WHY ARE PASSERINE EGGSHELLS SPOTTED? USING CALCIUM SUPPLEMENTATION AS A TOOL TO EXPLORE EGGSHELL PIGMENTATION

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by

Kaat Brulez

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ABSTRACT

The eggshells of many avian species are spotted in appearance but the functional significance of such maculation is poorly understood. Protoporphyrin, responsible for brownish-red colouring on eggshells, is postulated to reinforce the structural integrity of eggshells under conditions when dietary calcium (Ca) is scarce. Within the context of this hypothesis, this thesis documents the use of Ca supplementation of two common British passerine species, blue (*Cyanistes caeruleus*) and great tits (*Parus major*), to explore the relationships between eggshell Ca and protoporphyrin content and visible pigment spotting. It further assesses the diversity of avian eggshell coloration using museum eggshells of 73 British passerine species. Despite low soil Ca availability, females were not necessarily Ca-limited but Ca-supplements may still influence eggshell traits and breeding behaviour, possibly by providing females with more time to invest in other activities. The importance of quantifying eggshell pigment concentrations directly, rather than using a proxy, is highlighted. Finally, this thesis shows that passerine eggshell pigment concentrations are highly phylogenetically conserved, thereby encouraging future studies testing key hypotheses to compare eggshell pigmentation of closely related species. This phylogenetic association may be essential to explain the functional significance of eggshell coloration of avian species.

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AUTHOR'S DECLARATIONS

All of the chapters in this thesis were written by Kaat Brulez (KB) with comments and editing from Phill Cassey (PC), Andy G. Gosler (AGG) and S. Jim Reynolds (SJR). Further contributions for each chapter are detailed below.

Chapter Two

KB, PC and SJR conceived and designed the experiment. KB and Simone L. Webber (SLW) performed the fieldwork. KB and Ivan Mikšík (IM) performed the lab work. KB and PC analysed the data. All authors provided editorial advice.

Chapter Three

KB conceived and designed the experiment. KB and SLW performed the fieldwork. KB and IM performed the lab work. KB and Alan Meeson (AM) collected the colour data. KB analysed the data.

Chapter Four

KB, PC and SJR conceived and designed the experiment. KB and SLW performed the fieldwork. KB and IM performed the lab work. KB and AM collected the colour data. AGG visually scored the eggs. KB analysed the data. All authors provided editorial advice. A version of this chapter has been accepted in *The Journal of Avian Biology*.

Chapter Five

KB conceived and designed the experiment. KB and SLW performed the fieldwork. KB and IM performed the lab work. KB analysed the data.

Chapter Six

KB and PC conceived and designed the experiment. KB and IM performed the lab work. Steven J. Portugal (SJP) and Golo Maurer (GM) collected the species-specific data. GM photographed the eggs. P. George Lovell (PGL) collected the colour data. KB, PC and Chris Cooney (CC) analysed the data.

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CHAPTER 1

GENERAL INTRODUCTION

"I think if required on pain of death to name instantly the most perfect thing in the universe, I should risk my fate on a bird's egg"

(Higginson 1863)

1.1 The avian eggshell

The fundamental biological function of avian eggshells is to provide an all-encompassing incubation environment in which an embryo can develop (Roberts and Brackpool 1994). The eggshell acts as protection for the developing embryo and to exclude most bacteria. It must allow for adequate gaseous movement, including water vapour, and for the chick to release itself successfully from the shell once it is ready to hatch (Roberts and Brackpool 1994).

The avian eggshell is a protein matrix lined with mineral crystals, usually of a calcium (Ca) compound such as $CaCO_3$ (Ca carbonate) or $CaPO_4$ (apatite) (Weiner and Addadi 1991). Within 3 hours of its release from the ovary, the ovum, encapsulated in albumen and an immature membrane, reaches and remains in the shell gland pouch for approximately 20 hours, during which biomineralisation takes place (Board and Sparks 1991). The various layers of the shell are formed sequentially as the 'plumped' egg rotates within the shell (Lavelin et al. 2000). The foundation of the eggshell is provided by the inner and the outer shell membranes. At the onset of eggshell formation, mammillary knobs form on the outer membrane, which then develop into the main (palisade) layer. Between the palisade columns, narrow pores traverse the eggshell (Fig. 1.1), allowing for gaseous exchange (Hunton 2005). The shell is completed by the formation of a thin outer layer known as the cuticle.

Ca is the most prevalent mineral in the body. Ca homeostasis in birds is controlled by parathyroid hormone, calcitonin, vitamin D3 and sex hormones (reviewed in de Matos, 2008). The Ca necessary for shell formation is derived entirely from the blood (Simkiss and Taylor 1971). The increased demand for Ca during eggshell formation is met through increased intestinal absorption and resorption of Ca from the medullary bone (Klasing 1998). The medullary bone develops in egg-laying females in response to gonadal steroids and acts as a Ca storage-chamber required for eggshell formation (Dacke 2000). In the domestic chicken

(*Gallus gallus*), Ca from the medullary bone can donate as much as 40% of the Ca required for eggshell formation (Dacke et al. 1993), however in smaller birds, these endogenous Ca stores are insufficient to meet Ca demands required for eggshell formation (Pahl et al. 1997).

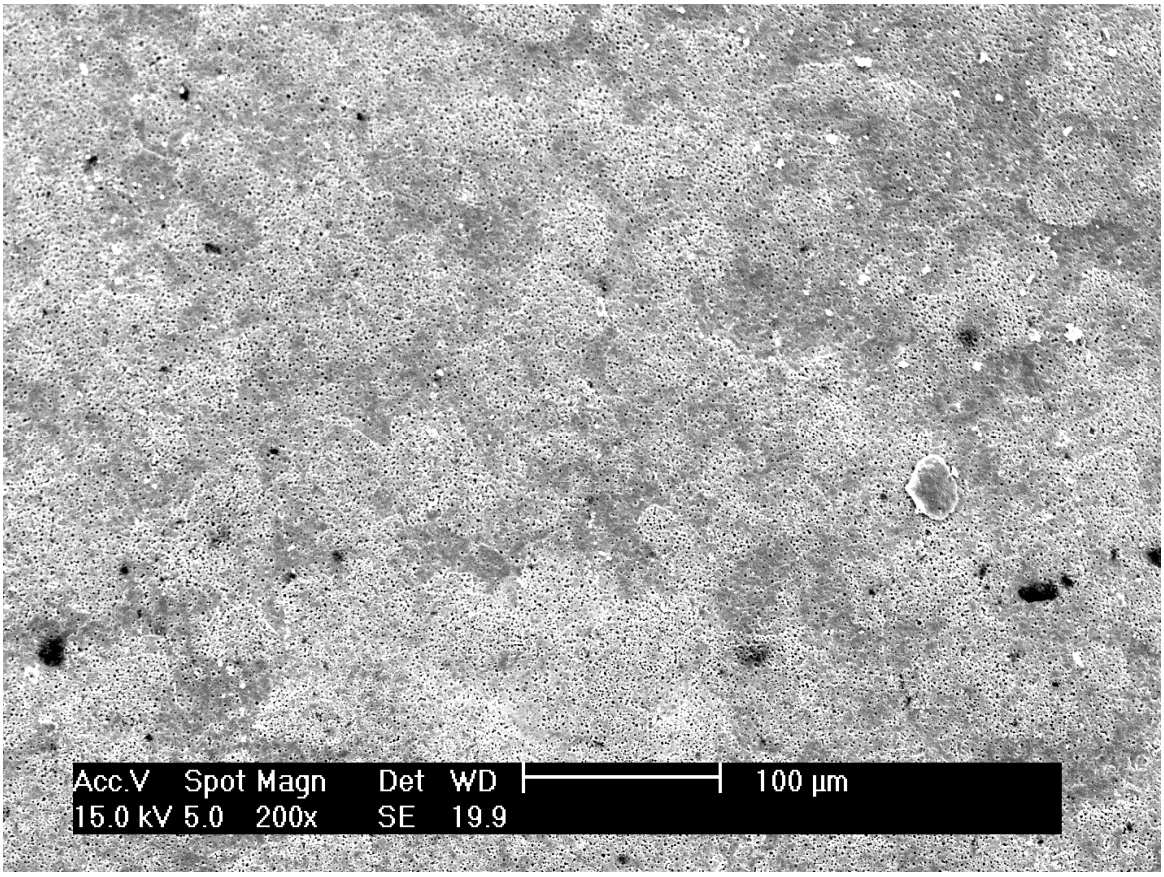


Figure 1.1. Photomicrograph (200×) of a great tit eggshell showing pores traversing the cuticular layer that allow gaseous exchange. (Photo: G. Maurer, University of Birmingham).

Eggshell pigments are deposited during the later stages of eggshell formation and therefore occur in the calcite and cuticle layers of the shell (Poole 1965). Pigments transfer to the eggshell via the surface epithelial cells while in the shell gland (Baird et al. 1975). There are two main types of pigments responsible for the coloration and patterning on eggshells. These are protoporphyrin IX, responsible for brownish hues, and biliverdin (IXα and zinc chelate), responsible for blue and green hues (Kennedy and Vevers 1973, 1976, Gorchein et

al. 2009). Both protoporphyrin and biliverdin are believed to be derived from a common precursor molecule which is likely to be haem (Wang et al. 2009) and are produced during the biosynthesis of blood. A survey examining the appearance and pigment content of eggshells of 108 avian species found that nearly half of them had eggshells containing only protoporphyrin with just over a third containing both protoporphyrin and biliverdin IXα (Kennedy and Vevers 1976). The eggshells of blue tits (*Cyanistes caeruleus*), the only Paridae species included in this survey, were found to contain only protoporphyrin. Another comparative study found that in eggs which had pigment present as a pattern, pigment was either restricted to the shell surface or found in various different layers of the eggshell (Harrison 1966). The majority (80-87%) of protoporphyrin is located within the calcarous layers of the eggshell, whilst a minority (13-20%) is located within the cuticle (Samiullah and Roberts 2013). Limited amounts of protoporphyrin deposited into the cuticle results in distinct spots, whereas large quantities of protoporphyrin deposited in the latter stages of shell formation result in large blotches or streaks (Solomon 1987). Most of the available information on eggshell formation comes from research into domestic fowl species such as chickens, however limited information suggests that smaller passerines show similar processes (e.g. Reynolds 2001). Surprisingly little is known about how eggshell pigments are synthesized, mobilized and deposited and how the presence of these pigments can affect eggshell Ca.

1.2 A synthesis of hypotheses for eggshell coloration

The great diversity of avian eggshell pigmentation and its possible adaptive significance has fascinated biologists for a long time. Poulton (1890) wrote "…any description of colour and

marking [of eggs] will be considered incomplete unless supplemented by an account of meaning and importance in the life of the species". Ancestrally, avian eggshells were most likely uniformly white (Kilner 2006). This trait has been retained by some species whose nests are secure from predators, while others with more vulnerable nests have developed darker and more patterned eggs. It is believed that these species may have evolved alternative functions for these adaptations, such as solar radiation or eggshell strengthening (Kilner 2006). The functional significance of eggshell coloration has acquired a range of hypotheses (for full reviews see Underwood and Sealy 2002, Kilner 2006, Reynolds et al. 2009, Maurer et al. 2011a). The main hypotheses are for crypsis to avoid predation (e.g. Götmark 1993) and brood parasitism (e.g. Davies and Brooke 1989a), thermoregulation (e.g. Bakken et al. 1978), and the recently introduced sexual-selection for the evolution of eggshell coloration (SSEC) hypothesis, which proposes that pigmentation is based upon females signalling their good body condition and thereby inducing higher investment by males in breeding attempts (Moreno and Osorno 2003; but see Reynolds et al. 2009). Finally, the structural-function hypothesis (Gosler et al. 2005) proposes that protoporphyrin pigmentation may increase shell strength by acting as a shock absorber (Solomon 1991). These hypotheses and how they relate to the eggs of blue and great tits (*Parus major*) are explained in more detail below.

1.2.1 Aposematism

Warning coloration is commonly used by animals to signal the unprofitability of a prey item to any potential predators, bright or conspicuous colours often being associated with toxic substances (Gittleman and Harvey 1980). The theory of colourful eggs being aposematic was based on two main studies. In the first the palatability of eggs was tested on three different predators as well as human (*Homo sapien*) subjects, and then compared to shell colour and

pigmentation (Swynnerton 1916). Egg palatability was not obviously correlated with eggshell coloration. A similar study using humans as egg predators (Cott 1948) found that cryptic eggs were more palatable but this study has more recently been discredited due to the subjective evaluation of egg crypsis (Lack 1958, Kilner 2006).

1.2.2 Thermoregulation and gas conductance

Incubation must occur under suitable physical conditions, such as nest temperature and water vapour pressures (Lundy 1969, Webb 1987), for avian embryos to develop and hatch successfully (Drent 1975, Deeming et al. 1987, Webb 1987). Prior to incubation, eggs should neither be too cold to kill the embryo nor too hot to initiate incubation prematurely (Rahn et al. 1977). During incubation, eggs vary in core temperature between 34° and 38°C with temperatures above 40°C placing embryos at risk of mortality from heat stress (Bennett and Dawson 1979, Burley and Vadhera 1989). Light-coloured eggs may reflect sunlight and protect eggs from over-heating. More than half of the sunlight that falls on eggshells is in the near-infra-red portion of the spectrum. Both protoporphyrin and biliverdin-pigmented eggs reflect more than 90% of light in the near-infra-red (Fig. 1.2), minimizing heating of the egg by the sun (Bakken et al. 1978). As both of these pigments have low absorbance in this near-infra-red range of wavelengths, they are unlikely to influence heat gain differentially (Bakken et al. 1978).

By reflecting incident sunlight, lightly pigmented eggs could protect embryos from hyperthermia when adults are away from the nest. Studies using artificially coloured eggs suggest that light-coloured eggs acquire heat more slowly than darker eggs (Montevecchi 1976, Bertram and Burger 1981). Naturally pigmented eggs exposed to full sunlight acquired

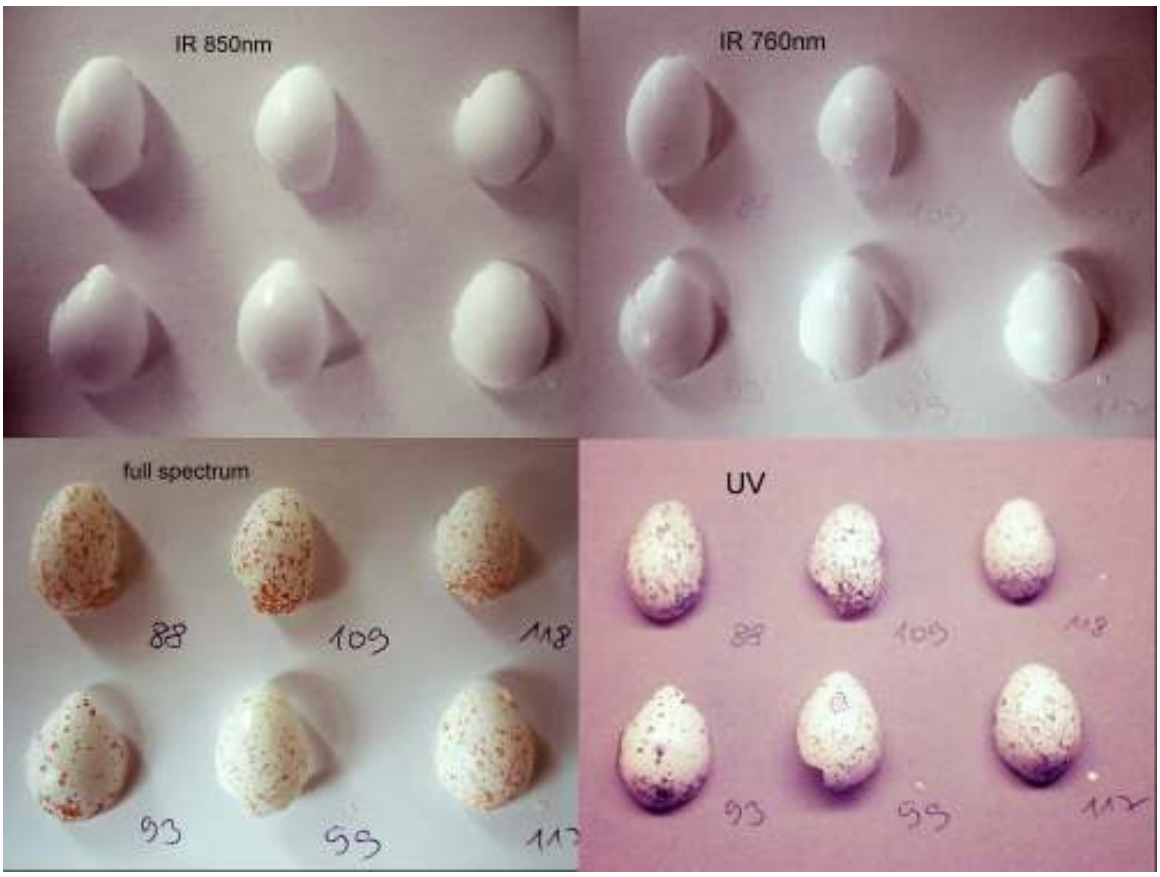


Figure 1.2. Eggshells of different great tit (numbered 88, 93, 99, 109 and 117) and blue tit (numbered 118) females photographed under infra-red spectra (R), ultra-violet (UV), and under the full spectrum of light. Protoporphyrin pigment spots are visible in the latter two only. (Photo: I. Mikšík, Academy of Sciences of the Czech Republic).

heat more rapidly than eggs in the shade but heat gain did not vary with eggshell colour in either environment (Westmoreland et al. 2007). The authors concluded that differences in reflectivity of eggshell pigments in the visible range (400-700 nm) do not result in different rates of heat acquisition, and therefore did not support the thermoregulation hypothesis. The use of artificially coloured eggs to study thermoregulation can be problematic as these artificial pigments (e.g. paint) likely do not exhibit the same thermal properties to natural pigments.

During incubation, the typical egg loses 18% of its initial mass (Rahn and Ar 1974) due to water loss, which occurs by diffusion of water vapour across the eggshell (Wangensteen and Rahn 1971). Total rate of water loss is determined both by intrinsic properties of the egg (e.g. eggshell thickness, egg size) and micro-environmental factors (e.g. temperature and ventilation of incubating female) (Rahn and Ar 1974). Protoporphyrin reflects strongly in the infra-red (Bakken et al. 1978; Fig. 1.2), potentially creating 'cold spots' on the eggshell and reducing permeability of the eggshell (Higham and Gosler 2006, Maurer et al. 2011b).

