharp-Tailed Grouse | [139, 167, 171] |
| *Numida meleagris* | Helmeted Guineafowl | [172, 173] |
| *Melanoperdix nigra* | Black Wood Partridge | [174, 175] |
| *Arborophila brunneopectus* | Bar-backed Partridge | [175] |
| *Gallus sonneratii* | Grey Junglefowl | [103, 175, 176] |
| *Lophura ignita* | Crested Fireback Pheasant | [174, 175] |
| *Crossoptilon crossoptilon* | White Eared Pheasant | [175] |
| *Callipepla californica* | California Quail | [4, 177, 178] |
| *Callipepla gambelii* | Gambel's Quail | [178, 179] |
| *Colinus virginianus* | Northern Bobwhite | [178, 180] |
| *Turnix tanki* | Yellow-legged Buttonquail | [103, 176, 181] |
| *Turnix nigricollis* | Madagascar Buttonquail | [181] |
| *Turnix sylvaticus* | Common Buttonquail | [103, 176, 181, 182] |
| *Turnix velox* | Australian Little Buttonquail | [8, 181] |
| *Grus canadensis* | Sandhill Crane | [183, 184] |
| *Grus antigone* | Sarus Crane | [8, 103, 176, 184] |
| *Anthropoides paradiseus* | Blue Crane | [184, 185] |
| *Aramus guarauna* | Limpkin | [186, 187] |

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| *Gallinula chloropus* | Common Moorhen | [45, 103, 111, 188, 189] |
| *Porzana carolina* | Sora | [73, 190-192] |
| *Porzana tabuensis* | Spotless Crake | [190] |
| *Rallus elegans* | King Rail | [8, 190, 193] |
| *Amaurornis flavirostra* | Black Crake | [96, 188, 190] |
| *Cariama cristata* | Red-legged Seriema | [194] |
| *Chlamydotis undulata* | Houbara Bustard | [45, 195-197] |
| *Otis tarda* | Great Bustard | [45, 196, 197] |
| *Tetrax tetrax* | Little Bustard | [45, 103, 196, 197] |
| *Podoica senegalensis* | African Finfoot | [96, 198, 199] |
| *Jacana spinosa* | Northern Jacana | [200, 201] |
| *Metopidius indicus* | Bronze-winged Jacana | [103, 176, 200, 202] |
| *Hydrophasianus chirurgus* | Pheasant-tailed Jacana | [103, 200] |
| *Burhinus recurvirostris* | Great Thick-knee | [103, 203] |
| *Haematopus palliatus* | American Oystercatcher | [204-206] |
| *Haematopus bachmani* | Black Oystercatcher | [204, 207] |
| *Charadrius alexandrinus* | Snowy Plover | [103, 128, 208, 209] |
| *Charadrius vociferus* | Killdeer | [210, 211] |
| *Charadrius montanus* | Mountain Plover | [211, 212] |
| *Charadrius hiaticula* | Ringed Plover | [8, 45, 211] |
| *Vanellus vanellus* | Northern Lapwing | [8, 45, 208, 211] |
| *Vanellus malarbaricus* | Yellow-wattled Lapwing | [103, 213, 214] |
| *Calidris maritima* | Purple Sandpiper | [213, 215, 216] |
| *Numenius americanus* | Long-billed Curlew | [213, 217] |
| *Actitis macularius* | Spotted Sandpiper | [73, 213, 218] |
| *Scolopax rusticola* | Woodcock | [45, 213] |
| *Gallinago stricklandii* | Fuegian Snipe | [9, 213] |
| *Larus argentatus* | Herring Gull | [219-221] |
| *Larus delawarensis* | Ring-billed Gull | [45, 220, |

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| *Xema sabini* | Sabine's Gull | [220, 223] |
| *Creagrus furcatus* | Swallow-tailed Gull | [220, 224] |
| *Stercorarius parasiticus* | Arctic Skua | [45, 225, 226] |
| *Sterna sandvicensis* | Sandwich Tern | [45, 227, 228] |
| *Hydroprogne caspia* | Caspian Tern | [229-231] |
| *Rynchops niger* | Black Skimmer | [7, 232-234] |
| *Uria lomvia* | Thick-billed Mure | [73, 235-237] |
| *Alle alle* | Dovekie | [237, 238] |
| *Synthliboramphus hypoleucus* | Xantus's Murrelet | [237, 239, 240] |
| *Columbina passerina* | Common Ground-Dove | [7, 241, 242] |
| *Columba picazuro* | Picazuro Pigeon | [241, 243] |
| *Columba flavirostris* | Red-billed Pigeon | [241, 244] |
| *Columba plumbea* | Plumbeous Pigeon | [241, 245] |
| *Columba inornata* | Plain Pigeon | [241] |
| *Ptilinopus coralensis* | Atoll Fruit- dove | [241] |
| *Ptilinopus porphyraceus* | Purple-capped Fruit Dove | [241] |
| *Zenaida asiatica* | White-winged Dove | [241, 246, 247] |
| *Zenaida macroura* | Mourning Dove | [241, 248] |
| *Petrophassa albipennis* | White-quilled Rock Pigeon | [241, 249] |
| *Leptotila jamaicensis* | Caribbean Dove | [241] |
| *Gallicolumba stairi* | Friendly Ground Dove | [241] |
| *Hemiphaga novaeseelandiae* | New Zealand Pigeon | [241, 249] |
| *Ducula rubricera* | Red-nobbed Imperial Pigeon | [241] |
| *Ducula badia* | Mountain Imperial Pigeon | [103, 174, 241] |
| *Corythaeola cristata* | Great Blue Turaco | [96, 250, 251] |
| *Crinifer zonurus* | Eastern Grey Plantain-eater | [250, 251] |
| *Ruwenzorornis johnstoni* | Ruwenzori Turaco | [250, 251] |
| *Tauraco leucolophus* | White-crested Turaco | [250, 251] |
| *Tauraco schuetti* | Black-billed Turaco | [250, 251] |
| *Clamator jacobinus* | Jacobin Cuckoo | [252, 253] |
| *Guira guira* | Guira Cuckoo | [253] |
| *Coccyzus americanus* | Yellow-billed Cuckoo | [253, 254] |
| *Coccyzus erythropthalmus* | Black-billed Cuckoo | [253, 255] |