1.2.3 Crypsis

It has long been known that birds nesting in cavities tend to lay white eggs (Newton 1893), leading naturalists to believe ancestral eggs were white and that all other forms of egg colour and patterning are adaptations to specific micro-environments, functioning to conceal eggs from predators (Wallace 1889). A study comparing nest structure and egg coloration of 27 non-passerine families showed that egg coloration in most open-nesting species is not sufficiently cryptic to be explained by camouflage (Götmark 1993). Amongst the Turdidae family, nest site of species explains some of the variation in colour and patterning on its eggshells. Hole-nesting species were more likely to lay immaculate white eggs while 80% of species which had exposed nests laid eggs covered in red or brown speckling (Lack 1958).

Many studies, through the use of artificially pigmented eggshells, have found no significant difference in predation rates between conspicuous eggs and those mimicking the natural appearance (e.g. Tinbergen et al. 1962, Montevecchi 1976; but see Underwood and Sealy 2002). However, studies using naturally pigmented eggs and nests have found that egg

coloration has a significant effect on predation rates and hence on nestling survival (Westmoreland and Kiltie 2007, Westmoreland 2008). In the South American tern (*Sterna hirundinacea*), a species which lays eggs with a large variation in background colour, green eggs were depredated less than other colour variations in areas where only mammalian predators were present. However, in areas where only avian species were present, rate of artificial nest predation was higher for eggs more conspicuous to the human eye than for eggs apparently resembling the nest substrate (Blanco and Bertellotti 2002). In Japanese quail (*Coturnix japonica*), females consistently selected laying substrates which maximised camouflage with the degree of maculation of their eggs (Lovell et al. 2013). This demonstrates that the selection for crypsis under fluctuating environmental conditions (e.g. variation in predatory behaviour, variation in choice of background substrate for breeding site) may be the main evolutionary force driving variation in eggshell coloration (Blanco and Bertellotti 2002).

1.2.4 Egg recognition/brood parasitism

Many species of birds have evolved within-clutch uniformity as well as individual distinctiveness in egg colour and spotting, a combination that facilitates identification of an individual's own eggs from those of a conspecific or a brood parasite (Baker 1913, Davies and Brooke 1989b). Eggshell patterning is genetically female sex-linked and subsequently inherited from mother to daughter (Gosler et al. 2000), thereby allowing for the evolution of individual-specific eggshell patterning. The ability to recognise an individual's own eggs has been shown in some studies (e.g. Pike 2011) but not in others (e.g. Cassey et al. 2009). Species breeding in colonies are able to distinguish conspecifics' foreign eggs from their own

(e.g. Bartholomew and Howell 1964, Buckley and Buckley 1972, Schaffner 1990). Female American coots (*Fulica americana*) combine egg recognition and counting to make clutch size decisions, thereby avoiding not only costs of incubating parasitic eggs but also those of wrongly discarding their own eggs (Lyon 2003).

Many species which are potential victims of brood parasites protect themselves through having evolved the ability to recognise and reject odd-looking eggs added to their clutch (Davies 2000). Brood parasites may lay eggs in conspecifics' nests (Yom-Tov 2001) or in those of heterospecifics, thereby transferring the costs of parental care to their chosen hosts (Rothstein 1990, Davies 2000). The cost of parasitism started an evolutionary arms race between parasite and host, in which the host evolves mechanisms to avoid being parasitized (e.g. egg recognition and within-clutch uniformity of egg appearance), and the parasite evolves the ability to exploit these newly-evolved strategies (Davies and Brooke 1989b, Davies 2000). Although a crypsis hypothesis could explain the evolution of complex eggshell colouring and patterning, existing evidence suggests otherwise. Eggs of pied wagtails (*Motacilla alba*) and meadow pipits (*Anthus pratensis*), taken from a population known to be parasitized by common cuckoos (*Cuculus canorus*), were equally spotty compared with those eggs taken from an un-parasitized population (Davies and Brooke 1989b). It has been suggested that hosts may use alternative strategies to avoid being parasitized such as the selection for odd-looking last eggs laid within the same clutch (Yom-Tov 1980), increased nest vigilance (Neudorf and Sealy 1994) and host aggression towards potential brood parasites (Robertson and Norman 1976).

1.2.5 The SSEC hypothesis

The SSEC hypothesis proposes that egg colour (with an emphasis on blue-green coloured eggs) acts as a sexually selected trait in females to display their genetic and phenotypic qualities to males as a post-mating selection mechanism (Moreno and Osorno 2003). Female ornaments have been documented to be important in male mate choice and paternal care (Amundsen 2000, Hill 2002). Moreno and Osorno (2003) argued that due to the costly deposition of biliverdin in eggshells, a female's capacity to control free radicals during an exceptionally stressful phase (e.g. due to food restriction) may act as an honest signal (Zahavi 1975). Subsequently, males cue on this signal to assess a female's body condition and genetic quality, and provide paternal care accordingly.

Relationships have been found between blue-green coloration (BGC) and female body condition at laying (Moreno et al. 2006a), immunocompetence during the nestling period (Moreno et al. 2005), and plasma antioxidant levels (Hanley et al. 2008). Other studies have found relationships between eggshell colour and nestling health and body condition (e.g. Morales et al. 2006, López-Rull et al. 2008, Soler et al. 2008; but see Stoddard et al. 2012). However, experimental support for a robust association between eggshell coloration and male provisioning effort is mixed (reviewed in Reynolds et al. 2009, Riehl 2011). Increased BGC has been linked to increased male parental care in some studies (e.g. Moreno et al. 2004, 2006b) but not in others (e.g. Krist and Grim 2007). Furthermore, distinguishing between egg and parental quality can be challenging and must not be neglected when interpreting the results of the afore-mentioned studies researching the association between eggshell colour and nestling health.

The SSEC hypothesis could be extended to include protoporphyrin-pigmented eggs because the accumulation of protoporphyrin within the liver can cause oxidative stress

(Afonso et al. 1999). The deposition of large amounts of protoporphyrin onto the eggshell may indicate a female's ability to cope with intense oxidative stress, or alternatively, may indicate that protoporphyrins have been successfully removed (Moreno and Osorno 2003, Holveck et al. 2010). Increased protoporphyrin deposition is found in females in lower body condition in some species (e.g. Japanese quail – Duval et al. 2013), but not in others (e.g. reed warbler [*Acrocephalus scirpaceus*] – Krištofík et al. 2013), and has not yet been related to an increase in male parental care (e.g. northern lapwing [*Vanellus vanellus*] – Bulla et al. 2012).

1.2.6 Structural-function hypothesis

The molecular structure of porphyrin, the pre-cursor to protoporphyrin, is similar to that of phthalocyanines which are used in solid-state engineering as lubricants, suggesting that protoporphyrin pigmentation may increase shell strength by acting as a shock absorber (Solomon 1991). This led to the structural-function hypothesis proposing that protoporphyrin is deposited onto the eggshell for structural strengthening when exogenous Ca is scarce (Gosler et al. 2005). Pigment spots have greater fracture toughness than un-pigmented shell, compensating for reduced eggshell thickness and increasing shell strength (Gosler et al. 2011).

In great tits, protoporphyrin-pigmented spots have been found to demarcate thinner areas of the shell, with darker spots covering thinner areas than paler spots (Gosler et al. 2005), and eggshell Ca content strongly positively related to local soil Ca content (Gosler et al. 2005). Similar results were found in the Eurasian sparrowhawk (*Accipiter nisus*), breeding in areas contaminated with DDT, a pollutant which blocks Ca uptake by the shell gland. Shell thinning was positively correlated with increasing DDT concentrations and to a greater extent with (internalised) pigmented spots (Jagannath et al. 2008).

1.2.7 Alternative hypotheses

Additional hypotheses include the blackmail hypothesis of Hanley et al. (2010), which proposes that sexual conflict load (Houston et al. 2005) may be imposed onto males if females lay conspicuous eggs, forcing males to increase paternal care. The anaemia hypothesis of De Coster et al. (2012) proposes that as protoporphyrin is derived from blood, an increase in anaemia would result in eggshells with reduced protoporphyrin pigmentation. Moreover, it has further been suggested that pigment spots may act as a defence against bacteria. Protoporphyrin, if activated by light, can reduce bacterial survival on eggshells (Ishikawa et al. 2010), large quantities of which can increase risk of mortality to the developing embryo (Cook et al. 2003, 2005).

1.2.8 Gaps in our knowledge

Although these hypotheses have received much attention in the past, surprisingly little is known about eggshell formation, especially in species not related to the poultry industry. In particular, the processes of how eggshell pigments are synthesized, mobilized and deposited and how the presence of these pigments can directly and/or indirectly affect eggshell Ca and other eggshell traits (i.e. shell thickness) are largely unknown.

1.3 Hypotheses explaining pigmentation of great tit and blue tit eggs

Maculated eggs are represented in all of the 22 passerine families of the Holarctic (Sibley and Monroe 1990). Eggs of great tits and blue tits have a dominant white background covered with protoporphyrin pigment spots (Fig. 1.3). Across the class Aves hole-nesting species tend

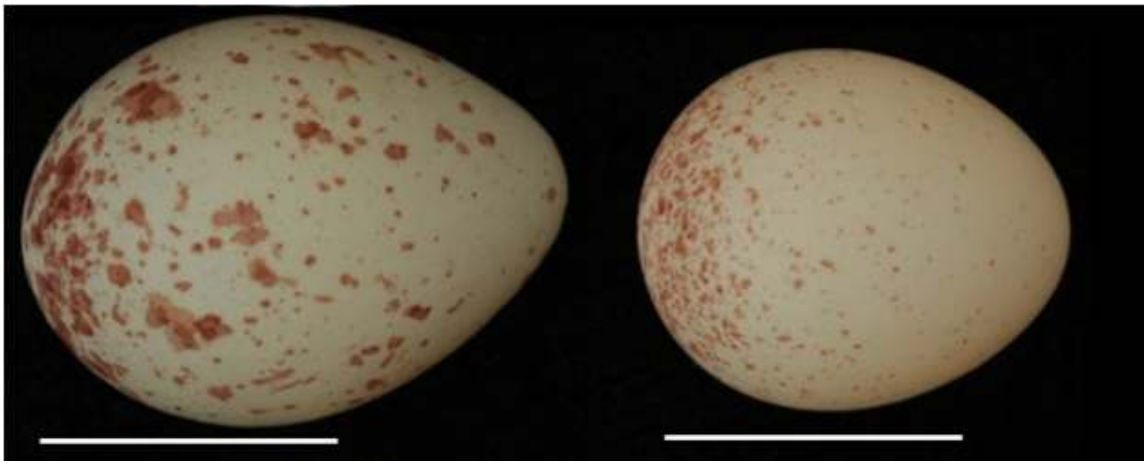


Figure 1.3. Example of typical eggs of a great tit (left) and a blue tit (right). Eggs of both species have been shown to reflect highly in the infra-red and protoporphyrin pigment spots can be seen in the ultra-violet and full spectra only (scale bar = 1 cm). (Photo: K. Brulez).

to lay un-pigmented eggs more often than open-nesting species (Lack 1968). It is unlikely that hole-nesting species have evolved eggshell coloration for the purpose of crypsis or aposematism as these species experience lower probability of predation (Martin 1995, Bennett and Owens 2002), although eggs of these two species may be spotted due to their evolutionary history.

Whilst the eggs of cavity-nesting species are not susceptible to over-heating caused by direct sunlight, they are sensitive to changes in heat and humidity within the nest-cavity. In cross-fostered great tit eggs, increased spotting resulted in an increased rate of water loss (Higham and Gosler 2006), whilst in blue tits, increased spotting resulted in shorter incubation periods (Sanz and García-Navas 2009), suggesting that pigmentation affects thermoregulatory properties of the eggshell. Whether this is an evolved effect or a secondary effect caused by reduced thickness of pigmented shell remains unclear.

Conspecific brood parasitism, whereby females lay eggs in the nests of other conspecifics (Yom-Tov 2001), occurs in over 200 species of birds (Lyon and Eadie 2008).

Eggs of both tit species vary greatly in their extent of spotting and spotting pattern between clutches, but not within clutches (Gosler et al. 2005). Evidence suggests that neither intraspecific brood parasitism, nor the ability to reject parasitic eggs, occurs in either blue or great tits (Kempenaers et al. 1995). However, in populations with high breeding densities, conspecific brood parasitism has been reported to occur in blue tits (Vedder et al. 2007; but see Griffith et al. 2009). The latter study contests the assumption that differently pigmented eggs are laid by a different female without the use of alternative methods (i.e. molecular genetic analysis). Parasitic eggs did not differ systematically in size or in quantities of yolk testosterone compared with host eggs, suggesting that a shortage of suitable nesting sites caused some females to use conspecific brood parasitism as a best-of-a-bad-job strategy (Vedder et al. 2007). Eggs are successfully incubated and hatched, and chicks fledge from nests of females of closely-related species (Slagsvold 1998), suggesting that there is the potential for conspecific brood parasitism to have evolved in parids.

A relationship between eggshell spottiness (i.e. protoporphyrin content) and female health and body condition has been suggested. In blue tits, females laying more spotted eggs were in lower body condition, with higher concentrations of stress proteins and lower concentrations of immunoglobulins (Martínez-de la Puente et al. 2007). Eggs with larger and less evenly distributed spots contained higher concentrations of antibodies, suggesting that eggshell pigmentation may reflect maternal investment of immune compounds into egg yolks (Holveck et al. 2012). In great tits, heavier females laid less maculated eggs, but neither male provisioning nor nestling growth rate was related to eggshell maculation (Stoddard et al. 2012). Furthermore, larger females and those with better constitutive innate immunity laid eggs with ‘darker’ pigment spots (De Coster et al. 2013). However, the ability of eggshell coloration to signal female body condition reliably to other birds has been brought into

question, especially as eggshell coloration may not be visible to cavity-nesting passerines (Reynolds et al. 2009, Holveck et al. 2010).

The 'structural-function' hypothesis may be the most applicable hypothesis to account for the pigmentation of the eggs of blue and great tits. Ca is a limiting resource for eggshell formation in breeding passerines (Reynolds and Perrins 2010). Total Ca content of a blue tit clutch constitutes 130% of the female's entire skeletal Ca content (Perrins and Birkhead 1983). For insectivorous and granivorous birds, their routine diet does not provide more than 25% of the Ca required for eggshell formation. Therefore, additional Ca-rich foods must be consumed (Graveland and van Gijzen 1994). Unlike larger birds that can store Ca in their skeletons (Larison et al. 2001; but see Reynolds 2003), small passerines must collect Ca daily during the egg-laying period to obtain sufficient resources for egg formation (Pahl et al. 1997, Reynolds 1997, 2003). The structural-function hypothesis was conjectured to explain the spotting on eggs of great tits, and has been tested on other species such as blue tits (e.g. García-Navas et al. 2011), and European pied flycatchers (*Ficedula hypoleuca*) (e.g. Tilgar et al. 1999).

1.4 Thesis objectives and structure

The primary aim of this thesis was to contribute to the existing knowledge that attempts to explain a functional and ecological role for eggshell coloration. It focuses on the structural-function hypothesis, as this seems most likely to apply to hole-nesting species such as blue tits and great tits. Within the context of this hypothesis, the thesis documents the use of Ca supplementation to explore the relationships between eggshell thickness and Ca content, protoporphyrin concentration and visible pigment spotting.

This study focuses on two closely related parid species: the blue tit which is a specialised arboreal forager (Slagsvold and Wiebe 2007), and the great tit which is a more generalist forager (Betts 1955, Gosler and Clement 2007). Eggs of these two species are expected to show similar relationships between eggshell traits, despite their different foraging techniques, due to the similarities in visible pigmentation. There are many benefits of studying these two species: both willingly breed in nestboxes (Perrins 1979), providing large sample sizes, and enabling researchers to record detailed data on life-history and breeding ecology traits; both exploit novel food sources (Soper 2006) and thus can be readily food-supplemented; both are determined breeders, readily replacing eggs removed from clutches (e.g. Oppliger et al. 1996) and unlikely to abandon breeding attempts in response to experimental manipulations; and both are short-lived enabling life-history traits to be studied over a short time-frame.

The fieldwork was conducted between 2010 and 2012 at Chaddesley Woods National Nature Reserve (NNR), Worcs., UK. Ca supplements were provided to the two species within the reserve that was divided into three distinct areas. One area was Ca-supplemented and one acted as the control (except for 2012 in which two areas acted as the control). Supplementation was rotated between feeding areas between years in an attempt to control for subtleties in habitat (including natural Ca availability) across the reserve. Two types of Ca supplements (chicken [*Gallus domesticus*] eggshell fragments and oystershell grit) were provided in 2010, but were changed to a single Ca supplement (i.e. oystershell grit) thereafter. Two eggshells were removed from a subset of clutches of both species in each of the three years (2010−2012 inclusive). Eggs collected in 2009 as part of another study, although not Ca-supplemented, were used in this thesis to investigate within-female repeatability of egg traits.

This thesis is structured as follows. **Chapter Two** uses Ca supplementation to study the interactions between eggshell characteristics (thickness, Ca and protoporphyrin concentration) focusing on the predictions outlined by the structural-function hypothesis (Gosler et al. 2005); **Chapter Three,** using data from three consecutive years, investigates the relationship between dietary Ca availability (natural and supplemented), eggshell maculation and other eggshell traits, and other breeding biology traits; **Chapter Four** considers whether protoporphyrin pigment spots can be used as a proxy for the total protoporphyrin content of eggshells by comparing two different eggshell spot scoring methods with measures of protoporphyrin concentration in eggshells from chemical analysis; **Chapter Five** uses Ca supplementation to study the flexibility of females in their expression of eggshell traits by examining within-female repeatability of eggshell traits under Ca supplementation and their heritability down the female line. We further look at whether these eggshell traits are heritable along the female line; **Chapter Six** investigates how pigment concentrations and colour diversity co-vary with phylogenetic affiliations among British passerine species, and how these vary with life-history and breeding ecology traits; and **Chapter Seven** discusses findings within the context of the functional significance of eggshell coloration, providing directions for future research.

CHAPTER 2

CALCIUM SUPPLEMENTATION DOES NOT INFLUENCE EGGSHELL PIGMENTATION IN CAVITY-NESTING TITS

2.1 Abstract

During egg production females of many species of small passerine birds shift their dietary preferences to include foods rich in Ca, a nutrient important in determining eggshell strength. Protoporphyrin, the main pigment that constitutes maculation of the eggshell of many avian species, is postulated to reinforce the structural integrity of eggshells under conditions when dietary Ca is scarce (as described by the structural-function hypothesis). Here, we used Ca supplementation to test the relationships between the thickness of the eggshell, its percentage Ca and protoporphyrin concentration in both great tits and blue tits. Ca-supplemented female great tits, but not blue tits, laid eggs with thicker shells and with higher Ca concentration than non-supplemented (control) females. Pigment spots occurred in thinner areas of the eggshell laid by control females but this was not the case with supplemented birds. In blue tits, pigmented eggshell was thinner than adjacent un-pigmented eggshell regardless of whether birds were supplemented. In both species, no significant relationship was found between the protoporphyrin and Ca concentrations of eggshells, although larger eggs laid by blue tits contained greater quantities of protoporphyrin relative to their size. Our results do not fully support the predictions outlined by the structural-function hypothesis, but indicate that protoporphyrin pigmentation may be consistently associated with thinner regions of eggshell. It may play an important structural role when natural dietary Ca is limited, but when Ca is abundant pigment spots may remain integral to the eggshell, possibly fulfilling alternative functions.

2.2 Introduction

The eggshells of many avian species are spotted in appearance but the functional significance of such maculation remains poorly understood (Wallace 1889, Kilner 2006). There are two key pigments believed to be responsible for the colouring and patterning of avian eggshells. These are protoporphyrin IX, responsible for brownish-red hues, and biliverdin, responsible for blue-green hues (Kennedy and Vevers 1976, Gorchein et al. 2009). Protoporphyrin, produced during the biosynthesis of blood haem (Burley and Vadhera 1989), occurs in both the calcite and cuticular layers of the eggshell (Roberts 2004), and is often localized as spots, either in distinct layers within or upon the eggshell (Kennedy and Vevers 1976). Internalized protoporphyrin spotting has been regarded as evidence for its structural function as opposed to one of external signalling or crypsis (reviewed in Cherry and Gosler 2010, Maurer et al. 2011a), as it is not necessarily visible from the outer surface. Solomon (1991) suggested that due to the similarities of the molecular structure of protoporphyrins to lubricants used in solid-state engineering, protoporphyrin deposition may increase eggshell strength by acting as a shock absorber within the eggshell matrix. This led to the structural-function hypothesis (Gosler et al. 2005), in which it is proposed that protoporphyrin is deposited into the eggshell for strengthening purposes when dietary Ca is scarce. The hypothesis was framed upon the great tit population at Wytham Woods, Oxfordshire, UK (Gosler et al. 2000), but has been extended to other species with mixed success (e.g. blue tits – Sanz and García-Navas 2009, black-headed gull [*Larus ridibundus*] – Maurer et al. 2011b, northern lapwing – Bulla et al. 2012). Although Ca supplementation has been applied to test the effects on eggshell spotting (e.g. García-Navas et al. 2011, Mägi et al. 2012), it has yet to be established whether it results in increased eggshell Ca concentration with a concomitant decrease in protoporphyrin concentration. If protoporphyrin pigmentation is important for structural strengthening of the

eggshell when dietary Ca is scarce, a negative relationship between eggshell Ca and protoporphyrin concentrations would be expected. To the best of our knowledge this relationship has not been tested. Investigating total protoporphyrin content rather than the position of pigment spots may be more effective in establishing the strengthening properties of protoporphyrin.

Unlike larger birds that can store Ca in their skeletons (domestic chicken – Simkiss 1967, white-tailed ptarmigan [*Lagopus leucurus*] – Larison et al. 2001; but see Reynolds 2003), small passerines must collect Ca daily during the egg-laying period to obtain sufficient resources for eggshell formation (Pahl et al. 1997, Reynolds 1997). Over 90% of Ca-rich foods, including snail shells and calcareous grit (Perrins 1996), are consumed immediately prior to, or during, egg-laying (Graveland and Berends 1997). This Ca-specific foraging behaviour has been observed in a variety of species (reviewed by Reynolds and Perrins 2010). In great tits, for example, eggshell mineral (predominantly Ca) content is strongly influenced by soil Ca content, with eggshells having a higher mineral content when females were nesting in areas where soils had higher Ca contents (Gosler et al. 2005).

Eggshell strength is determined by a combination of shell thickness ($CaCO_3$ content) and eggshell matrix organization (Tyler 1969, Ar et al. 1979), and is important in minimising damage to the integrity of the eggshell, as this could likely cause embryonic dehydration and death (Rahn and Ar 1980). Ca-supplemented female blue tits laid eggs with thicker eggshells and with more widely distributed pigmented spots, but there was no effect on spot size or intensity (García-Navas et al. 2011). Protoporphyrin pigmented spots have been found in areas of the eggshell where structural strengthening is more important (i.e. where eggs are thinner) (Gosler et al. 2005).

Here, we investigate the effects of Ca supplementation on eggshell thickness, Ca and protoporphyrin concentrations of eggshells of great tits and blue tits, focusing on the predictions arising from the structural-function hypothesis of Gosler et al. (2005). We compared eggshells laid by Ca-supplemented females to those of un-supplemented (control) females. According to the structural-function hypothesis, we predicted that supplemented females of both species would lay eggs with shells that were (1) thicker, (2) higher in mineral (i.e. Ca) content, and (3) with pigmented areas of the eggshells that would be thinner than non-pigmented areas. In addition, (4) we quantified protoporphyrin content of eggshells and predicted from the structural-function hypothesis that it would be negatively related to both Ca concentration and to eggshell thickness.

2.3 Materials and methods

2.3.1 Study site

This study was conducted in the 2010 breeding season at Chaddesley Woods NNR, a 101-hectare mixed woodland in Worcestershire (UK Ordnance Survey Grid Reference: SO914736, 52°36'N, 2°14'W, Fig. 2.1a), UK. The woodland containing this study area consisted of predominantly ancient tree species (e.g. oak [*Quercus* spp.] and ash [*Fraxinus excelsior*]), intermixed with some planted species (e.g. Scots pine [*Pinus sylvestris*] and European larch [*Larix decidua*]). The study area consisted of two treatment blocks (Ca and control), each of which contained 96 nestboxes, positioned on a 40 m × 40 m grid (Fig. 2.1b).

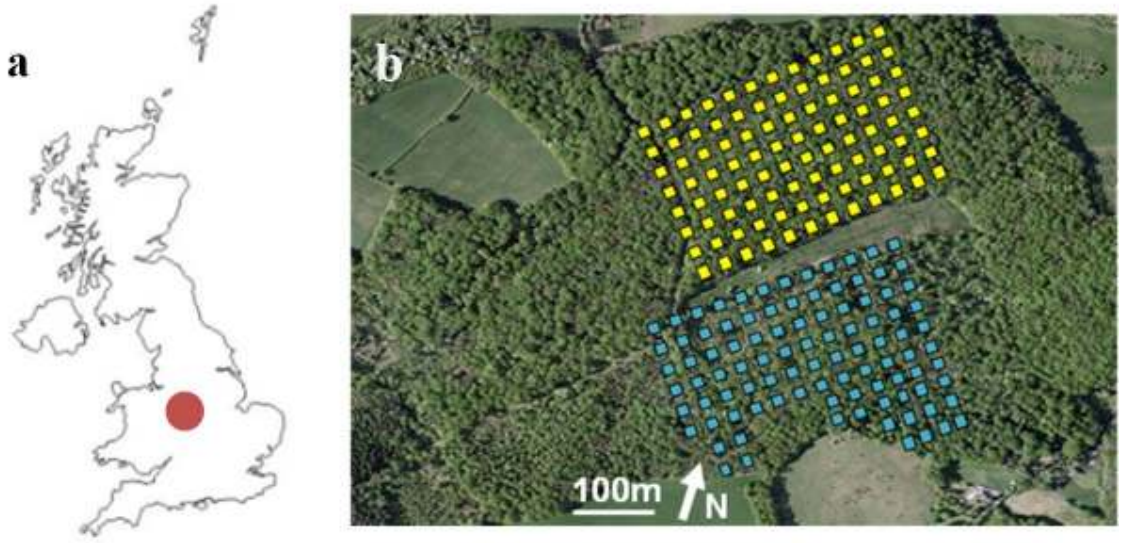


Figure 2.1. (a) The location of Chaddesley Woods NNR, Worcs., UK. (b) Schematic diagram showing the nestbox arrangements in the two treatment blocks (blue: supplemented; yellow: controls) at Chaddesley Woods NNR. (Reproduced from Webber 2012).

Wooden nestboxes (Fig. 2.2) were mounted on tree trunks approximately 2 m off the ground, and each had a 32 mm entrance hole facing NE, away from the prevailing SW winds

Figure 2.2. Wooden nestbox with Ca supplements placed in feed trays on either side. In this photo, chicken eggshell fragments are placed to the left and oystershell grit to the right of the nestbox (see text for further details). (Photo: K. Brulez).

(see Harrison et al. 2010 for more details). Both great tits and blue tits are territorial from January, becoming increasingly so as the breeding season approaches (Gosler and Clement 2007). Therefore, access to supplementary Ca provided at nestboxes was likely to be substantially lower for control females than for Ca-supplemented females.

2.3.2 Field methods

Nestboxes were checked every 3-5 days for signs of nest building and then checked daily from the half-nest stage onwards (after Smith et al. 2013). At the first signs of nest lining (determined from the appearance of the first piece of lining material, which was usually a feather or fur), Ca supplements were placed into feeder trays on the sides of nestboxes (Fig. 2.2). The supplements consisted of a mixture of chicken eggshell fragments (98.8% Ca, Fresh Thinking Catering, Birmingham, UK) and oystershell grit (97.8% Ca, CJ Wildlife Ltd., Upton Magna, UK). Both species exploit novel food sources (Soper 2006) and thus can be readily food-supplemented. Both Ca supplements have been used in previous supplementation experiments of great tits and blue tits (e.g. Graveland 1996, Ramsay and Houston 1999). Through the use of video recording, both species were seen to consume the Ca supplements. The assimilation of ingested Ca into the eggshell is believed to be rapid, taking place in a matter of hours (Comar and Driggers 1949, Graveland and Berends 1997). In egg-laying birds, Ca can be incorporated into the skeletons within 8 hours of ingestion (Simkiss 1967, Reynolds 1997).

Nestboxes, Ca-supplemented and control, were checked daily to ensure accurate egg-laying dates. The mean time delay (± 1 SE) between the start of manipulation (at first signs of nest-lining) and first egg date was 4.97 ± 0.39 days (n = 37) for great tits and 5.59 ± 0.43 days

(n = 34) for blue tits. This was not significantly different from the time delay between the first sign of nest lining and first egg date in the control areas (great tit [n = 50]: 4.76 ± 0.30 days, t_{85} = 0.44, P = 0.66; blue tit [n = 37]: 5.73 ± 0.5 days, t_{69}= 0.21, P = 0.84). Eggs 1, 2, and 3 were numbered according to laying order using a waterproof marker pen. Eggs 4 and 5 were removed under licence (Natural England Permit 20100857) on the day of laying.

2.3.3 Egg sampling

Roughly 12 hours after collection, the length and breadth of eggs 4 and 5 were measured (to the nearest 0.1 mm) using dial callipers and weighed (to the nearest 0.0001 g) on an electronic balance (Sartorius, Goettingen, Germany) in the laboratory. Egg volume was calculated from length (L) and breadth (Br) following the equation of Hoyt (1979):

$$\text{Egg volume (mm}^3\text{)} = 0.51 \times \text{L (mm)} \times \text{Br}^2 \text{ (mm}^2\text{)} \qquad \text{(Eqn 2.1)}$$

Eggs were cut longitudinally into halves using a disposable razor-blade, their contents removed and eggshells were washed in water. Shell membranes were left intact.

Eggshell thickness was measured to an accuracy of 1 μm using a modified digital micrometer (series 227-203, Absolute Digimatic, Mitutoyo, Kawasaki, Japan) at a constant pressure setting of 1.5 N (see Maurer et al. 2011b for further details). Thickness was measured twice on both halves of the eggshell at three pre-defined regions of the eggshell (Fig. 2.3), known as the blunt end (B), the equator (E) (i.e. the widest point), and the pointed end (P), amounting to a total of 12 B, E, and P measurements per egg. These replicate measurements (n = 4) were repeatable at each of the three areas of the egg (great tit – B: r = 0.67, P < 0.0001; E: r = 0.72, P < 0.0001; P: r = 0.59, P = 0.0007; blue tit – B: r = 0.51, P = 0.005; E: r = 0.65, P < 0.0001; P: r = 0.66, P < 0.0001) for the initial 10 eggs sampled.

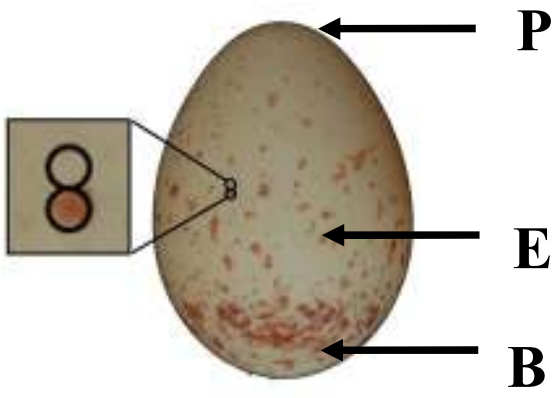


Figure 2.3. Eggshell thickness was measured at three distinct areas of the egg of great and blue tits. Areas are the blunt end (B), the equator (E) and the pointed end (P). Thickness was further measured at a pigmented spot and an immediately adjacent un-pigmented background area. (Photo: K. Brulez).

For the remaining eggs, only one randomly chosen half was measured for eggshell thickness. To quantify differences in thickness between pigmented and un-pigmented (background) eggshell, two pigmented spots and their immediately adjacent un-pigmented background areas were measured (Fig. 2.3).

One randomly chosen half per eggshell was oven-dried (Mino, Genlab, Widnes, UK) to constant mass at 60°C, weighed to the nearest 0.0001 g on an electronic balance before being placed into pre-weighed, individually labelled porcelain crucibles. The eggshells were subsequently reduced to ash at 650ºC in an electric muffle furnace (AAF 1100; Carbolite, Hope, UK) for 25 hours. Crucibles were removed from the furnace and then cooled prior to being weighed to the nearest 0.0001 g. Ashing vaporised any inorganic materials leaving ash which is predominantly $CaCO_3$ (Rivera et al. 1999). Ash mass data are presented as ash/eggshell surface area (g mm^{-2}) (after Gosler et al. 2005). Egg surface area was calculated from volume (vol) following the equation of Hoyt (1979):

$$\text{Egg surface area } (mm^3) = 4.951 \times \text{vol}^{\,0.066}\,(mm) \qquad \text{(Eqn 2.2)}$$

2.3.4 Pigment analysis

The amount of pigments (i.e. protoporphyrin IX and biliverdin) present in the eggshell was quantified by chromatography as described by Mikšík et al. (1996). Briefly, eggshells were extracted (and esterified) in the dark in 5 ml absolute methanol (LiChrosolv, gradient grade for chromatography, Merck, Darmstadt, Germany) containing 5% concentrated sulphuric acid at room temperature under N_2 for 24 hours. Extracts were decanted and 4 ml of chloroform (Merck) and 4 ml of distilled water were added and then shaken. The lower (chloroform) phase was collected, and the higher (water) phase was again extracted with chloroform (chloroform phases from both extractions were collected). These phases were washed with 2 ml of 10% NaCl followed by distilled water until the phase was of neutral pH. Extracts were evaporated to dryness and reconstituted in 0.5 ml of chloroform with an internal standard (5,10,15,20-tetra (4-pyridyl)-21H,23H-porphine, Aldrich, Sigma-Aldrich, St. Louis, MO, USA; 0.01 mg ml^{-1}). Standards for quantification (product of MP Biomedicals, LLC, Eschwege, Germany) were treated using the same procedures.

Pigments were determined and quantified by reversed-phase high-performance liquid chromatography (HPLC) using an Agilent 1100 LC system (Agilent, Palo Alto, CA, USA) using multi-wavelength detector and coupled to an ion-trap mass spectrometer (Agilent LC-MSD Trap XCT-Ultra; Agilent, Palo Alto, CA, USA). Chromatographic separation was conducted in a Gemini 5u C18 110A column (250 × 2.0 mm I.D., Phenomenex, Torrence, CA, USA). The 10 µl sample was injected into the column and eluted using a linear gradient (X = water with 0.1% formic acid, and Y = acetonitrile with 0.085% formic acid), a flow rate of 0.35 ml min^{-1} and a temperature of 55°C. The gradient started at X/Y 80:20 reaching 10:90 ratios after 15 minutes and reaching 100% Y after 5 minutes. For the next 10 minutes the elution was isocratic. Elution was monitored by absorbance at 410 nm. Atmospheric pressure

ionization-electrospray ionization (API-ESI) positive mode ion-trap mass spectrometry at MRM (multiple reaction monitoring) mode was used when precursor ions were 619 m/z (internal standard), 611 m/z (biliverdin), and 591 m/z (protoporphyrin IX).

The amount of error in pigment quantification was estimated in two ways. First, in instances of high concentrations of protoporphyrin (e.g., 15,000-15 ng/ml), absorbance at 410 nm was used when calibration curves were linear with regression coefficients in the range of R^2 = 0.9979 and 0.9947. Error of quantification (relative standard deviations - RSD), of the whole sample preparation procedure (i.e., methylesterification, extraction, analysis) was calculated based on standards using six independent measures and did not exceed 11%. Samples were re-analysed a month after the first analysis, and compared to each other for repeatability. The RSD values were lower than 5% for all samples, indicating the good repeatability of results from the HPLC methodology. Pigment contents of eggshells are expressed as their mass per g of eggshell ($\mu g\ g^{-1}$).

2.3.5 Statistical analysis

All statistical analyses were performed in R 2.14.0 (R Development Core Team 2011), applying General Linear Models (GLMs) with normal error structures, and Generalised Linear Mixed Effects Models (GLMMs), using likelihood ratio chi-squared tests, when including random effects. Subsequent to a non-significant result of the Shapiro-Wilk test for normality, model simplification was performed using backward stepwise regression to find the minimal adequate model using *F*-tests (GLMs) or Chi-squared (GLMMs) to compare the residual deviance of models including and excluding explanatory variables. Interactions between explanatory variables were initially included in models, but due to non-significance

were subsequently disregarded for the purpose of further statistical analysis. Tukey *post-hoc* (95% CIs) tests were implemented for significant factors with multiple levels to compare confidence intervals on the differences (diff) between the observed means, and upper (upr) and lower (lwr) bounds of the confidence intervals. Eggshell thickness was expressed as either regional thickness (i.e. B, E and P) mean values or as a single mean thickness value. The distribution of the protoporphyrin content data was normalised by square-root transformation.