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| *Geococcyx californianus* | Greater Roadrunner | [253, 256] |
| *Chrysococcyx basalis* | Horsfield Bronze-Cuckoo | [253, 257] |
| *Chrysococcyx klaas* | Klaas's Cuckoo | [96, 252, 253] |
| *Centropus nigrorufus* | Sunda Coucal | [253] |
| *Centropus grillii* | African Black Coucal | [96, 252, 253] |
| *Crotophaga ani* | Smooth-billed Ani | [139, 253, 258] |
| *Opisthocomus hoazin* | Hoatzin | [4, 7, 245, 259, 260] |
| *Tyto alba* | Barn Owl | [174, 261] |
| *Tyto rosenbergii* | Sulawesi Owl | [262] |
| *Bubo virginianus* | Great Horned Owl | [262, 263] |
| *Bubo bengalensis* | Rock Eagle Owl | [103, 176, 264] |
| *Micrathene whitneyi* | Elf Owl | [264, 265] |
| *Megascops asio* | Eastern Screech-Owl | [264, 266] |
| *Otus rutilus* | Madagascar Scops Owl | [264] |
| *Scotopelia peli* | Pel's Fishing Owl | [96, 264, 267] |
| *Glaucidium passerinum* | Eurasian Pygmy Owl | [45, 264] |
| *Caprimulgus vociferus* | Whip-poor-will | [73, 268, 269] |
| *Phalaenoptilus nuttallii* | Common Poorwill | [269-271] |
| *Caprimulgus tristigma* | Freckled Nightjar | [96, 269, 272] |
| *Chordeiles acutipennis* | Lesser Nighthawk | [269, 273] |
| *Chordeiles minor* | Common Nighthawk | [4, 139, 269, 274] |
| *Chaetura pelagica* | Chimney Swift | [275, 276] |
| *Chaetura vauxi* | Vaux's Swift | [275, 277] |
| *Apus apus* | Common Swift | [45, 275, 278] |
| *Tachymarptis melba* | Alpine Swift | [275, 278] |
| *Apus pallidus* | Pallid Swift | [45, 275, 278] |
| *Streptoprocne zonaris* | White-collared Swift | [275] |
| *Cypsiurus balasiensis* | Asian Palm Swift | [174, 176, 275] |
| *Archilochus alexandri* | Black-chinned Hummingbird | [279-281] |
| *Archilochus colubris* | Ruby-throated Hummingbird | [279, 282] |
| *Calypte anna* | Anna's Hummingbird | [279, 283] |
| *Phaethornis longuemareus* | Little Hermit Hummingbird | [73, 279] |

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| *Amazilia tzacatl* | Rufous-tailed Hummingbird | [73, 279, 284-287] |
| *Coeligena torquata* | Collared Inca | [279] |
| *Metallura tyrianthina* | Tyrian Metaltail | [279] |
| *Chlorostilbon mellisugus* | Blue-tailed Emerald | [279] |
| *Loddigesia mirabilis* | Marvelous Spatuletail | [279] |
| *Selasphorus rufus* | Rufous Hummingbird | [279, 288] |
| *Colius striatus* | Speckled Mousebird | [96, 289] |
| *Trogon elegans* | Elegant Trogon | [290-292] |
| *Trogon viridis* | White-tailed Trogon | [290, 291, 293] |
| *Megaceryle alcyon* | Belted Kingfisher | [294, 295] |
| *Alcedo atthis* | Common Kingfisher | [45, 103, 295] |
| *Alcedo euryzona* | Blue-banded Kingfisher | [174, 295] |
| *Chloroceryle americana* | Green Kingfisher | [295, 296] |
| *Momotus momota* | Blue-crowned Motmot | [297] |
| *Momotus mexicanus* | Russet-crowned Motmot | [297] |
| *Merops apiaster* | European Bee-eater | [73, 103, 195, 298, 299] |
| *Merops persicus* | Blue-cheeked Bee-eater | [45, 298, 299] |
| *Merops bulocki* | Red throated bee-eater | [298, 299] |
| *Merops oreobates* | Cinnamon-chested Bee-eater | [298, 299] |
| *Merops superciliosus* | Madagascar Bee-eater | [96, 298, 299] |
| *Coracias benghalensis* | Indian Roller | [174, 176, 300] |
| *Coracias garrulus* | European Roller | [45, 300, 301] |
| *Upupa epops* | Hoopoe | [45, 302, 303] |
| *Bycanistes bucinator* | Trumpeter Hornbill | [80, 304, 305] |
| *Bucorvus leadbeateri* | Southern Ground-hornbill | [304, 305] |
| *Indicator exilis* | Least Honeyguide | [96, 306, 307] |
| *Colaptes auratus* | Northern Flicker | [308-310] |
| *Melanerpes lewis* | Lewis's Woodpecker | [310-312] |
| *Melanerpes erythrocephalus* | Red-headed Woodpecker | [310, 313] |
| *Picoides pubescens* | Downy Woodpecker | [310, 314, 315] |
| *Picoides scalaris* | Ladder-backed Woodpecker | [310, 316] |