All statistical analyses were performed using the fourth-laid egg from each female, except when looking at within-egg differences between spotted and adjacent un-spotted eggshell, when data from fifth-laid eggs were also included. It is possible that the fourth- and fifth-laid eggs are not representative of all eggs laid in the clutch. However, within-female repeatability of eggshell spotting (spot intensity, distribution and spot size) has been shown to be high in both great tits (Gosler et al. 2000) and blue tits (Sanz and García-Navas 2009). Other egg traits (e.g. yolk androgen concentration, egg mass, and yolk mass) have also been shown to be highly repeatable within females in other passerine species (Tschirren et al. 2009). Therefore, we assume that the traits examined in this study are also repeatable within females and hence the fourth- and fifth-laid eggs are representative of all eggs laid in the clutch.

2.4 Results

Two eggs were removed from each of 59 great tit clutches (37 un-supplemented and 22 Ca-supplemented), and 38 blue tit clutches (14 un-supplemented and 24 Ca-supplemented). Protoporphyrin IX and biliverdin were detected in eggshells of both species. Protoporphyrin

was present in all eggshells but biliverdin was only found in a subset of eggshells (great tits: 41.8%; blue tits: 8.1%), often in very small quantities (mean [±1 SE]: 0.0013 ± 0.0024 μg g^{-1} of eggshell). A Chi-squared test with Yates' continuity correction found no significant difference in the presence of biliverdin in eggshells (presence/absence) of great tits (χ^2_1 = 0.45, P = 0.50) or blue tits (χ^2_1 = 0.005, P = 0.95). Because of the very low quantities discovered, biliverdin was disregarded for the purpose of further statistical analyses.

2.4.1 Variation in thickness across the eggshell

Mean (± 1 SE) eggshell thickness for great tit eggs was 85.00 ± 0.54 μm, and for blue tit eggs it was 76.00 ± 0.64 μm. Within eggs of both species, thickness of un-pigmented eggshell varied between different egg regions when controlling for egg volume (great tit: χ^2_2 = 27.76, P < 0.0001; blue tit: χ^2_2 = 27.16, P < 0.0001; Fig. 2.4).

In great tits, Tukey *post-hoc* comparisons (95% CIs) of eggshell regional thickness revealed that the P region of the egg was significantly thicker than the B region (P = 0.002, diff = 2.64 μm, lwr = 0.83, upr = 4.44), and E region (P = 0.02, diff = 2.11 μm, lwr = 0.31, upr = 3.91) (Fig. 2.4). In blue tits shell thickness at the P region of the egg was significantly thicker than the B region (P = 0.001) and the E region (P = 0.04) (Fig. 2.4). Comparison between the other two areas of the eggshell revealed no significant differences.

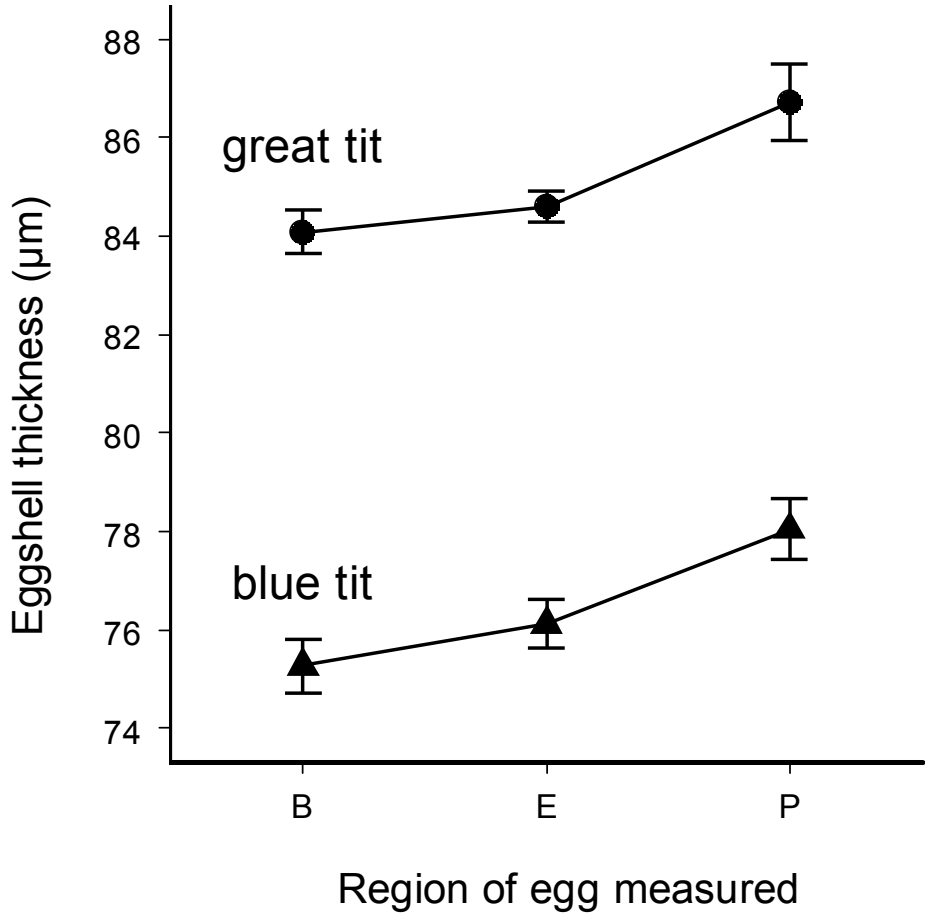


Figure 2.4. Thickness (mean ± 1 SE) of un-pigmented eggshell of great tits (n = 59) and blue tits (n = 38) at Chaddesley Woods NNR, Worcs., UK in 2010. Thickness was measured in three distinct regions of the shell − B: blunt end; E: equator; and P: pointed end.

2.4.2 Variation in eggshell thickness

In great tits, mean eggshell thickness was related to dietary Ca treatment and eggshell Ca concentration (Fig. 2.5; Table 2.1). Ca-supplemented females laid eggs with thicker eggshells than those laid by un-supplemented females (difference in eggshell thickness = 2.10 ± 0.37 µm; Fig. 2.5). Great tit eggshells containing more Ca were thicker (Fig. 2.5).

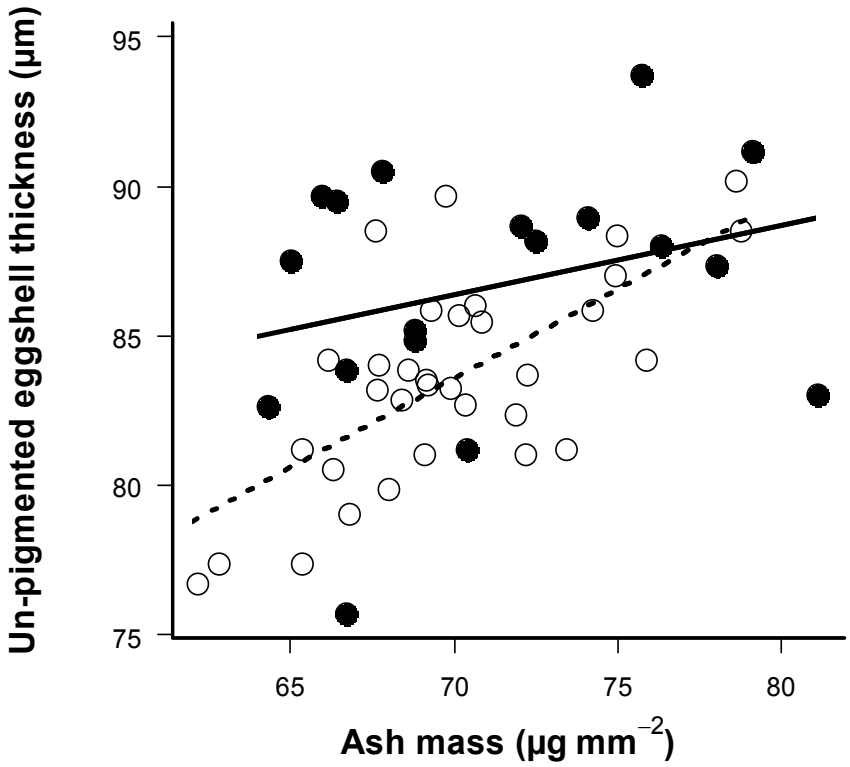


Figure 2.5. Positive association between mean un-pigmented eggshell thickness and ash content of eggs laid by Ca-supplemented (filled circles and solid line) and un-supplemented female (open circles and dotted line) great tits.

In blue tits, mean eggshell thickness was related to dietary Ca availability with a strong effect but in the opposite direction than predicted; supplemented females laid eggs with thinner eggshells (difference in eggshell thickness = 3.10 ± 0.08 µm) than those laid by un-supplemented females (Table 2.1).

Table 2.1. Statistical outputs from models of reproductive parameters (*F* and associated *P* values). Response variables are given in bold in the column on the left with the explanatory variables included in the model given below. Model simplification was performed using backward stepwise regression to find the minimal adequate model using *F*-tests to compare the residual deviance of models including and excluding explanatory variables. Interactions between explanatory variables were initially included in models, but due to non-significance were subsequently disregarded for the purpose of further statistical analysis. Bold text indicates a term that is significant at the α threshold of 0.05.

	Great tits				Blue tits			
	Model effect estimate ± SE	**df**	***F***	***P***	**Model effect estimate ± SE**	**df**	***F***	***P***
Thickness (µm)								
Treatment area	2.67 ± 0.96	1,50	7.70	**0.0078**	-3.08 ± 1.22	1,36	6.35	**0.02**
Volume (mm^3)	0.0013 ± 0.0046	1,49	0.08	0.79	0.0075 ± 0.0077	1,35	0.53	0.47
Ca ($g\ mm^{-2}$)	0.21 ± 0.052	1,50	15.74	**0.00024**	-0.017 ± 0.053	1,34	0.11	0.75
Protoporphyrin (µg g^{-1})								
Treatment area	-4.59 ± 4.37	1,51	1.10	0.30	2.44 ± 1.68	1,34	2.11	0.16
Volume (mm^3)	0.0099 ± 0.021	1,49	0.22	0.64	0.029 ± 0.01	1,35	7.64	**0.009**
Ca ($g\ mm^{-2}$)	0.20 ± 0.28	1,50	0.53	0.47	-0.0065 ± 0.073	1,31	0.00	0.99
Thickness (µm)	0.48 ± 0.51	1,52	0.38	0.69	0.24 ± 0.24	1,32	2.31	0.14

2.4.3 Relationship between eggshell thickness, maculation and pigment concentration

GLMMs, controlling for nest identity and multiple measures per egg, showed that the difference in eggshell thickness (background shell - pigmented shell) varied between dietary treatment areas in eggs laid by great tits ($\chi^2_1 = 4.89$, $P = 0.03$, effect estimate ± SE: Ca-supplemented: -0.52 ± 0.23 μm; un-supplemented: 0.86 ± 0.14 μm), but not blue tits ($\chi^2_1 = 3.21$, $P = 0.07$; Fig. 2.6).

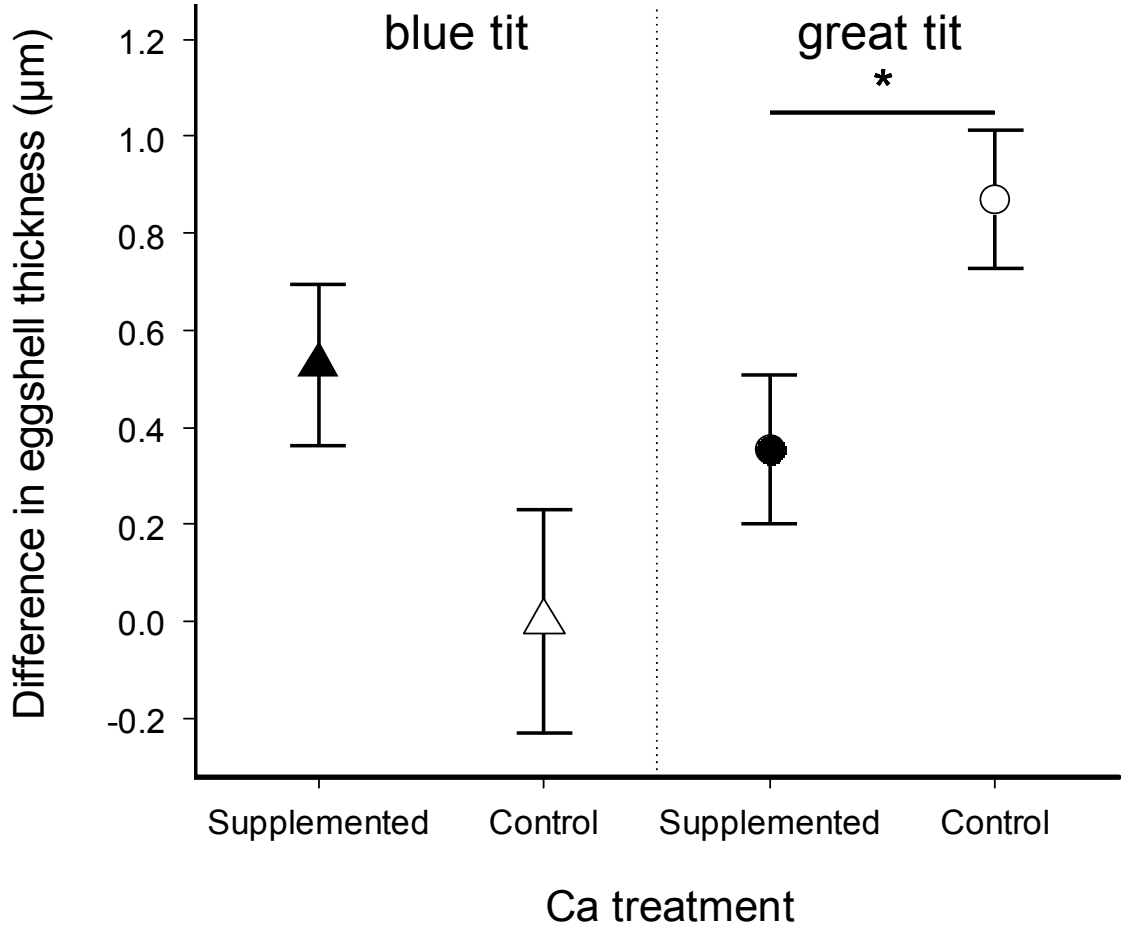


Figure 2.6. Difference between pigmented and adjacent un-pigmented eggshell thickness (mean ± 1 SE) from eggs laid by un-supplemented (open points) and Ca-supplemented (solid points) great tits (filled triangles) and blue tits (filled circles) in Chaddesley Woods NNR, Worcs., UK in 2010. The asterisk denotes a significant finding at the $P < 0.05$ level.

In great tits, protoporphyrin concentration in eggshells was not associated with dietary treatment, egg volume, Ca concentration, or eggshell thickness (Table 2.1). In blue tits, larger eggs contained significantly more protoporphyrin ($F_{1,35} = 7.64$, $P = 0.009$; Table 2.1).

2.5 Discussion

In accordance with the structural-function hypothesis, Ca-supplemented female great tits (but not blue tits) laid eggs with thicker shells (prediction 1) and with higher Ca concentration (prediction 2) than non-supplemented (control) females. In both species, pigment spots occurred in thinner areas of the eggshell in eggs laid by un-supplemented females (prediction 3). However, in eggs laid by Ca-supplemented great tit females no such difference in shell thickness was found. Larger eggs laid by blue tits contained greater quantities of protoporphyrin relative to their size. In conflict with the structural-function hypothesis, no significant relationship was found between the protoporphyrin and Ca concentrations of eggshells in either species (prediction 4).

2.5.1 Variation in thickness across the eggshell

Within eggs of both species, the B and E regions were found to be thinner than the P region of the eggshell (Fig. 2.4). This increase in thickness from the B to the P region of the shell is compatible with findings from many other bird species (e.g. mute swan [*Cygnus olor*] – Booth 1989, mallard [*Anas platyrhynchos*] – Balkan et al. 2006). The thickness of an eggshell is vital for normal embryonic development: embryos in thin-shelled eggs experience reduced hatchability due to dehydration during incubation (Ar et al. 1974, Ar and Rahn 1980, Bennett

1992). Thin eggshells are more prone to breakage (Mallory and Weatherhead 1990, Boersma et al. 2004), exposing the embryo to external influences such as pathogens and fluctuations in environmental conditions. However, there is an upper limit to eggshell thickness due to the need for gaseous exchange (Ar et al. 1974, Gonzalez et al. 1999), pipping and successful hatching (Honza et al. 2001). The thickness of an eggshell must, therefore, be an evolutionary compromise between opposing selective forces.

2.5.2 Causes of variation in eggshell thickness

In accordance with previous studies (e.g. Graveland and van Gijzen 1994, Tilgar et al. 1999, Mora et al. 2011), great tit eggs with thicker eggshells had higher total Ca concentrations (Fig. 2.5). Thicker shells increase pore length, decreasing the water conductance and overall functional pore area (Ar et al. 1974). Therefore, an increase in eggshell thickness should coincide with an increase in the quantity, size and length of pores (Ancel and Girard 1992, Zimmermann et al. 2007). Unfortunately this was not examined in this study. It is interesting that while both dietary treatment and Ca concentration (i.e. ash mass) positively affected eggshell thickness, they were unrelated. This suggests that the birds in this study population were either not Ca deficient or may not be employing sources of dietary Ca from supplements in constructing their eggshell.

Contrary to what was predicted, Ca-supplemented blue tit females laid eggs which had thinner shells than those laid by un-supplemented females. There is an indication that eggshell microstructure (e.g. crystal size, shape and orientation) may influence eggshell mechanical properties (Rodriguez-Navarro et al. 2002). Eggshell strength is dependent upon modification of the microstructure rather than upon an increase of the inorganic constituents (e.g. $CaCO_3$).

Eggshells with smaller calcite crystals, and a reduction in the degree of their orientation, are more solid and have increased mechanical strength (Ahmed et al. 2005). This might explain why blue tit eggs from the Ca-supplemented area of the woodland had thinner shells because calcite crystals could be more compact within the shell's microstructure, allowing an increase in crystal density. However, if this is the case, we must reconsider the scenario that thin eggshells result from a lack of dietary Ca.

Nonetheless, this conflicting result could be due to limitations of the experimental design. This experiment did not take into account natural dietary Ca availability. Control females may not have been more Ca-limited than Ca-supplemented birds. Furthermore, females may have crossed between treatment areas, allowing control birds access to supplemented Ca. This may have resulted in Ca-supplemented females investing an increased amount of time into defending this food source rather than on other activities.

2.5.3 Relationship between eggshell thickness and maculation

In accordance with the findings of Gosler et al. (2005), spotting on the eggshells of great tits demarcated thinner areas (Fig. 2.6). However, although maculation was still present on eggshells laid by Ca-supplemented great tits, no difference in eggshell thickness between spotted and un-spotted shell was found, suggesting that visible protoporphyrin on these eggs may be superficially deposited and, thus, might have a functional significance beyond structural strengthening. Females may deposit protoporphyrin onto eggshells, with a specific function or as a by-product (e.g. De Coster et al. 2012), regardless of Ca concentration of the eggshell. This function may safeguard the eggs in periods of Ca deficiency and an effect would only be observed when dietary Ca availability is severely restricted but not when it is

abundant. Soil Ca levels at the study site ranged from 41.3 – 2,413.0 mg 100 g^{-1} of soil (Copley 2009, Johnson 2009). Although this is low compared to other sites (e.g. Wytham Woods [63 to 21,000 mg Ca 100 g^{-1} of soil] – Dawkins and Field 1978, Farmer 1995), females may have had time to adapt to these conditions and are therefore able to obtain sufficient Ca from natural sources (Ramsay and Houston 1999).

Although significantly different, the disparity between spotted and un-spotted eggshell thickness in eggs laid by un-supplemented females corresponds to only 1.04% of the mean eggshell, only slightly greater than the variance shown in this population, and substantially less than what other studies have reported (e.g. 7.5% – Gosler et al. 2005). Could this difference in shell thickness be enough to cause any disadvantages to the egg, structurally or otherwise? The spotted parts of the eggshell may simply be thinner because they are not covered by the outer Ca layer and so reveal the protoporphyrin pigment layer underneath.

Contrary to great tits, Ca-supplemented blue tits laid eggs similar to those of un-supplemented conspecifics with their spotting demarcating thinner areas of the eggshell compared with adjacent un-pigmented areas (Fig. 2.6). It is possible that protoporphyrin may be more internal to the shell matrix in eggs laid by blue tits and therefore may not be susceptible to eggshell thickness changes caused by dietary Ca availability.

2.5.4 Causes of variation in protoporphyrin concentration

In great tits, eggshell protoporphyrin concentration was independent of its Ca concentration and thickness. This is surprising as pigmented eggshell was thinner than adjacent un-pigmented areas (but not for Ca-supplemented great tits; Fig. 2.6), suggesting that the localisation of protoporphyrin may be more important than the quantity deposited. The B and

E regions of the eggshell tend to be more heavily spotted than the P region (Gosler et al. 2005). It is these B and E regions that were thinner in great tit and blue tit eggshells (Fig. 2.4), indicating that these regions may need more structural support than the pointed region and, therefore, may be more sensitive to dietary Ca availability. However, pigmentation in thinner areas of the eggshell could purely be as a result of the eggshell formation process rather than a specific structural function. Surface pigmentation is deposited in the latter stages of egg formation so the B region of the shell, which tends to be thinnest (but see Gosler et al. 2005), is in direct contact with the shell gland where protoporphyrin is secreted for the longest amount of time. Hence, it tends to be most pigmented. Heavier females lay less speckled eggs implying that eggshell protoporphyrin may be related to a female's ability to remove harmful protoporphyrin (Stoddard et al. 2012) or as a consequence of enhanced red blood cell production in response to anaemia (De Coster et al. 2012). This could mean that regardless of dietary Ca availability, protoporphyrin may still be required for purposes related to female body condition and/or egg quality.

In blue tit eggshells, Ca concentration was not related to eggshell thickness. However, larger eggs contained greater protoporphyrin concentrations, despite no difference in their Ca concentration. This would be expected if females deposited more protoporphyrin into larger eggshells to increase their strength in the absence of greater quantities of Ca. Larger eggs are advantageous if they produce larger nestlings which in turn have higher survival rates (Schifferli 1973; but see Williams 1994, Christians 2002). Ca and protoporphyrin share a carrier protein during eggshell formation (Gosler et al. 2005, Jagannath et al. 2008) and the deposition of protoporphyrin into an eggshell may therefore result in Ca deficits. In addition to increasing eggshell strength, protoporphyrin may have alternative functions (see Maurer et al. 2011b for more details). For example, protoporphyrin, if activated by light, can reduce

bacterial survival on eggshells (Ishikawa et al. 2010), large quantities of which can increase risk of mortality to the developing embryo (Cook et al. 2003, 2005). Protoporphyrin may further affect the developing embryo by influencing gas conductance across the eggshell by physically blocking pathways for gaseous exchange (Higham and Gosler 2006; but see Maurer et al. 2011b). External protoporphyrin pigmentation on eggshells is negatively related to female anaemia (De Coster et al. 2012), health (Martínez-de la Puente et al. 2007) and body condition (Stoddard et al. 2012). However, the relationship between protoporphyrin and female health indices is poorly understood due to our inability to relate the production of protoporphyrin to its function. Protoporphyrin is a pro-oxidant that may induce oxidative stress (Shan et al. 2000), and hence heavily spotted eggs may indicate a female's poor health status due to her inability to remove harmful free radicals. Alternatively, heavily spotted eggs may indicate a female's good health status due to her ability either to function under high oxidative stress or to remove free radicals (Moreno et al. 2006a).

2.5.5 Interspecific differences

Eggshell thickness varies with egg shape in great tits (Gosler et al. 2005). A spherical egg shape provides the highest resistance against external forces (Bain 1991), optimal gas exchange between the developing embryo and the external environment (Ar et al. 1974), and is the most conservative in the use of shell materials (Gosler et al. 2005). It has further been suggested that optimal egg shape may depend on clutch size for optimal fit under the incubating parent, with eggs in clutches of greater than seven eggs being the most spherical (Barta and Székely 1997; but see Encabo et al. 2001). In our study population, blue tits lay larger clutches (10.1 ± 0.4, n = 59) than great tits (7.2 ± 0.3, n = 38), and thus, they may have

evolved eggshell parameters (e.g. thinner shells of lower Ca concentration) to compensate for increased spherical egg shape. Alternatively, it would be worth testing whether due to large clutch sizes, blue tits may have adapted their eggshell thickness and maculation to promote lower pore density so that nest humidity and the rate of water loss remain constant in clutches of different egg numbers (Hargitai et al. 2011).

2.6 Conclusions

Protoporphyrin is found in the eggs of over 100 avian species (Kennedy and Vevers 1976, Gorchein et al. 2009, Cassey et al. 2012a), and as eggshell morphology of each species has evolved under different selection pressures (Rahn and Ar 1974), it is unlikely that one hypothesis will explain pigment function in all species (Reynolds et al. 2009). This study suggests that protoporphyrin pigment spots have an important structural role when structural strengthening is required, but when Ca is abundant, pigment spots remain integral to the eggshell, possibly fulfilling alternative functions. Can females with limited protoporphyrin achieve similar results by strategically placing pigment spots where they are most required, structurally or otherwise? If eggshell patterning is under genetic control (Gosler et al. 2000), how much flexibility do females have in responding to environmental variability, including local Ca availability? Further insight into the structural-function hypothesis needs to be gained, especially into the importance of localisation of pigment. We encourage cross-disciplinary approaches when researching the functional significance of eggshell pigmentation as the structure of the eggshell and pigment must be considered when researching the ecological functions.

Having explored how Ca supplementation affects eggshell characteristics in this chapter, the following chapter will look at how the combination of natural Ca availability and Ca supplementation affects eggshell traits, including eggshell maculation, using data from three consecutive years.

CHAPTER 3

THE CONSEQUENCES OF CALCIUM AVAILABILITY ON EGGSHELL AND LIFE-HISTORY TRAITS

3.1 Abstract

Egg formation is costly, both in terms of nutritional and energetic requirements. Small passerines must collect Ca daily during the egg-laying period to obtain sufficient resources for eggshell formation. Availability of Ca-rich food items is vital for habitat quality which affects breeding performance in many bird species. Ca supplementation of breeding birds will most likely be ineffective if natural dietary Ca availability provides sufficient Ca for the egg-laying female. Snail diversity, abundance and size are strongly correlated with local soil Ca levels. Here, we investigate the combined effects of local soil Ca levels and Ca supplementation on the breeding ecology of free-living blue and great tits over a 3-year period. We examine the consequences of Ca availability (natural and supplemented) on physical eggshell traits (Ca concentration, protoporphyrin concentration, percentage spot cover, spot intensity and thickness) and examine whether females adapt their breeding behaviour (lay date, clutch size, incubation initiation) in response to changes in Ca availability. We found that Ca supplementation had a greater effect on eggshell characteristics of both species than local soil Ca concentration, suggesting that females in this population are not suffering from severe Ca limitation but have adapted to these conditions using different strategies without displaying obvious reproductive problems. The two species reacted differently to changes in Ca availability with great tits showing changes in physical eggshell traits and blue tits showing changes in both physical eggshell traits and breeding behaviour.

3.2 Introduction

Egg formation is costly, both in terms of nutritional and energetic requirements (Walsberg 1983). Breeding is timed so that the peak in nestling provisioning coincides with the peak in seasonal food availability (e.g. caterpillars), but this means that females must start laying when food is not maximally available (Perrins 1970). The 'constraint hypothesis' suggests that early egg-laying is prevented by food availability (Perrins 1970, 1996), including micronutrients such as Ca required for eggshell formation (Reynolds and Perrins 2010).

Much evidence of Ca-limited reproduction exists (reviewed by Reynolds and Perrins 2010), with the first well-documented case in The Buunderkamp Forest in The Netherlands (Table 3.1), where female great tits laid eggs with very thin shells or no shells at all (Drent and Woldendorp 1989). The defects in eggshells were attributed to the low availability of soil

Table 3.1. Comparison of soil Ca concentration between the focal study site area (Chaddesley Woods NNR, Worcs., UK) and those in Wytham Woods (Oxford, UK) and The Buunderkamp Forest (The Netherlands).

Soil Ca (mg Ca 100 g^{-1} soil)	Chaddesley Woods NNR*	Wytham Woods†	The Buunderkamp Forest‡
Minimum	37.7	63	300
Maximum	2,413.3	23,000	1,180

Data from: * (Johnson 2009); † (Farmer 1995); ‡(Graveland and van Gijzen 1994)

Ca (Table 3.1) as a consequence of acid precipitation. Increased soil acidity causes advanced leaching of Ca (Graveland 1998), resulting in decreased Ca concentration of dietary foods such as plants or herbivorous arthropods (Drent and Woldendorp 1989).

Small passerines are unable to store Ca in their skeletons (Simkiss 1967) and therefore must collect Ca daily during the egg-laying period to obtain sufficient resources for eggshell

formation (Pahl et al. 1997, Reynolds 1997). Over 90% of Ca-rich foods, including snail shells and calcareous grit (Perrins 1996), are consumed immediately prior to, or during, egg-laying (Graveland and Berends 1997). Availability of Ca-rich foods is vital for habitat quality which affects breeding performance in many bird species (Scheuhammer et al. 1991, Graveland et al. 1994, Tilgar et al. 1999). In captive common pheasants (*Phasianus colchicus*), dietary Ca availability has been shown to decrease reproductive success by causing reduced egg production, eggshell thinning and osteoporosis in egg-laying females (Chambers et al. 1966). Similar results have been found in wild passerines breeding on poor soils (Graveland et al. 1997). Females breeding in acidified habitats are especially prone to Ca deficiency as invertebrates high in Ca are particularly sensitive to acidification (Scheuhammer et al. 1997). Females can potentially adapt their breeding behaviour to compensate for changes in Ca availability including, for example, adjusting clutch size (Patten 2007) or lay date (Mänd et al. 2000b). Anthropogenic Ca can be a particularly important Ca source to egg-laying females breeding on base-poor soils (Graveland et al. 1994).

Females may cope with reduced dietary Ca availability by depositing increased amounts of pigment onto or into the eggshell (Gosler et al. 2005). If protoporphyrin pigmentation has a structural strengthening function in the eggshell when dietary Ca is scarce, a negative relationship between eggshell Ca and protoporphyrin concentrations would be expected. The efficacy of protoporphyrin may depend on its total content or on its position within the shell. An increased amount of spotting may also reduce eggshell permeability which necessitates a change in incubation behaviour of the female (Higham and Gosler 2006).

The past decade has witnessed a range of studies investigating the importance of Ca by looking at the effects of Ca supplementation on breeding in both acidified and non-acidified habitats (reviewed in Reynolds et al. 2004, Reynolds and Perrins 2010). Ca

supplementation has been shown to influence eggshell traits such as eggshell thickness (García-Navas et al. 2011), as well as life-history traits such as clutch size (Tilgar et al. 2002). However, not all studies have found positive effects of Ca supplementation on egg and breeding parameters (but see Ramsay and Houston 1999).

Ca supplementation of breeding birds will most likely be ineffective if natural dietary Ca availability provides sufficient Ca for the egg-laying female (Reynolds et al. 2004). Snail diversity, abundance and size are strongly correlated with local soil Ca content (Mänd et al. 2000b, Jubb et al. 2006). Snail shells are the main source of Ca for small passerines (Graveland et al. 1994), and therefore soil Ca content is a good indicator of the amount of Ca available to breeding females (Pabian and Brittingham 2011). Increased soil Ca content results in an increase in both individual and species abundance of snails (Johannessen and Solhøy 2001). Both plants and soil organisms absorb nutrients from the soil and leaf litter (Tyler 1954). However, snail abundance is not just related to soil Ca content but can also be influenced by soil properties such as such as moisture levels and soil pH (Martin and Sommer 2004).

Local soil Ca positively influences eggshell ash mass (i.e. Ca content) of great tits (Gosler et al. 2005), and eggshell maculation decreases with increasing soil Ca, with both spot intensity and distribution relating to eggshell thickness (Gosler et al. 2005). Studies providing Ca supplementation in order to observe effects on life-history traits of breeding birds, specifically eggshell attributes, must therefore consider natural Ca availability.

Here, we investigate the combined effects of local soil Ca concentration (i.e. a proxy for dietary Ca availability) and Ca supplementation on the breeding ecology of free-living blue and great tits over a 3-year period. We predict that Ca-supplementation will have a greater effect on birds breeding in territories with low soil Ca concentration. We predict that

increased Ca availability (natural and supplemented) will have a positive influence on physical eggshell traits such as Ca concentration and eggshell thickness, but a negative influence on protoporphyrin concentration, percentage spot cover and spot intensity. We further predict that changes in physical eggshell attributes will coincide with changes in females' breeding behaviour. Eggshells with thinner shells and a greater extent of spotting will be laid in larger clutches which have later lay dates and incubation initiation dates.

3.3 Materials and methods

3.3.1 Study site, field methods and egg sampling

Please refer to sections 2.3.1 to 2.3.3 of Chapter Two.

3.3.2 Ca supplementation

This study was conducted in three consecutive breeding seasons (2010-2012) at Chaddesley Woods NNR. The study site is divided into three distinct areas: Santery Hill Wood, Coalpit Coppice and Chaddesley Wood. During the first two breeding seasons, Ca was supplemented in one of these three areas, while a second area acted as the control with both areas rotated annually (see Fig. 3.1). In 2012, two areas were used as the control area. In 2010, both oystershell grit and eggshell fragments were provided as Ca supplements but only oystershell grit was provided in subsequent years.

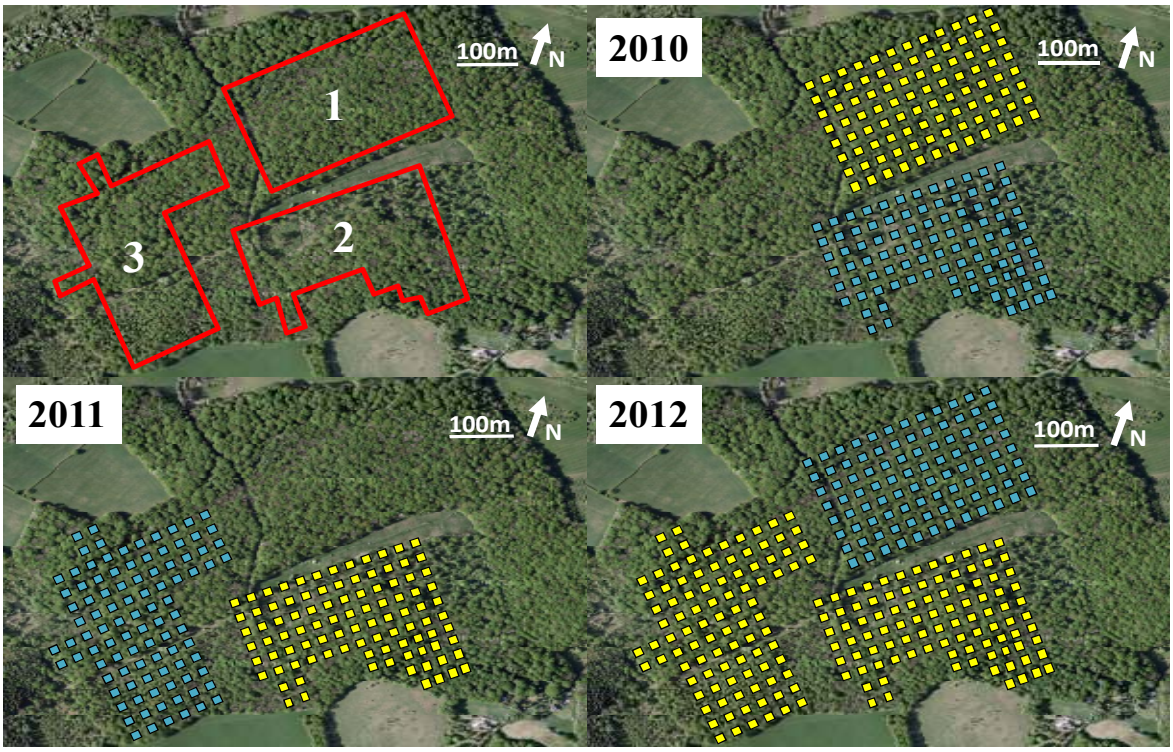


Figure 3.1. Distribution of Ca treatments (blue: supplemented; yellow: controls) between three woodland nestbox blocks (1: Coalpit Coppice; 2: Santery Hill Wood; and 3: Chaddesley Wood) in Chaddesley Woods NNR, Worcs., UK between 2010 and 2012, inclusive. (Reproduced from Webber 2012).

3.3.3 Breeding parameters

Nestboxes were checked daily until the start of incubation to ensure accurate recording of clutch size and lay date. The start of incubation was defined as occurring after eggs were uncovered and warm, and/or the female was observed sitting on eggs for two consecutive days, the first day being counted as incubation day 0. Subsequently, hatching checks were carried out on a daily basis 10 days after clutch completion until the first egg hatched (with day 0 being day of hatching). A mean incubation length of *c.* 12-13 days was expected for both species (Perrins 1979, Cresswell and McCleery 2003).

Adults were caught on nestling day 10 using nestbox spring traps, ringed/identified and measured. If un-ringed, adults were ringed with a unique BTO metal ring on the right leg (under ringing license C5707). If already ringed, the BTO ring number was recorded. Measurements taken included body mass (to the nearest 0.1 g) using a Pesola spring balance, and wing length and tarsus length (to the nearest 0.01 mm) using dial callipers.