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| *Sphyrapicus varius* | Yellow-bellied Sapsucker | [310, 317] |
| *Sasia ochracea* | White-browed Piculet | [103, 174, 310] |
| *Veniliornis passerinus* | Little Woodpecker | [9, 310] |
| *Dryocopus pileatus* | Pileated Woodpecker | [310, 318] |
| *Meiglyptes tristis* | Buff-rumped Woodpecker | [174, 310] |
| *Campephilus magellanicus* | Magellanic Woodpecker | [310] |
| *Myiopsitta monachus* | Monk Parakeet | [319] |
| *Psittacus erithacus* | Grey Parrot | [96, 319] |
| *Prosopeia personata* | Masked Shining Parrot | [319] |
| *Alisterus scapularis* | Australian King Parrot | [257, 319] |
| *Alisterus amboinensis* | Moluccan King Parrot | [319] |
| *Strigops habroptilus* | Kakapo | [257, 319] |
| *Ara ararauna* | Blue and Yellow Macaw | [319] |
| *Pionopsitta pileata* | Pileated Parrot | [319] |
| *Phleocryptes melanops* | Wren-like Rushbird | [320] |
| *Furnarius rufus* | Rufous Hornero | [320, 321] |
| *Upucerthia certhioides* | Chaco Earthcreeper | [320] |
| *Cranioleuca pyrrhophia* | Stripe Crowned Spinetail | [320] |
| *Syndactyla rufosuperciliata* | Buff-browed Foliage Gleaner | [320] |
| *Thripadectes holostictus* | Striped Treehunter | [320, 322] |
| *Glyphorynchus spirurus* | Wedge-billed Woodcreeper | [73, 323] |
| *Lepidocolaptes angustirostris* | Narrow-billed Woodcreeper | [323] |
| *Formicaria analis* | Black-faced Anttrush | [324] |
| *Grallaria ruficapilla* | Chestnut Crowned Antpitta | [324] |
| *Thamnophilus caerulescens* | Variable Antshrike | [325] |
| *Thamnophilus ruficapillus* | Rufous-capped Antshrike | [325] |
| *Taraba major* | Great Antshrike | [73, 325, 326] |
| *Thamnomanes ardesiacus* | Dusky Throated Antshrike | [325] |
| *Myrmeciza longipes* | White-bellied Antbird | [325, 327] |
| *Empidonax traillii* | Willow Flycatcher | [328] |
| *Phylloscartes ventralis* | Mottle-cheeked Tyrannulet | [328] |
| *Tyrannus tyrannus* | Eastern Kingbird | [328] |
| *Myiozetetes similis* | Social Flycatcher | [328, 329] |
| *Contopus virens* | Eastern Wood-Pewee | [328] |
| *Myiarchus tyrannulus* | Brown-crested Flycatcher | [328] |
| *Myiarchus crinitus* | Great Crested Flycatcher | [328] |
| *Pachyramphus aglaiae* | Rose-throated Becard | [73, 326, 328] |
| *Hemitriccus granadensis* | Black-throated Tody Tyrant | [328] |
| *Todirostrum sylvia* | Slate Headed Tody Flycatcher | [73, 328, 329] |

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| *Myiopagis viridicata* | Greenish Elaenia | [328] |
| *Tolmomyias sulphurescens* | Yellow-olive Flycatcher | [328, 329] |
| *Anairetes flavirostris* | Yellow-billed Tit Tyrant | [328, 330] |
| *Myiophobus fasciatus* | Bran-colored Flycatcher | [328] |
| *Attila spadiceus* | Bright-rumped Attila | [73, 328] |
| *Phibalura flavirostris* | Swallow-tailed Cotinga | [331] |
| *Perissocephalus tricolor* | Capuchinbird | [331] |
| *Pipreola riefferi* | Green and Black Fruiteater | [331, 332] |
| *Machaeropterus regulus* | Eastern Striped Manakin | [245, 332, 333] |
| *Alauda arvensis* | Sky Lark | [45, 334-338] |
| *Eremophila alpestris* | Horned Lark | [338, 339] |
| *Mirafra africana* | Rufous-naped Lark | [96, 335, 338] |
| *Mirafra africanoides* | Fawn Coloured Lark | [96, 335, 338] |
| *Melanocorypha maxima* | Tibetan Lark | [103, 338] |
| *Melanocorypha yeltoniensis* | Black Lark | [45, 338] |
| *Spizocorys conirostris* | Pink-billed Lark | [335, 338] |
| *Galerida cristata* | Crested Lark | [45, 103, 335, 338] |
| *Lullula arborea* | Woodlark | [45, 335, 338] |
| *Tachycineta bicolor* | Tree Swallow | [340, 341] |
| *Tachycineta thalassina* | Violet-green Swallow | [340, 342] |
| *Hirundo rustica* | Barn Swallow | [103, 340, 343, 344] |
| *Progne subis* | Purple Martin | [340, 345] |
| *Riparia riparia* | Bank Swallow | [340, 343, 346] |
| *Petrochelidon pyrrhonota* | Cliff Swallow | [340, 347] |
| *Stelgidopteryx ruficollis* | Southern Rough-winged Swallow | [73, 327, 329, 340] |
| *Stelgidopteryx serripennis* | Northern Rough-winged Swallow | [340, 348] |
| *Anthus rubescens* | American Pipit | [349, 350] |
| *Anthus richardi* | Richard's Pipit | [45, 103, 349, 351, 352] |
| *Anthus campestris* | Tawny Pipit | [45, 103, 349, 351, 353] |
| *Anthus nyassae* | Woodland Pipit | [349, 351] |
| *Motacilla alba* | White Wagtail | [45, 176, |

|  |  | 349, 351, 354] |
| *Prionochilus percussus* | Crimson-breasted Flowerpecker | [343, 355] |
| *Dicaeum australe* | Red-striped Flowerpecker | [355] |
| *Anthreptes anchietae* | Anchieta's Sunbird | [355] |
| *Nectarinia verticalis* | Green-headed Sunbird | [355, 356] |
| *Nectarinia rubescens* | Green-throated Sunbird | [355, 356] |
| *Arachnothera chrysogenys* | Yellow-eared Spiderhunter | [343, 355] |
| *Pycnonotus barbatus* | Common Bulbul | [357, 358] |
| *Pycnonotus urostictus* | Yellow-wattled Bulbul | [357] |
| *Chlorocichla simplex* | Simple Greenbul | [357-359] |
| *Ixos philippinus* | Phillipine Bulbul | [357] |
| *Bombycilla cedrorum* | Cedar Waxwing | [360, 361] |
| *Bombycilla garrulus* | Bohemian Waxwing | [45, 360, 361] |
| *Phainopepla nitens* | Phainopepla | [362, 363] |
| *Cinclus mexicanus* | American Dipper | [364, 365] |
| *Cinclus cinclus* | White-throated Dipper | [4, 45, 364, 366] |
| *Regulus regulus* | Goldcrest | [45, 367] |
| *Regulus satrapa* | Golden-crowned Kinglet | [367, 368] |
| *Campylorhynchus brunneicapillus* | Cactus Wren | [369-372] |
| *Campylorhynchus gularis* | Spotted Wren | [245, 370, 372] |
| *Campylorhynchus griseus* | Bicoloured Wren | [370, 372-374] |
| *Troglodytes aedon* | House Wren | [370, 375] |
| *Thryothorus ludovicianus* | Carolina Wren | [4, 370, 376] |
| *Thryothorus genibarbis* | Moustached Wren | [370, 377, 378] |
| *Thryothorus modestus* | Plain Wren | [329, 370] |
| *Thryothorus longirostris* | Long-billed Wren | [370] |
| *Cistothorus platensis* | Sedge Wren | [4, 369, 370, 379, 380] |
| *Cistothorus palustris* | Marsh Wren | [370, 381] |
| *Mimus polyglottos* | Northern Mockingbird | [73, 382] |
| *Dumetella carolinensis* | Gray Catbird | [382, 383] |
| *Oreoscoptes montanus* | Sage Thrasher | [382, 384] |
| *Toxostoma rufum* | Brown Thrasher | [382, 385] |
| *Toxostoma crissale* | Crissal Thrasher | [382, 386] |
| *Toxostoma bendirei* | Bendire's Thrasher | [382, 387] |