3.3.4 Pixel pigment scoring

Eggs were photographed on the day of laying prior to egg sampling, using a Canon 450D digital camera with a 105 MM Sigma AF lens under standardised conditions following Cassey et al. (2010a). The camera was mounted on a Kaiser camera stand, surrounded by two Calumet photographic umbrellas with silver-white (AU3046) and flat white (AU3045) lining. Eggs were alluminated to the right and front using two Osram 11 W energy saving light bulbs. Photographs were taken at ISO 400 with an aperture of f16 and the exposure was set to automatic. To ensure that the whole eggshell was recorded, four photographs were taken per eggshell, rotating the egg 90º between photographs. Eggs were cut longitudinally into halves using a disposable razor-blade, their contents removed and eggshells were washed in water, and dried to constant mass.

Eggshell images were used to quantify eggshell coverage by, and intensity of, pigment spots. Spot cover (%) was defined as the amount of spotting in the foreground compared to the background (based on number of pixels). Spot pigment intensity was defined as the darkness of the spotting based on greyscale intensity (on a scale of 0 [black] to 1 [white]). The mean pigment scores were calculated from multiple images per eggshell.

Analysis of the eggshell images was conducted in MATLAB (The MathWorks, Natick, MA, USA). Each image was loaded and processed individually. Processing comprised

two main phases: selection of the regions of the image to analyse, and calculation of the image statistics. In order to select the regions of the image, the image was first partitioned into egg and background regions using a simple binary threshold on a greyscale version of the image. The threshold level was determined using the method of Otsu (1990), to locate where the intra-class variance is minimized, and the inter-class variance is maximized. All images were checked visually to ascertain that the eggs were separated from their background correctly, and if incorrect, were removed from the analysis when detected.

Having identified the egg region of the image, two square sections (square 1 at the B region [crown] and square 2 at the E region [shoulder] – Fig. 3.2) were taken along the long axis of the egg. The squares were equal in size which was determined so that each side was 20% of the total egg length. The squares were placed such that each square fell entirely within the perimeter of the eggshell in the image, and were separated from each other by a distance of 10% of the total egg length. This had the effect of excluding pixels found near the edge of the egg, thereby avoiding parts of the image where the pigment spots may have been distorted due to eggshell curvature.

Finally, pixels in the image were categorised as either maculated or non-maculated using the same greyscale threshold and the method of Otsu (1990). The percentages of squares 1 and 2 that were maculated were then calculated. In addition, the mean greyscale intensity was calculated for maculated and non-maculated eggshell in each square.

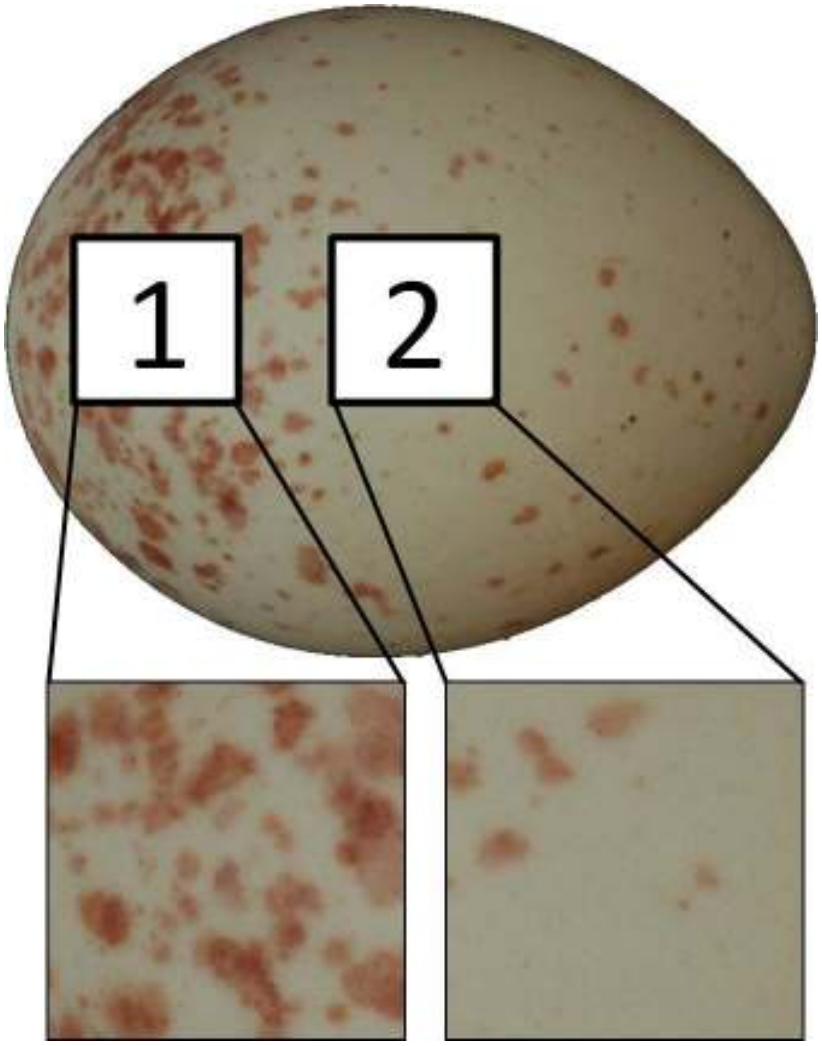


Figure 3.2. Two squares per egg were used to analyse eggshell pigment spotting of blue and great tits. One was centred on (1) the B region (crown) and the other on (2) the E region (i.e. the widest point) of the eggshell.

3.3.5 Soil Ca survey

Soil samples were taken between March and May 2009 from all three experimental areas in Chaddesley Woods NNR, as well as from the meadow (i.e. Black Meadow) situated between Santery Hill Wood and Coalpit Coppice (Fig. 3.3).

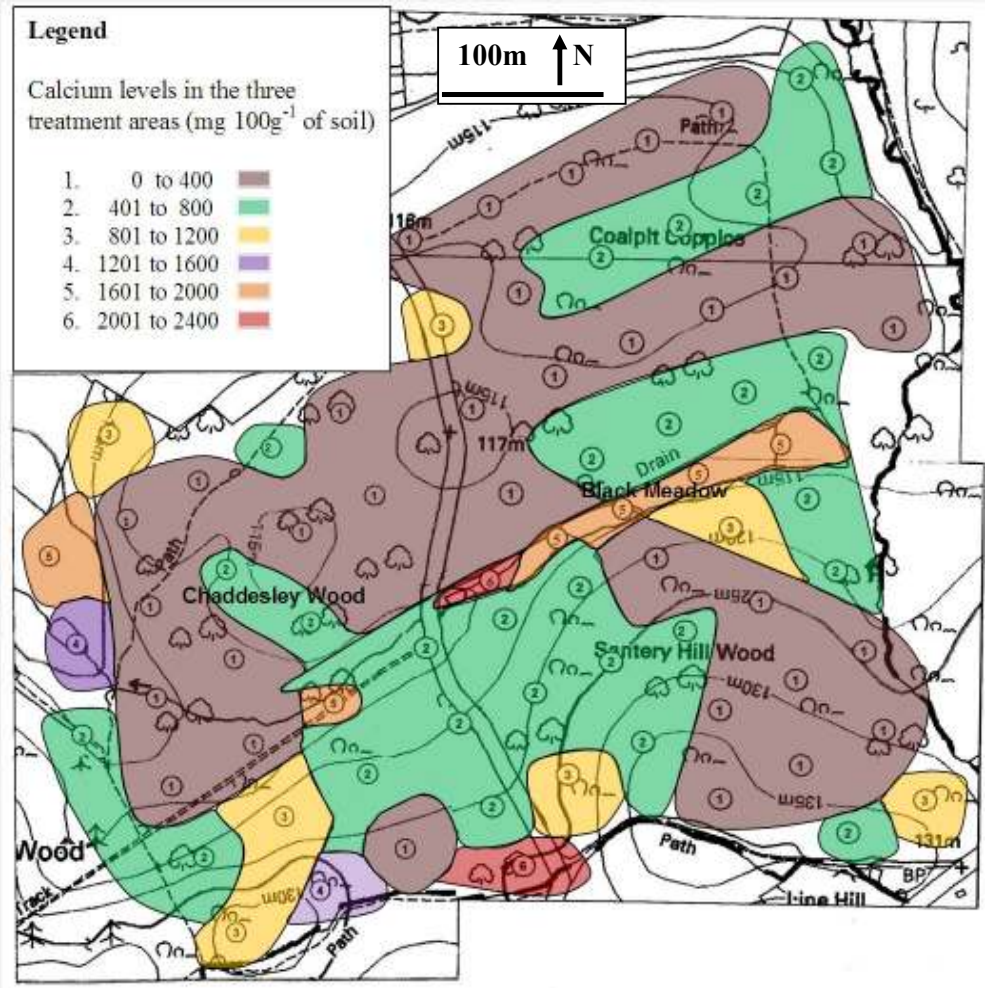


Figure 3.3. Map of Chaddesley Woods NNR, Worcs., UK showing the three supplementary feeding areas and their respective soil Ca availability (after Johnson 2009). Circled numbers represent sampling sites, with the number within referring to the soil-Ca level found at that specific sampling site. The different coloured areas are a rough representation of the soil Ca levels throughout. Ca classes are based on the top 10 cm of soil only. Soil Ca ranged from 37.7 to 2,413.0 mg 100 g^{-1} of soil, with Coalpit Coppice having the lowest overall levels (41.3-1,003.7 mg 100 g^{-1} of soil) and Santery Hill Wood having the highest levels (49.7-2,413.3 mg 100 g^{-1} of soil).

Twenty-four sets of samples were taken from each treatment area and five additional sets of samples were taken from the Black Meadow. Sample sites were situated in a grid pattern, *c.* 80 m apart, so that each nestbox was a maximum distance of *c.* 28 m away from a

sampling site. The soil Ca survey was carried out as part of two MSc projects (Copley 2009, Johnson 2009).

Soil Ca concentration at Chaddesley Woods NNR are low and highly variable. The majority of survey plots contained low Ca concentrations (i.e. 0 to 1,000 mg 100 g^{-1} of soil), inter-mixed with several smaller areas of higher soil Ca concentrations (2,000 to 2,400 mg 100 g^{-1} of soil; see Fig. 3.3). Top-soil Ca content is highly correlated with snail abundance (Juřičková et al. 2008) and hence only results from the top soil layer were used in all subsequent analyses. Two values for soil Ca concentration were used. The 'local soil Ca' calculated as the maximum soil Ca concentration of the nearest sampled site from the nestbox (*c*. 28 m), and 'the regional soil Ca' calculated as the maximum soil Ca concentration within 100 m from the nestbox. No significant correlations were found when including regional soil Ca and thus this variable was excluded from any further analyses.

3.3.6 Statistical analysis

Two eggs were removed from a total of 159 great tit clutches (98 control and 61 Ca supplemented), and 160 blue tit clutches (84 control and 76 Ca supplemented; see Table 3.2 for details).

All statistical analyses were performed in R 2.14.0 (R Development Core Team 2011). Generalised Linear Mixed Effects Models (GLMMs), using a likelihood ratio chi-squared test, were performed using the LME4 package (R Development Core Team 2011). GLMMs allow statistical models to control for random effects. Model simplification was performed using backward stepwise regression to find the minimal adequate model using Chi-squared (GLMMs) to compare the residual deviance of models including and excluding explanatory

variables. Chi-squared values are derived from Wald tests which examine the relative contribution of each explanatory variable in the model. Full interactions between explanatory variables were initially included in statistical models but only those deemed significant remained in the model and are shown below. After term deletion using ANOVA, *P*-values were calculated for the remaining significant variables in the minimal adequate model using Markov Chain Monte Carlo (MCMC) simulations by means of the 'pvals.fnc' from the 'languageR' package (R Development Core Team 2011).

Table 3.2. Sample sizes of eggs removed from Ca-supplemented and control great and blue tit females throughout the three years of the study at Chaddesley Woods NNR, Worcs., UK.

Year	Great tits			Blue tits		
	Ca	Control	Total	Ca	Control	Total
2010	22	37	59	24	14	38
2011	21	16	37	29	29	58
2012	19	43	62	23	41	64

GLMM full models used the following eggshell traits as response variables: Ca concentration (μg mm^{-2}) of individual eggs, protoporphyrin concentration (μg g^{-1}), spot cover (%), spot intensity, and mean eggshell thickness (μm). Two GLMM models were run to test the effects of two sets of variables: Ca availability (natural and supplemented), and life-history traits. Ca availability included: local soil Ca concentration (mg 100 g^{-1} of soil), experimental treatment (Ca-supplemented or control), and eggshell Ca concentration (μg mm^{-2}) of individual eggs. Life-history traits included: female wing length (mm), female tarsus length (mm), incubation initiation date, clutch size (including those eggs removed), and egg-laying date (of the fourth laid egg). Eggshell Ca concentration was included as both a

response and an explanatory variable as we were interested in how eggshell Ca concentration was related to dietary treatment and additionally how Ca concentration is correlated to other eggshell properties (e.g. protoporphyrin concentration). Full models included two random factors to control for 'egg volume' and 'year in which eggs were laid' as we were not interested in the direct effects of these terms on the response variable. Total clutch Ca content was calculated by multiplying the Ca content found in the fourth laid egg by the number of eggs laid. Eggshell Ca content has been shown to be constant across the laying sequence in zebra finches fed on *ad libitum* Ca (Reynolds 2001) and in wild, free-ranging blue-winged teals (*Anas discors*, Rohwer 1986). Other egg traits (e.g. yolk androgen concentration, egg mass and yolk mass) are highly repeatable within females in other passerine species (Tschirren et al. 2009). We therefore assume that eggshell Ca content is also repeatable within females and hence the fourth laid egg is representative of all eggs laid in the clutch.

All statistical analyses were performed using the fourth-laid egg from each female. The distribution of the protoporphyrin concentration data was normalised by square-root transformation. Outliers were removed. Dates and clutch size were treated as continuous variables as we were looking for trends and not interested in differences between specific dates. Spot cover and intensity were calculated from the B region of the eggshell only, as this area is the most heavily spotted.

3.4 Results

A GLMM controlling for egg volume showed that blue tits had a higher mean (± 1 SE) clutch Ca content of 714.50 ± 13.34 μg than great tits (661.90 ± 11.58 μg, Chisq = 8.85, $P = 0.0029$).

There was no difference in clutch Ca content between treatment areas for great tits (t = 0.16, df = 135.51, P = 0.88) or blue tits (t = -1.46, df = 136.04, P = 0.15).

3.4.1 Does Ca availability influence eggshell traits?

Ca availability (natural and supplemented) was not associated with eggshell Ca concentrations but was associated with other eggshell traits (Table 3.3) such as eggshell spotting (Fig. 3.4).

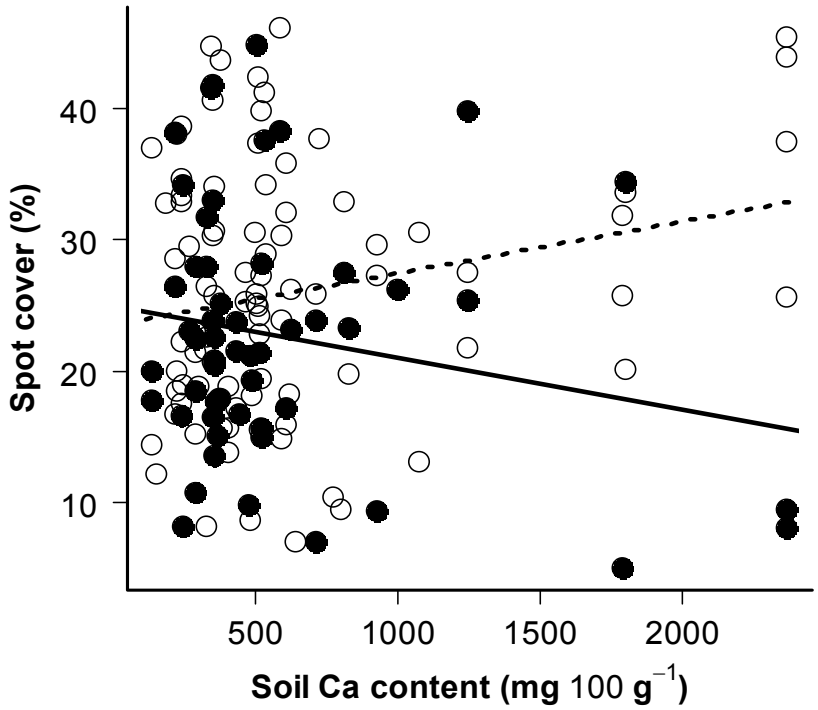


Figure 3.4. The relationship between local soil Ca concentration and eggshell spot cover of eggs laid by Ca-supplemented (filled circles and solid line) and un-supplemented (control) (open circles and dotted line) great tits at Chaddesley Woods NNR, Worcs., UK. Lines of best fit are estimated by ordinary least squares regression.

Table 3.3. Statistical outputs from models of the effects of Ca availability on eggshell traits (χ^2 and associated *P* values [two sets of *P* values are given: *P* – calculated using ANOVA, and *P* mcmc – calculated using MCMC simulations]) of great tits and blue tits at Chaddesley Woods NNR, Worcs., UK, during 2010-2012. Model simplification was performed using backward stepwise regression to find the minimal adequate model using Chisq-tests to compare the residual deviance of models including and excluding explanatory variables. Response variables are given in bold in the column on the left with the significant ($\alpha = 0.05$) explanatory variables in the model given below. 'N/A' signifies a non-significant output.