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| *Toxostoma lecontei* | Le Conte's Thrasher | [382, 388] |
| *Toxostoma redivivum* | California Thrasher | [382, 389] |
| *Toxostoma cinereum* | Grey Thrasher | [382, 390] |
| *Toxostoma curvirostre* | Curve-billed Thrasher | [382, 391] |
| *Toxostoma longirostre* | Long-billed Thrasher | [382, 392] |
| *Prunella modularis* | Dunnock | [45, 351, 393] |
| *Catharus fuscescens* | Veery | [394, 395] |
| *Sialia mexicana* | Western Bluebird | [394, 396] |
| *Hylocichla mustelina* | Wood Thrush | [394, 397, 398] |
| *Turdus migratorius* | American Robin | [4, 73, 394, 399, 400] |
| *Turdus merula* | Blackbird | [45, 337, 394, 401] |
| *Turdus philomelos* | Song Thrush | [45, 337, 394, 402] |
| *Rhinomyias umbratilis* | Grey-chested Jungle Flycatcher | [343, 403] |
| *Stiphrornis erythrothorax* | Forest Robin | [394, 404] |
| *Hippolais icterina* | Icterine Warbler | [45, 405] |
| *Hippolais polyglotta* | Melodius Warbler | [45, 402, 405, 406] |
| *Acrocephalus scirpaceus* | Eurasian Reed-warbler | [405] |
| *Hylia prasina* | Green Hylia | [405, 406] |
| *Malacocincla abbotti* | Abotts Babbler | [407] |
| *Malacopteron magnum* | Rufous-crowned Babbler | [343, 407] |
| *Rimator malacoptilus* | Long-billed Wren Babbler | [407] |
| *Alcippe cinereiceps* | Grey-hooded Fulvetta | [103, 407] |
| *Alcippe rufogularis* | Rufous-throated Fulvetta | [103, 407] |
| *Alcippe morrison* | Rufous-winged Fulvetta | [103, 343, 407] |
| *Alcippe nipalensis* | Nepal Fulvetta | [103, 407] |
| *Heterophasia melanoleuca* | Black-backed Sibia | [407] |
| *Paradoxornis brunneus* | Brown Winged Parrotbill | [408] |
| *Sylvia nisoria* | Barred Warbler | [45, 405] |
| *Sylvia layardi* | Layard's Warbler | [405, 406] |
| *Polioptila caerulea* | Blue-gray Gnatcatcher | [409] |
| *Oenanthe oenanthe* | Northern Wheatear | [45, 394] |
| *Erithacus rubecula* | European Robin | [45, 394, 404] |
| *Cossypha dichroa* | Chorister Robin Chat | [96, 394, 404] |
| *Cichladusa guttata* | Spotted Morning Thrush | [394, 404] |

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| *Copsychus saularis* | Magpie Robin | [103, 176, 343, 394] |
| *Cercomela familiaris* | Familiar Chat | [96, 394, 404] |
| *Luscinia megarhynchos* | Common Nightingale | [45, 394, 404] |
| *Phoenicurus phoenicurus* | Common Redstart | [45, 394, 404] |
| *Petroica phoenicea* | Flame Robin | [410] |
| *Poecilodryas superciliosa* | White-browed Robin | [410] |
| *Clytorhynchus pachycephaloides* | Southern Shrikebill | [411] |
| *Clytorhynchus vitiensis* | Lesser Shrikebill | [411] |
| *Pachycephala pectoralis* | Golden Whistler | [412] |
| *Chamaea fasciata* | Wrentit | [407, 413] |
| *Psaltriparus minimus* | Bushtit | [414] |
| *Aegithalos caudatus* | Northern Long-tailed Tit | [45, 414] |
| *Parus atricapillus* | Black-capped Chickadee | [415-417] |
| *Parus bicolor* | Tufted Titmouse | [415, 418] |
| *Parus carolinensis* | Carolina Chickadee | [415, 419, 420] |
| *Parus varius* | Varied Tit | [415] |
| *Certhia familiaris* | Eurasian Treecreeper | [45, 421] |
| *Donacobius atricapillus* | Black-capped Donacobious | [73, 370] |
| *Remiz consobrinus* | Chinese Penduline Tit | [422, 423] |
| *Auriparus flaviceps* | Verdin | [422, 424] |
| *Cisticola chiniana* | Rattling Cisticola | [96, 406, 425] |
| *Cisticola juncidis* | Zitting Cisticola | [337, 343, 406, 425] |
| *Cisticola dambo* | Cloud-scraping Cisticola | [96, 406, 425] |
| *Prinia somalica* | Pale Pinea | [406, 425] |
| *Prinia maculosa* | Karoo Pinea | [96, 406, 425] |
| *Apalis flavida* | Yellow-breasted Apalis | [96, 406, 425] |
| *Camaroptera brevicaudata* | Grey-backed Camaroptera | [406, 425] |
| *Zosterops conspicillatus* | Bridled White-eye | [426] |
| *Zosterops chloris* | Lemon-bellied White-eye | [426] |
| *Malurus cyaneus* | Superb Fairy Wren | [73, 257, 427] |
| *Phylidonyris novaehollandiae* | New Holland Honeyeater | [257, 428] |
| *Lichmera indistincta* | Brown Honeyeater | [257, 428] |
| *Meliphaga gracilis* | Graceful Honeyeater | [257, 428] |

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| *Lichenostomus flavescens* | Yellow-tinted Honeyeater | [257, 428] |
| *Philemon argenticeps* | Silver-crowned Friarbird | [257, 428] |
| *Phylidonyris albifrons* | White-fronted Honeyeater | [428] |
| *Manorina melanocephala* | Noisy Miner | [257, 428] |
| *Dasyornis broadbenti* | Rufous Bristlebird | [429] |
| *Sericornis magnirostris* | Large-billed Scrubwren | [430] |
| *Oriolus chinensis* | Black-naped Oriole | [103, 343, 431] |
| *Oriolus oriolus* | Eurasian Golden Oriole | [45, 103, 431, 432] |
| *Lanius ludovicianus* | Loggerhead Shrike | [433] |
| *Lanius schach* | long-tailed Shrike | [176, 433] |
| *Lanius collurio* | Red -backed Shrike | [45, 433, 434] |
| *Lanius minor* | lesser Grey Shrike | [45, 433] |
| *Lanius tigrinus* | Tiger Shrike | [433] |
| *Lanius souzae* | Souza's Shrike | [96, 433, 435] |
| *Artamus cinereus* | Black-faced Woodswallow | [337, 436] |
| *Gymnorhina tibicen* | Australasian Magpie | [337, 437] |
| *Manucodia atra* | Glossy-mantled Manucode | [438] |
| *Paradisaea apoda* | Greater Bird-of-paradise | [438] |
| *Paradisaea rudolphi* | Blue Bird-of-paradise | [438] |
| *Epimachus meyeri* | Brown Sicklebill | [438] |
| *Amblyornis macgregoriae* | Macgregor's Bowerbird | [439] |
| *Corvus brachyrhynchos* | American Crow | [440, 441] |
| *Corvus corax* | Common Raven | [45, 442] |
| *Aphelocoma ultramarina* | Mexican Jay | [440, 443] |
| *Aphelocoma coerulescens* | Florida Scrub-Jay | [440, 444] |
| *Pyrrhocorax pyrrhocorax* | Red-billed Chough | [45, 440, 445] |
| *Cyanocitta cristata* | Blue Jay | [440, 446] |
| *Cyanocitta stelleri* | Steller's Jay | [440] |
| *Pica pica* | Black-billed Magpie | [440, 445, 447] |
| *Turnagra capensis* | South Island Piopio | [337] |
| *Gymnorhinus cyanocephalus* | Pinyon Jay | [440, 448] |
| *Cyanocorax sanblasianus* | San Blas Jay | [440, 449] |
| *Cyanocorax caeruleus* | Azure Jay | [440, 450] |
| *Cyanocorax affinis* | Black Chested Jay | [327, 440] |
| *Dendrocitta bayleyi* | Andaman Treepie | [103, 440] |
| *Podoces panderi* | Turkestan (Panders) Ground Jay | [440, 451] |
| *Corvus splendens* | Indian House Crow | [103, 214, |

|  |  | 440, 445] |
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| *Corvus enca* | Slender-billed Crow | [80, 343, 440] |
| *Astrapia rothschildi* | Huon Astrapia | [438] |
| *Strepera graculina* | Pied Currawong | [337, 437] |
| *Lalage leucopyga* | Long-tailed Triller | [337, 452] |
| *Pericrocotus igneus* | Fiery Minivet | [103, 343, 452] |
| *Pericrocotus brevirostris* | Short-billed Minivet | [176, 214, 452] |
| *Sturnus vulgaris* | European Starling | [45, 453, 454] |
| *Aplonis tabuensis* | Polynesian Starling | [454] |
| *Aplonis cantorides* | Singing Starling | [454] |
| *Aplonis metallica* | Metallic Starling | [337, 454] |
| *Acridotheres tristis* | Common Myna | [80, 337, 453, 454] |
| *Passer domesticus* | House Sparrow | [103, 455-457] |
| *Passer montanus* | Eurasian Tree Sparrow | [45, 455, 457, 458] |
| *Petronia petronia* | Rock Petronia | [45, 455, 457] |
| *Estrilda troglodytes* | Black-rumped Waxbill | [459] |
| *Estrilda astrild* | Common Waxbill | [45, 359, 459, 460] |
| *Poephila acuticauda* | Long-tailed Finch | [337] |
| *Vidua macroura* | Pin-tailed Whydah | [359, 461] |
| *Brachycope anomala* | Bob-tailed Weaver | [462] |
| *Ploceus cucullatus* | Village Weaver | [359] |
| *Ploceus ocularis* | Spectacled Weaver | [463] |
| *Ploceus rubiginosus* | Chestnut Weaver | [359] |
| *Ploceus nelicourvi* | Nelicourvi Weaver | [464, 465] |
| *Ploceus bicolor* | Forest Weaver | [80, 463] |
| *Vireo griseus* | White-eyed Vireo | [466] |
| *Vireo olivaceus* | Red-eyed Vireo | [467] |
| *Vireo solitarius* | Solitary Vireo | [468, 469] |
| *Vireo bellii* | Bell's Vireo | [470, 471] |
| *Cyclarhis gujanensis* | Rufous-browed Peppershrike | [472] |
| *Hylophilus aurantiifrons* | Golden-fronted Greenlet | [327] |
| *Carpodacus cassinii* | Cassin's Finch | [473] |
| *Carpodacus mexicanus* | House Finch | [474] |
| *Fringilla coelebs* | Chaffinch | [45, 475] |