	Great tits					Blue tits				
	Model effect estimate ± SE	**df**	***Chisq***	***P***	***P* mcmc**	**Model effect estimate ± SE**	**df**	***Chisq***	***P***	***P* mcmc**
Ca (µg mm^{-2})										
Local soil Ca:Treatment	0.0032 ± 0.0019	7	2.69	0.10	N/A	$-3.95\text{x}10^{-5} \pm 2.97\text{x}10^{-5}$	7	0.0002	0.98	N/A
Local soil Ca	$1.82\text{x}10^{-4} \pm 8.84\text{x}10^{-3}$	5	5.24	0.02	0.89	0.00018 ± 0.0015	6	0.02	0.90	N/A
Treatment	-0.52± 0.93	6	0.31	0.58	N/A	-1.04 ± 1.23	5	0.70	0.40	N/A
Protoporphyrin (µg g^{-1})										
Local soil Ca:Treatment	-0.00036 ± 0.00053	8	0.44	0.51	N/A	$-7.91\text{x}10^{-5} \pm 5.10\text{x}10^{-4}$	8	0.024	0.88	N/A
Local soil Ca	0.00028 ± 0.00026	6	3.68	0.055	N/A	0.00019 ± 0.00025	7	0.53	0.47	N/A
Treatment	-0.050 ± 0.26	7	0.035	0.85	N/A	**-0.46 ± 0.20**	**6**	**4.51**	**0.034**	**0.018**
Ca (g mm^{-2})	-0.036 ± 0.020	6	12.41	<0.001	0.097	0.00047 ± 0.015	5	9.45	<0.001	0.93

Spot cover (%)										
Local soil Ca:Treatment	**0.0073± 0.0033**	**8**	**4.91**	**0.027**	**0.03**	0.0028 ± 0.0039	8	0.51	0.47	N/A
Local soil Ca	-0.0035 ± 0.0026		N/A	N/A	0.18	-0.0013 ± 0.0019	7	0.42	0.52	N/A
Treatment	-0.81 ± 2.60		N/A	N/A	0.76	1.26 ± 1.68	6	0.55	0.46	N/A
Ca (g mm^{-2})	0.16 ± 0.12	8	46.22	<0.0001	0.18	-0.17 ± 0.11	5	31.74	<0.0001	0.13
Spot intensity										
Local soil Ca:Treatment	$7.27x10^{-6}$ ± $1.57x10^{-5}$	7	0.22	0.64	N/A	$6.08x10^{-6}$ ± $1.53x10^{-5}$	7	0.15	0.70	N/A
Local soil Ca	$-1.25x10^{-5}$ ± $7.49x10^{-6}$	6	0	1	N/A	$-9.16x10^{-6}$ ± $7.62x10^{-6}$	5	1.44	0.23	N/A
Treatment	0.011 ± 0.0080	5	1.81	0.18	N/A	$-1.15x10^{-3}$ ± $6.52x10^{-3}$	6	0.031	0.86	N/A
Ca (g mm^{-2})	$4.12x10^{-4}$ ± $5.80x10^{-4}$	8	0	1	N/A	$-1.54x10^{-4}$ ± $4.25x10^{-4}$	8	0	1	N/A
Thickness (µm)										
Local soil Ca:Treatment	-0.0011 ± 0.0013	8	0.69	0.41	N/A	0.00068 ± 0.0015	8	0.231	0.65	N/A
Local soil Ca	0.0004 ± 0.0006	6	5.15	0.02	0.47	-0.0005 ± 0.0007	7	6.22	0.47	N/A
Treatment	-0.51 ± 0.61	7	0.67	0.41	N/A	**1.56 ± 0.62**	**6**	**5.94**	**0.015**	**0.01**
Ca (g mm^{-2})	**0.36 ± 0.0044**	**6**	**94.90**	**<0.0001**	**<0.001**	**0.23 ± 0.041**	**6**	**47.66**	**<0.0001**	**0.0001**

No difference was found between treatment areas in spot coverage of eggs laid by females in territories containing low soil Ca concentration, but in territories containing high soil Ca concentration, a lower percent of spot cover was correlated with Ca-supplementation (Fig. 3.4). In concurrence with findings in Chapter Two, eggshells containing greater ash mass (Ca) contents had thicker shells.

In blue tits, natural Ca availability was not correlated to any of eggshell traits included in the model. However, Ca supplementation was positively correlated with eggshell thickness and eggshell protoporphyrin concentration (Table 3.3, Fig. 3.5). Furthermore, eggshell thickness was positively correlated with greater Ca concentration.

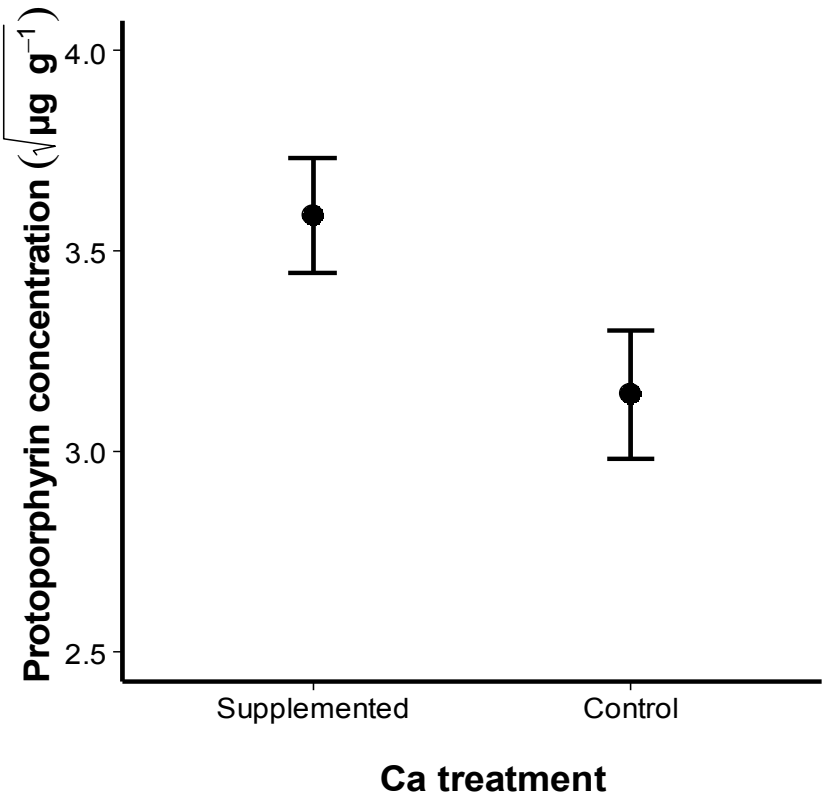


Figure 3.5. The relationship between Ca treatment and mean (± 1 SE) eggshell protoporphyrin concentration (μg g^{-1}) in eggs laid by blue tits at Chaddesley Woods NNR, Worcs., UK.

This relationship was more pronounced in eggs laid by control females than those laid by Ca-supplemented females (Fig. 3.6).

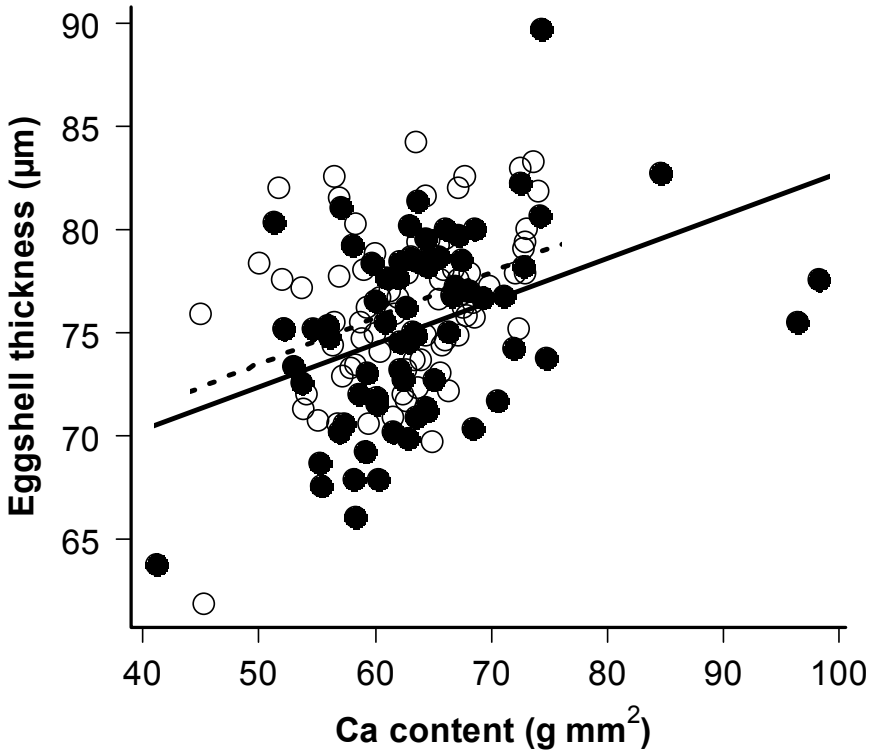


Figure 3.6. The relationship between eggshell Ca concentration and thickness of eggs laid by Ca-supplemented female (filled circles and solid line) and un-supplemented control (open circles and dotted line) blue tits at Chaddesley Woods NNR, Worcs., UK. Lines of best fit are estimated by ordinary least squares regression.

3.4.2 Do life-history traits influence eggshell parameters?

Life-history traits were not associated with most eggshell traits studies of eggs laid by either species (Table 3.4). In great tits, clutch size was positively correlated with both eggshell thickness (Fig. 3.7a) and protoporphyrin concentration (Fig. 3.7b).

Table 3.4. Statistical outputs from models of the effects of life-history traits on eggshell traits (χ^2 and associated *P* values [two sets of *P* values are given: *P* – calculated using ANOVA, and *P* mcmc – calculated using MCMC simulations]) of great tits and blue tits at Chaddesley Woods NNR during 2010-2012. Model simplification was performed using backward stepwise regression to find the minimal adequate model using Chisq-tests to compare the residual deviance of models including and excluding explanatory variables. Interactions between explanatory variables were initially included in models but only those deemed significant remained in the model and are shown below. Response variables are given in bold in the column on the left with the significant ($\alpha = 0.05$) explanatory variables in the model given below. 'N/A' signifies a non-significant output.

	Great tits					**Blue tits**				
	Model effect estimate ± SE	**df**	***Chisq***	***P***	***P* mcmc**	**Model effect estimate ± SE**	**df**	***Chisq***	***P***	***P* mcmc**
Ca (g mm^{-2})										
Wing length:Tarsus length	-0.12 ± 0.75	10	0.02	0.90	N/A	2.70 ± 1.52	9	2.94	0.09	N/A
Tarsus length (mm)	0.70 ± 1.23	7	16.89	<0.0001	0.57	-0.22 ± 1.77	8	0.02	0.90	N/A
Wing length (mm)	-0.013 ± 0.31	7	49.89	<0.0001	0.99	0.072 ± 0.80	7	221.29	<0.0001	0.80
Incubation initiation	-0.18 ± 0.09	7	121.69	<0.0001	0.15	-0.22 ± 0.14	7	95.45	<0.0001	0.25
Clutch size	-0.43 ± 0.32	8	1.68	0.20	N/A	-0.40 ± 0.41	7	17.48	<0.0001	0.39
Lay date	-0.03 ± 0.17	9	0.019	0.89	N/A	-0.32 ± 0.16	10	3.58	0.06	N/A
Protoporphyrin (µg g^{-1})										
Wing length:Tarsus length	- 0.28 ± 0.16	10	54.76	<0.0001	0.086	-0.51 ± 0.23	10	0.049	0.82	N/A
Tarsus length (mm)	0.20 ± 0.12	10	N/A	N/A	N/A	0.21 ± 0.25	9	0.71	0.40	N/A
Wing length (mm)	5.59 ± 3.31	10	N/A	N/A	N/A	-0.092 ± 0.14	7	89.50	<0.0001	0.55
Incubation initiation	-0.0058 ± 0.032	10	97.23	<0.0001	0.86	-0.030 ± 0.034	7	22.51	<0.0001	0.67
Clutch size	**-0.19 ± 0.083**	**10**	**52.23**	**<0.0001**	**0.027**	0.0024 ± 0.034	7	5.58	0.018	0.96
Lay date	0.082 ± 0.044	10	53.39	<0.0001	0.068	0.067 ± 0.035	8	2.89	0.089	N/A

Spot cover (%)										
Wing length:Tarsus length	1.86 ± 1.50	10	1.52	0.22	N/A	3.48 ± 1.96	10	270.57	<0.0001	0.10
Tarsus length (mm)	3.09 ± 2.48	7	15.89	<0.0001	0.22	-2.18.16 ± 121.51	N/A	N/A	N/A	0.10
Wing length (mm)	-1.02 ± 0.64	7	52.30	<0.0001	0.12	-58.75 ± 33.06	N/A	N/A	N/A	0.10
Incubation initiation	0.036 ± 0.15	7	169.9	<0.0001	0.79	**0.65 ± 0.23**	**10**	**106.00**	**<0.0001**	**0.047**
Clutch size	-0.26 ± 0.67	9	0.15	0.70	N/A	**-1.78 ± 0.56**	**10**	**16.62**	**<0.0001**	**0.012**
Lay date	-0.14 ± 0.29	10	0.24	0.62	N/A	**-0.76 ± 0.24**	**10**	**7.50**	**<0.01**	**0.008**
Spot intensity										
Wing length:Tarsus length	0.0022 ± 0.0054	8	0.88	0.35	N/A	0.0084 ± 0.0071	7	0.49	0.48	N/A
Tarsus length (mm)	0.00034 ± 0.0073	6	0	1	N/A	-0.011 ± 0.0077	6	0	1	N/A
Wing length (mm)	-0.0027 ± 0.0025	7	0	1	N/A	-0.011 ± 0.0066	5	0	1	N/A
Incubation initiation	0.0010 ± 0.00085	10	0	1	N/A	0.0017 ± 0.0008	9	0	1	N/A
Clutch size	-0.0027 ± 0.0027	9	0	1	N/A	-0.0029 ± 0.0022	10	0	1	N/A
Lay date	0.0013 ± 0.00067	5	3.64	0.056	0.14	0.00063 ± 0.0006	8	0.97	0.32	N/A
Thickness (µm)										
Wing length:Tarsus length	-0.43 ± 0.57	9	0.59	0.44	N/A	-0.12 ± 0.79	10	0.02	0.89	N/A
Tarsus length (mm)	-0.27 ± 0.92	8	15.23	<0.0001	0.78	0.001 ± 0.88	9	0.00	0.99	N/A
Wing length (mm)	0.20 ± 0.26	8	43.87	<0.0001	0.48	0.57 ± 0.38	8	208.63	<0.0001	0.33
Incubation initiation	-0.05 ± 0.06	8	124.37	<0.0001	0.27	**-0.18 ± 0.08**	**8**	**75.86**	**<0.0001**	**0.038**
Clutch size	**-0.56 ± 0.26**	**8**	**4.53**	**0.03**	**0.042**	**0.46 ± 0.20**	**8**	**18.24**	**<0.0001**	**0.046**
Lay date	0.058 ± 0.11	10	0.28	0.60	N/A	0.19 ± 0.078	8	5.64	0.018	0.072

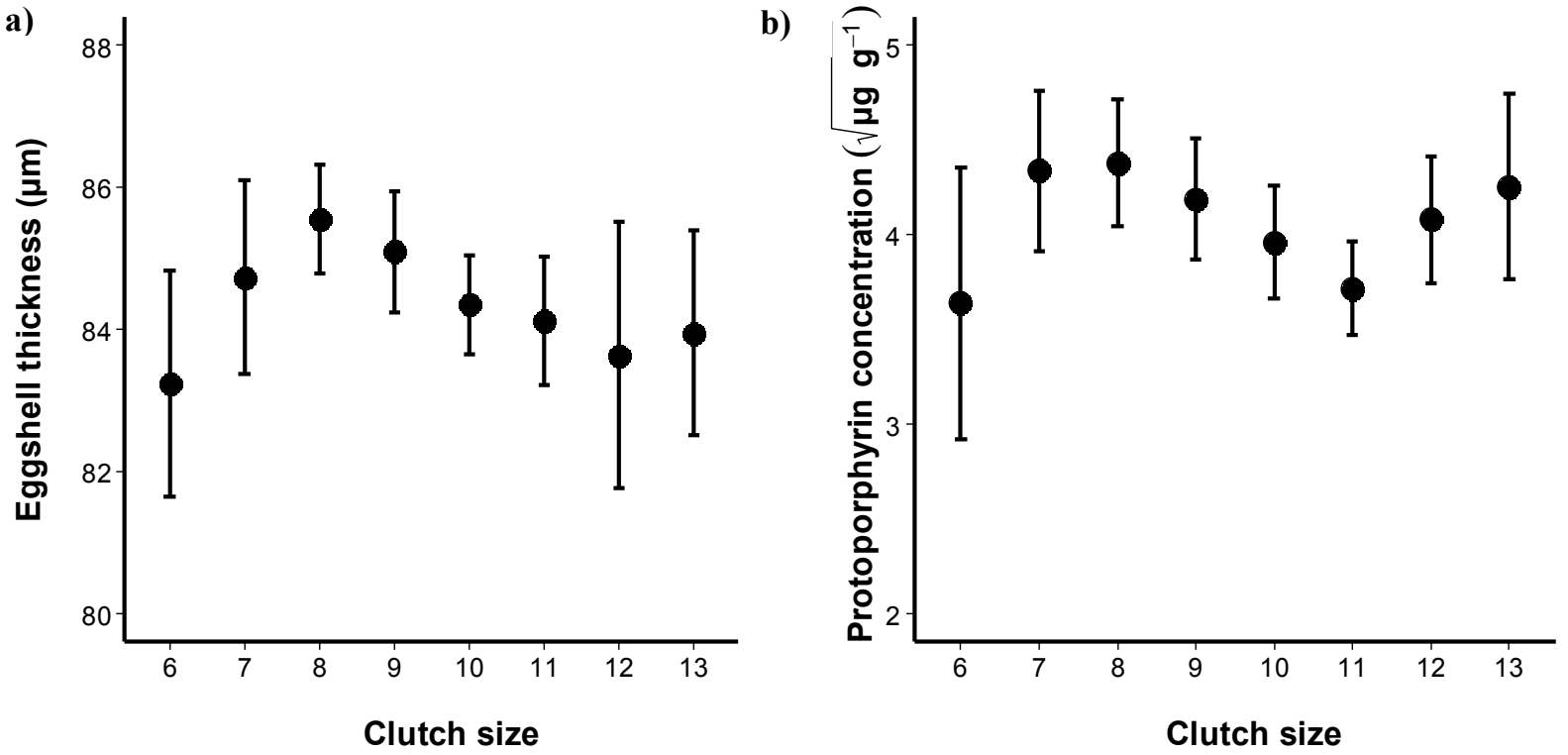


Figure 3.7. The relationship between clutch size (including those eggs removed) and (a) mean (± 1 SE) eggshell thickness, and (b) mean (± 1 SE) protoporphyrin concentration of eggs laid by great tits at Chaddesley Woods NNR, Worcs., UK.

In eggs laid by blue tits, percentage spot cover was negatively influenced by lay date (Table 3.4, Fig. 3.8) and clutch size (Table 3.4, Fig. 3.9), and positively influenced by incubation initiation date (Table 3.4, Fig. 3.10). In eggs laid by blue tits, eggshell thickness was negatively influenced by incubation initiation date (Table 3.4, Fig. 3.11), and positively influenced by clutch size (Table 3.4, Fig. 3.12).