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| *Loxia curvirostra* | Red Crossbill | [45, 476] |
| *Leucosticte tephrocotis* | Gray-crowned Rosy-Finch | [477] |
| *Carduelis hornemanni* | Hoary Redpoll | [478] |
| *Pyrrhula pyrrhula* | Eurasian Bullfinch | [45] |
| *Serinus leucopygius* | White-rumped Seedeater | [479] |
| *Serinus alario* | Alario Finch | [479] |
| *Carduelis tristis* | American Goldfinch | [480] |
| *Poospiza nigrorufa* | Black and Rufous Warbling Finch | |
| *Sicalis luteiventris* | Misto Yellow Finch | [7, 9, 321, 332, 481] |
| *Oryzoborus angolensis* | Lesser Seed Finch | [327, 482] |
| *Diglossa caerulescens* | Bluish Flower Piercer | [483-485] |
| *Psarocolius guatimozinus* | Black Oropendola | [327, 486] |
| *Conirostrum sitticolor* | Blue-backed Conebill | [332, 487] |
| *Arremon aurantiirostris* | Orange-billed Sparrow | [73, 287, 327, 484, 488] |
| *Atlapetes brunneinucha* | Chestnutcapped Brush Finch | [472] |
| *Telespiza cantans* | Laysan Finch | [489] |
| *Himatione sanguinea* | Laysan Apapane | [490] |
| *Pinicola enucleator* | Pine Grosbeak | [491] |
| *Pitohui ferrugineus* | Rusty Pitohui | [412] |
| *Pitohui dichrous* | Hooded Pitohui | [412, 492] |
| *Dendroica kirtlandii* | Kirtland's Warbler | [493] |
| *Dendroica petechia* | Yellow Warbler | [494] |
| *Dendroica cerulea* | Cerulean Warbler | [495] |
| *Dendroica pensylvanica* | Chestnut-sided Warbler | [496] |
| *Geothlypis trichas* | Common Yellowthroat | [497] |
| *Icteria virens* | Yellow-breasted Chat | [498] |
| *Myioborus pictus* | Painted Redstart | [499] |
| *Vermivora celata* | Orange-crowned Warbler | [500] |
| *Vermivora luciae* | Lucy's Warbler | [501] |
| *Vermivora pinus* | Blue-winged Warbler | [502] |
| *Limnothlypis swainsonii* | Swainson's Warbler | [73, 503] |
| *Helmitheros vermivorum* | Worm-eating Warbler | [504] |
| *Protonotaria citrea* | Prothonotary Warbler | [505] |
| *Wilsonia citrina* | Hooded Warbler | [506] |
| *Passerina caerulea* | Blue Grosbeak | [507] |
| *Passerina ciris* | Painted Bunting | [139, 508] |
| *Passerina cyanea* | Indigo Bunting | [509] |
| *Pheucticus ludovicianus* | Rose-breasted Grosbeak | [510] |
| *Pheucticus melanocephalus* | Black-headed Grosbeak | [511, 512] |

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| *Spiza americana* | Dickcissel | [513] |
| *Cardinalis cardinalis* | Northern Cardinal | [514] |
| *Cardinalis sinuatus* | Pyrrhuloxia | [515] |
| *Ramphocelus dimidiatus* | Crimson-backed Tanager | [484, 485] |
| *Thraupis episcopus* | Blue-gray Tanager | [73, 327, 484] |
| *Thraupis cyanocephala* | Blue-capped Tanager | [245, 483, 485] |
| *Thraupis palmarum* | Palm Tanager | [12, 245, 327, 485] |
| *Piranga olivacea* | Scarlet Tanager | [485, 516] |
| *Piranga rubra* | Summer Tanager | [517] |
| *Piranga flava* | Hepatic Tanager | [518] |
| *Piranga ludoviciana* | Western Tanager | [519] |
| *Tangara guttata* | Speckled Tanager | [73, 327, 485] |
| *Tangara cucullata* | Lesser Antillean Tanager | [485] |
| *Tangara vitriolina* | Scrub Tanager | [485] |
| *Tachyphonus rufus* | White-lined Tanager | [327, 485] |
| *Chlorospingus ophthalmicus* | Common Bush Tanager | [327, 485] |
| *Cnemoscopus rubrirostris* | Gray-hooded Bush Tanager | [332, 485] |
| *Habia gutturalis* | Sooty Ant Tanager | [485, 520, 521] |
| *Chlorornis riefferi* | Grass Green Tanager | [485] |
| *Euphonia xanthogaster* | Orange-billed Euphonia | [245, 327] |
| *Melospiza lincolnii* | Lincoln's Sparrow | [522] |
| *Ammodramus caudacutus* | Saltmarsh Sharp-tailed Sparrow | [80, 139, 523] |
| *Ammodramus nelsoni* | Nelson's Sharp-tailed Sparrow | [80, 139, 523] |
| *Aimophila aestivalis* | Bachman's Sparrow | [524, 525] |
| *Amphispiza belli* | Sage Sparrow | [526] |
| *Amphispiza bilineata* | Black-throated Sparrow | [527] |
| *Calcarius mccownii* | McCown's Longspur | [528] |
| *Calcarius lapponicus* | Lapland Longspur | [45, 529] |
| *Calcarius ornatus* | Chestnut-collared Longspur | [530] |
| *Chondestes grammacus* | Lark Sparrow | [531] |
| *Passerella iliaca* | Fox Sparrow | [532] |
| *Plectrophenax nivalis* | Snow Bunting | [45, 533] |
| *Pooecetes gramineus* | Vesper Sparrow | [534, 535] |
| *Spizella pallida* | Clay-colored Sparrow | [536] |
| *Spizella pusilla* | Field Sparrow | [535, 537] |
| *Spizella passerina* | Chipping Sparrow | [538] |

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| *Sporophila torqueola* | White-collared Seedeater | [12, 484, 539] |
| *Tiaris bicolor* | Black-faced Grassquit | [540, 541] |
| *Zonotrichia albicollis* | White-throated Sparrow | [542] |
| *Zonotrichia leucophrys* | White-crowned Sparrow | [543, 544] |
| *Paroaria coronata* | Red-crested Cardinal | [7, 481] |
| *Paroaria capitata* | Yellow-billed Cardinal | [332, 545] |
| *Arremonops rufivirgatus* | Olive Sparrow | [546] |
| *Calamospiza melanocorys* | Lark Bunting | [547] |
| *Junco hyemalis* | Dark-eyed Junco | [548] |
| *Junco phaeonotus* | Yellow-eyed Junco | [549] |
| *Pipilo aberti* | Abert's Towhee | [550] |
| *Pipilo fuscus* | Canyon Towhee | [551] |
| *Icterus cucullatus* | Hooded Oriole | [552] |
| *Icterus galbula* | Baltimore Oriole | [553] |
| *Icterus chrysater* | Yellow-backed Oriole | [327, 486, 554] |
| *Molothrus aeneus* | Bronzed Cowbird | [555] |
| *Molothrus ater* | Brown-headed Cowbird | [556] |
| *Dolichonyx oryzivorus* | Bobolink | [557] |
| *Agelaius phoeniceus* | Red-winged Blackbird | [558, 559] |
| *Agelaius humeralis* | Tawny-shouldered Blackbird | [560, 561] |
| *Sturnella magna* | Eastern Meadowlark | [562] |
| *Euphagus cyanocephalus* | Brewer's Blackbird | [563] |
| *Quiscalus quiscula* | Common Grackle | [564] |

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**APPENDIX 2 – PHYLOGENETIC RECONSTRUCTION**

**Placement decisions**

We reconstructed a composite supertree using Mesquite (v2.6), with the major

phylogenetic relationships following a recent hypothesis for the phylogenic relationships

among birds [1]. The relationships within Passeriformes generally relate to the

phylogentic positions suggested by a large-scale (1723 extant species) super tree [2].

Previous literature has provided extensive evidence for the relative placement of species

and therefore the information we present here will not duplicate that. Instead, for each

order, we provide references that we used to resolve contentious taxonomic issues where

we relied on better resolved or more recent information.

Struthioniformes [3], Tinamiformes [4], Sphenisciformes [5], Gaviiformes [6],

Podicepidiformes [7], Procellariformes [8], Pelecaniformes [1, 9-11], Ciconiiformes [12-

14], Phoenicopteriformes [1], Anseriformes [15, 16], Falconiformes [1, 17, 18],

Galliformes [19], Gruiformes [20], Charadriformes [1, 21-23], Columbidae [24-28],

Pscittaciformes [1, 29-32], Cuculiformes [33, 34], Strigiformes [1, 35],

Camprimulgiformes [36, 37], Apodiformes [38, 39], Coliiformes [29, 40], Trogoniformes

[1], Coraciformes [1, 41], Galbuliformes [40, 42], Bucerotiformes [1], Piciformes [40,

42, 43], Passeriformes (*Subocines*) [44, 45], Passeriformes (*Oscines*) [46-83].

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**APPENDIX 3 - BOX-COX TRANSFORMATIONS**

**BOX-COX TRANSFORMATIONS**

We used Box-Cox transformations to improve the normality of our non-normal continuous variables (Box and Cox 1964). This method of transformation provides the best fit of the input data to normality and simplifies back transformation. For this purpose we used the *box.cox.powers* function in the R statistical package 'car' (Fox and Weisberg 2010) to determine the unconditional power transformation ($\lambda_1$) for each non-normal variable. This power transformation ($\lambda_1$) is determined through a maximum likelihood procedure that selects the value that makes the data maximally normal. Box-Cox transformations are only appropriate for non-negative values; therefore we performed linear shifts with a second parameter ($\lambda_2$) when a variable contained a negative value. Therefore, all variables were transformed as follows:

where

In addition to normalizing our data, these parameters allowed for back transformation, which may be useful in interpretation.

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**APPENDIX 4 – SUPPELEMENTARY VIDEO (SEE CD)**

**APPENDIX 5 - SUPPLEMETARY MATERIAL FOR CHAPTER 6**

**Supplementary Materials and Methods**

We recorded the presence and absence of eggshell phosphorescence while measuring eggs in a darkened room using a PX2 light source (Ocean Optics, USA). We only recorded the presence or absence of visually detectable phosphorescence when we were confident in our ability to detect its presence, as certain factors such as the size of the egg impeded our ability to detect phosphorescence (it is easier to detect in large eggs). In cases of uncertainty, neither presence nor absence was recorded. We compiled these data in a taxonomically sorted list along with a general description of egg colour and pigment composition, if known (Table S1).

To determine whether phosphorescence influences egg reflectance spectra, we measured egg reflectance with different combinations of light sources that either included or excluded excitation wavelengths in the UV. We first measured eggs using a DH2000 light source that illuminates across the range from 215 – 2200 nm through the combination of a deuterium bulb and a halogen bulb (Ocean Optics, USA). We then turned off the deuterium light source, recalibrated the spectrometer, and measured the same eggs using only the halogen bulb, which excluded most ultraviolet irradiance and illuminated from 360 – 2000 nm. This appeared to be an appropriate treatment because phosphorescence was not visually detectable when only the halogen bulb illuminated the eggs. Each egg was measured three times, once on the equator, and once at each pole. For a subset of eggs, we took these measurements using two different light conditions: deuterium bulb only (D) that illuminates over the same range as the combined output of the halogen and deuterium lamps (215 – 2200 nm) and can therefore serve as an estimate of measurement error, and a separate pulsed xenon light source that provides illumination

from 220 – 750 nm (PX2, Ocean Optics, USA). It is important to note that this configuration does not allow us to distinguish between luminescence caused by fluorescence or phosphorescence, and thus any measureable effect of luminescence on reflectance could be a combination of both fluorescence and phosphorescence.

Table S1

Presence or absence of visually detectable phosphorescence in avian eggs. Species

exhibiting phosphorescence represent a broad taxonomic range. Generally, eggs that

exhibited phosphorescence were white or brown in coloration. We provide a general

description of colour for each egg (1= white, 2 = white with markings 3 = brown, 4 =

brown with markings 5 = blue-green, 6 = blue-green with markings) and indicate whether

the pigment composition is known and the source of this information († = Kennedy and

Vevers (1976), ‡ = Miksik et al. (1996)). Names follow Clements Checklist of Birds of

the World 6th edition (Clements 2007).

| **Species exhibiting phosphorescence** | | |
|---|---|---|
| Tinamidae | *Crypturellus cinnamomeus* | 3 |
| | *Crypturellus noctivagus* | 3 |
| | *Crypturellus obsoletus* | 3 |
| | *Crypturellus parvirostris* | 3 |
| | *Crypturellus soui* | 3 |
| | *Crypturellus undulatus* | 3 |
| | *Tinamus major* | 5 |
| | *Tinamus osgoodi* | 5 |
| Podicipedidae | *Aechmophorus clarkii* | 1 |
| | *Podiceps auritus* | 1 |
| | *Podiceps grisegena* | 1 |
| | *Podilymbus podiceps* | 1 |
| Procellariidae | *Fulmarus glacialis* | 1† |
| Fregatidae | *Fregata ariel* | 1 |
| | *Fregata magnificens* | 1 |
| | *Fregata minor* | 1 |
| Sulidae | *Morus bassanus* | 1† |
| Pelecanidae | *Pelecanus occidentalis* | 1 |

| | | |
|---|---|---|
| Anhingidae | *Anhinga anhinga* | 1 |
| | *Anhinga melanogaster* | 1 |
| Phalacrocoracidae | *Phalacrocorax auritus* | 5 |
| Ciconiidae | *Mycteria americana* | 1 |
| Phoenicopteridae | *Phoenicopterus ruber* | 1† |
| Threskiornithidae | *Ajaia ajaja* | 2 |
| | *Eudocimus ruber* | 4 |
| | *Eudocimus albus* | 2 |
| Megapodiidae | *Megacephalon maleo* | 1 |
| | *Megapodius cumingii* | 1 |
| | *Megapodius freycinet* | 1 |
| | *Megapodius pritchardii* | 1 |
| | *Megapodius reinwardt* | 1 |
| Anatidae | *Branta leucopsis* | 1† |
| Anhimidae | *Anhima cornuta* | 1 |
| | *Chauna chavaria* | 1 |
| Opisthocomidae | *Opisthocomus hoazin* | 2 |
| Cathartidae | *Coragyps atratus* | 2 |
| Accipitridae | *Sagittarius serpentarius* | 1 |
| | *Circus cyaneus* | 1 |
| | *Elanoides forficatus* | 2 |
| | *Ictinia mississippiensis* | 1 |
| | *Parabuteo unicinctus* | 1 |
| | *Haliaeetus leucocephalus* | 1 |
| Falconidae | *Caracara cheriway* | 2 |
| Pandionidae | *Pandion haliaetus* | 4 |
| Aramidae | *Aramus guarauna* | 4 |
| Cariamidae | *Cariama cristata* | 2 |
| Otidae | *Ottis tarda* | 3 |
| Cracidae | *Ortalis vetula* | 1 |
| Odontophoridae | *Colinus virginianus* | 2 |
| Rallidae | *Gallinula chloropus* | 4† |
| | *Rallus elegans* | 4 |
| Turnicidae | *Turnix sylvatica* | 2† |
| Charadriidae | *Charadrius montanus* | 4 |
| Haematopodidae | *Haematopus bachmani* | 4 |

|  | *Haematopus palliatus* | 4 |
| Alcidae | *Alle alle* | 5 |
| Laridae | *Larus delawarensis* | 4 |
| Rynchopidae | *Rynchops nigra* | 2 |
| Psittacidae | *Psittacus erithacus* | 1 |
| Apodidae | *Apus apus* | 1† |
|  | *Tachymarptis melba* | 1 |
|  | *Chaetura vauxi* | 1 |
| Cuculidae | *Guira guira* | 6 |
| Trogonidae | *Trogon elegans* | 1 |
| Alcedinidae | *Alcedo atthis* | 1 |
|  | *Ceryle alcyon* | 1 |
| Cinclidae | *Cinclus cinclus* | 1† |
| Bucerotidae | *Bucorvus leadbeateri* | 1 |
|  | *Bycanistes bucinator* | 1 |
| Sylviidae | *Sylvia nisoria* | 2 |
| Upupidae | *Upupa epops* | 1 |
| Mimidae | *Toxostoma bendirei* | 2 |
|  | *Toxostoma cinereum* | 2 |
| Oriolidae | *Oriolus oriolus* | 2 |
| Sturnidae | *Acridotheres tristis* | 5 |
|  | *Aplonis vitensis* | 5 |
|  | *Sturnus vulgaris* | 5‡ |
| Fringillidae | *Leucosticte tephrocotis* | 1 |
| **Species lacking phosphorescence** | | |
| Threskiornithidae | *Plegadis chihi* | 5 |
|  | *Plegadis falcinellus* | 5† |
| Turdidae | *Turdus migratorius* | 5† |
| Mimidae | *Toxostoma crissale* | 5 |

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EDUCATION:           The Millbrook School, Millbrook, NY
1996 – 2000, diploma

Skidmore College, Saratoga, NY
2000 – 2002, transfer

Cornell University, Ithaca, NY
2002 – 2004, B.Sc., cum laude
Honours and distinction in research

Bucknell University, Lewsiburg, PA
M.Sc. Biological Sciences

University of Windsor, Windsor, ON
Ph.D Biological Sciences

EXPERIENCE         Volunteer bird bander, Millbrook, NY & Glendobbin, VA
1st North American Canopy banding operation, mist netting, banding, measuring songbirds. All season banding. Preparation of study skins and mounts
1996 – 2002

Lab assistant, Skidmore College
Assistance in running PCRs for a study examining Plasmodium infection in Savannah Sparrow (*Passerculus sandwichensis*)
2002

NSF Research Experience for Undergraduates Intern, University of Virginia. Examining the effect light and canopy stratification on the growth rate of a leaf mining moth (*Cameraria hamadryadella*)
2002

Research Assistant, Cornell Lab of Ornithology
Radio tracking a House Finch (*Carpodacus mexicanus*) population in Ithaca, NY. Banding and bleeding of House

Finches, as well as behavioural observations at Liddell Laboratory
2002 – 2003

Special Species Symposium participant, the College of Veterinary Medicine at Cornell University, Trained in using ultrasound on animal subjects
2003

Graduate level Research Assistant, Iowa State University & Cornell University, Construction of manoeuvrability flight tunnel for video analysis, ultrasound measurements of furculum depth
2003

Curator's Assistant, Cornell University's Museum of Vertebrates, Cataloguing, installation, organized collection for move, preparation: study skins, skeletons, and fluid specimens
2004

Wilderness First Aid, Wilderness Medical Associates, Canada Trained in Wilderness First Aid, Basic Life Support, and Anaphylaxis Emergency Response
2008

Instructional Skills Workshop, CTL University of Windsor
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