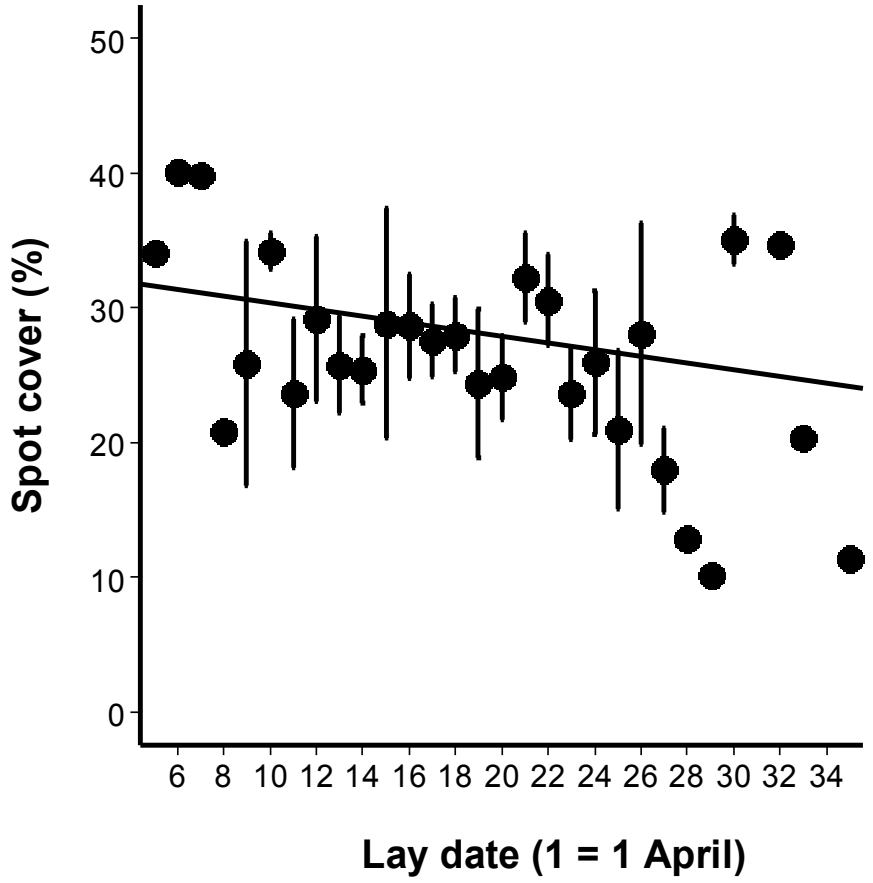


Figure 3.8. The relationship between lay date and mean (± 1 SE) spot cover of eggs laid by blue tits at Chaddesley Woods NNR, Worcs., UK. Date is treated as continuous because we were looking for trends in the variable (intercept = 31.84, slope = -0.25). Lines of best fit are included for visual comparison as estimated by ordinary least squares regression.

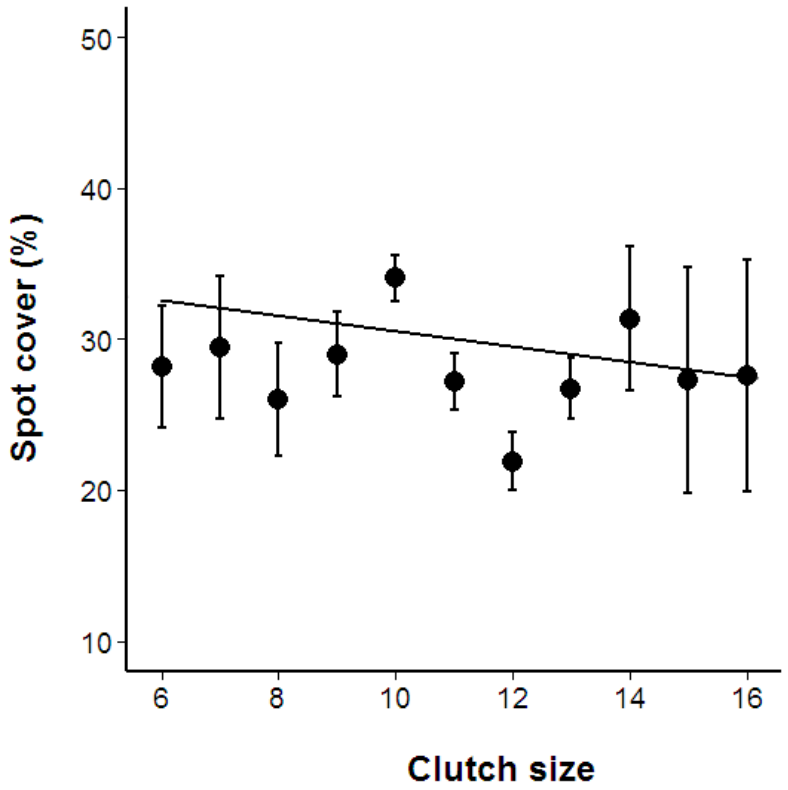


Figure 3.9. The relationship between clutch size (including those eggs removed) and mean (± 1 SE) spot cover of eggs laid by blue tits at Chaddesley Woods NNR, Worcs., UK. Date is treated as continuous because we were looking for trends in the variable (intercept = 32.86, slope = -0.49). Lines of best fit are included for visual comparison as estimated by ordinary least squares regression.

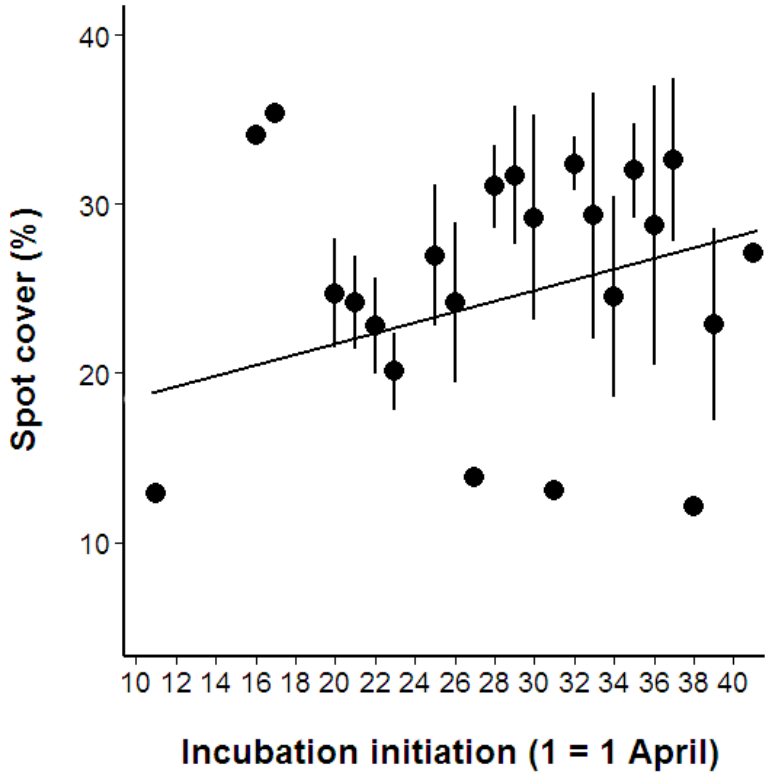


Figure 3.10. The relationship between incubation initiation date and mean (± 1 SE) spot cover of eggs laid by blue tits at Chaddesley Woods NNR, Worcs., UK. Date is treated as continuous because we were looking for trends in the variable (intercept = 18.26, slope = 0.32). Lines of best fit are included for visual comparison as estimated by ordinary least squares regression.

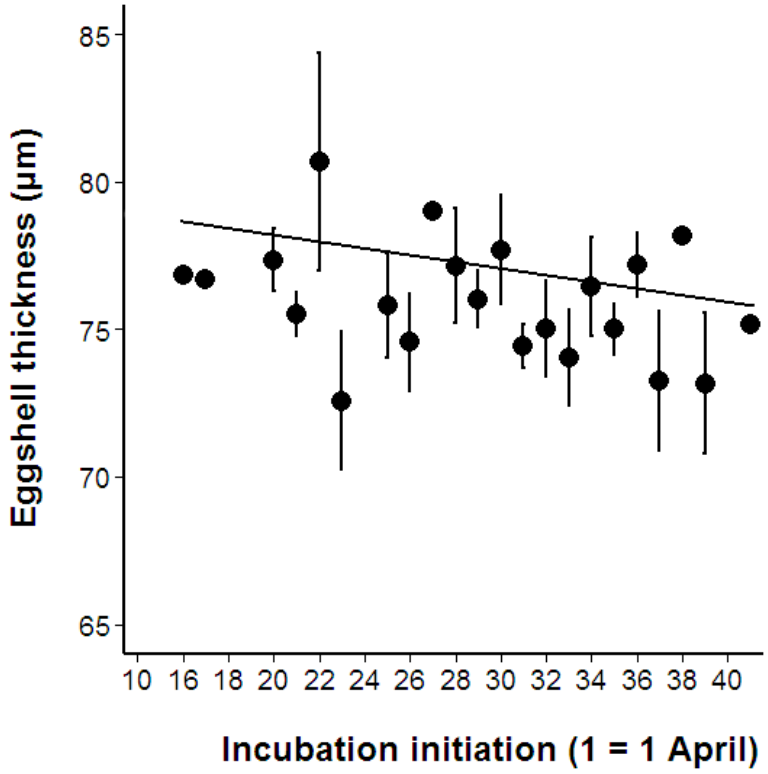


Figure 3.11. The relationship between incubation initiation date and mean (± 1 SE) eggshell thickness of eggs laid by blue tits at Chaddesley Woods NNR, Worcs., UK. Date is treated as continuous because we were looking for trends in the variable (intercept = 79.00, slope = -0.11). Lines of best fit are included for visual comparison as estimated by ordinary least squares regression.

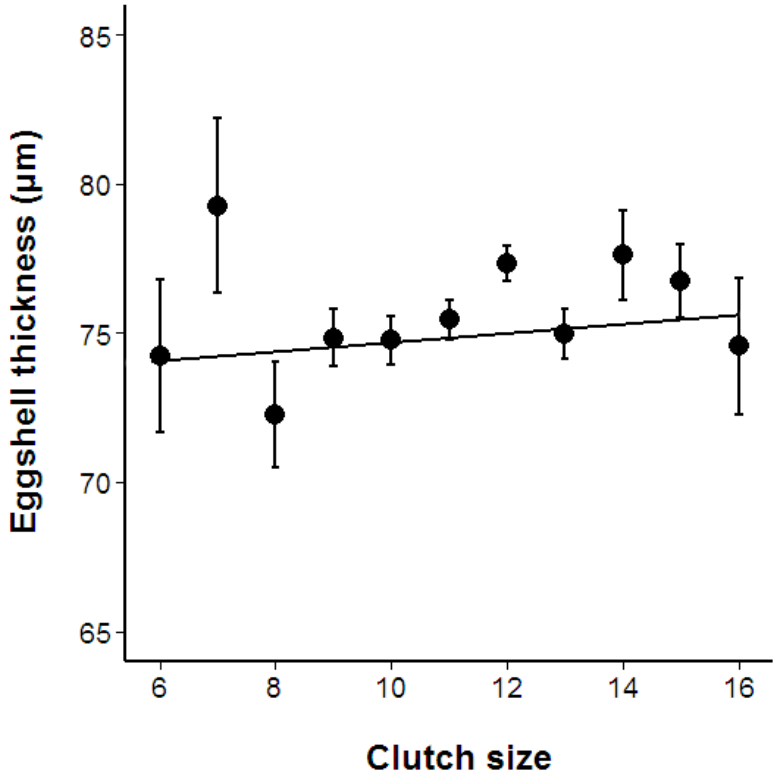


Figure 3.12. The relationship between clutch size (including those eggs removed) and mean (± 1 SE) eggshell thickness of eggs laid by blue tits at Chaddesley Woods NNR, Worcs., UK. Date is treated as continuous because we were looking for trends in the variable (intercept = 73.91, slope = 0.16). Lines of best fit are included for visual comparison as estimated by ordinary least squares regression.

3.5 Discussion

In accordance with our predictions, increased Ca availability was positively associated with eggshell thickness in eggs laid by both species. Contrary to our predictions, Ca supplementation was positively associated with protoporphyrin content of eggs laid by blue tits. As predicted, changes in physical eggshell attributes coincided with changes in females' breeding behaviour. In great tits, clutch size was negatively associated with mean eggshell thickness and protoporphyrin content (of the fourth laid egg). In blue tits, percentage spot cover was positively associated with incubation initiation but negatively associated with lay date and clutch size. Eggshell thickness was positively associated with clutch size but negatively associated with incubation initiation.

3.5.1 Does Ca availability influence eggshell traits?

Eggshell Ca concentration was not influenced by either local soil Ca concentration or Ca supplementation in either species, suggesting that breeding females in both populations do not suffer from Ca-deficiency, despite Chaddesley Woods NNR being known as an acidic woodland (Ragg et al. 1984) and the corresponding low levels of soil Ca found in some areas (Table 3.1, Fig. 3.3). In an area severely affected by acid precipitation Ramsay and Houston (1999) found no significant effects of Ca supplementation on the breeding parameters of blue tits. Despite very low snail densities in their study area (i.e. 0.36 snails m^{-2} compared to 92-214 snails m^{-2} found in a deciduous woodland with nutrient-rich soils – Tilgar et al. 1999, Mänd et al. 2000a), the gizzards of control females were found to contain sufficient Ca for eggshell formation. Female passerines have been recorded to forage over 400 m away from home territories when food is scarce (Wilkin et al. 2009a, van Overveld and Matthysen 2010)

suggesting that local soil Ca concentration may not be that informative in understanding foraging behaviour of breeding females.

In blue tits, Ca supplementation increased eggshell thickness (Fig. 3.6) and decreased protoporphyrin concentration (Table 3.3, Fig. 3.5). Both of these findings strongly support the structural-function hypothesis (Gosler et al. 2005) indicating that blue tit females in this population may be Ca-deficient. However, eggshell traits were not influenced by local soil Ca concentration. Despite not influencing eggshell Ca concentration, local soil Ca concentration and Ca supplementation did influence spot cover on eggshells laid by great tits, but not as expected (Fig. 3.4). No difference was found between treatment areas in spot coverage of eggs laid by females in territories containing low soil Ca concentration, but in territories containing high soil Ca concentration, un-supplemented females laid eggs with a higher percent of spot cover than Ca-supplemented females (Fig. 3.4). Ca-supplemented females breeding on territories containing high soil Ca concentrations may have reduced energetic costs to obtain sufficient nutrients for eggshell formation compared to un-supplemented females or those breeding in territories containing low soil Ca concentrations. During egg-laying, female energy requirements might be more important than Ca-specific foraging (Perrins 1970). Great tits in this population reduced reproductive investment in conditions of low food availability to maximise their own survival prospects until their next breeding attempt (Webber 2012). When food (supplementary) availability increased, females experienced reduced energetic costs of egg-laying, with those females in low body condition benefitting most (Webber 2012). Providing Ca supplements at the nest could therefore greatly reduce a female's cost of Ca-specific foraging, explaining why the Ca supplements had greater influence on eggshell traits compared with local soil Ca concentration. Clutch size and recruitment are positively related to soil Ca content at spatial scales over 300 m from each

nestbox, and fledgling mass (at day 15) is positively related to soil Ca content at a distance of 58 m from the nestbox, suggesting that local soil Ca may be more important to foraging adults during nestling stages (Wilkin et al. 2009a).

Birds in a naturally base-poor environment may have had time to adapt to low Ca availability, whereas soil acidification is largely caused by anthropogenic inputs and, therefore, is too recent to have influenced adaption (Graveland and Drent 1997, Mänd et al. 2000b). Ca supplementation has a larger impact on the reproductive biology of birds when breeding conditions are less optimal (Mänd et al. 2000b) and the trade-off between time spent searching for food and Ca is greater (Mänd et al. 2000a). Ca limitation in the UK may not be as problematic as in some other countries (e.g. The Netherlands – Graveland and Berends 1997), as blue and great tits only lay single clutches due to shorter breeding seasons in the UK (Crick et al. 1993).

3.5.2 Do life-history traits influence eggshell parameters?

In blue tits, spot cover on eggshells was associated with a delay in incubation initiation date (Table 3.4, Fig. 3.10). Food availability increases with later date, suggesting that later-laying females are in higher body condition (Van Noordwijk and de Jong 1986). Potentially, females delay clutch initiation so as to find sufficient nutrients to produce larger eggs containing more egg nutrients and hence potentially resulting in larger chicks. However, females that lay early tend to raise more young (Perrins 1991, Verhulst and Tinbergen 1991) but early laying can be halted when environmental conditions (e.g. temperature, food availability) are sub-optimal (Dhondt et al. 1983). Females that lay early do not necessarily start incubation early. Eggshell thickness was negatively associated and spot cover was positively associated with later

incubation initiation dates (Table 3.4, Fig. 3.11). An increased amount of spotting might compensate for reduced eggshell thickness to increase shell strength (Gosler et al. 2005) and/or for decreasing gas conductance (Higham and Gosler 2006; but see Maurer et al. 2011b). Eggs with thicker shells require a prolonged incubation period (Rahn and Ar 1974) which may have been allowed for by females adjusting laying and incubation initiation dates.

Blue tits laying larger clutches were associated with eggs with thicker shells (Fig. 3.12) and a lower percentage spot cover (Table 3.4, Fig. 3.9). Larger clutches could directly influence nest microclimate experienced by embryos during incubation (Reid et al. 2000), and so thicker eggshells might reduce excess water loss due to increased temperatures. Moreover, eggs from larger clutches potentially remain un-incubated for longer and therefore thicker eggshells may prevent excessive water loss prior to the start of incubation (Tazawa and Whittow 2000). This would mean that eggs should have thinner shells as the laying sequence progresses. Unfortunately, this was not investigated in the present study but has been found in other studies (e.g. Reynolds 2001). In Spain, pigment 'spread' was positively related to clutch size in blue tits (Sanz and García-Navas 2009), so that eggs from larger clutches tended to be maculated evenly, whereas those from smaller clutches tended to be maculated at one end of the shell only. It is simply possible that females capable of laying larger clutches are also more capable of foraging for all the nutrients required for eggshell formation.

Great tits lay smaller clutches than blue tits so eggs likely do not encounter the same extent of changes in nest microclimate caused by larger clutches, hence explaining the different relationships found between clutch size and eggshell thickness in the two focal species.

3.5.3 Inter-specific differences in response to Ca requirements

Results from the present study suggest that the two focal species react to the Ca demands of egg production differently. Both species altered the physical properties (i.e. eggshell thickness, pigment concentration) of the eggshell to cope with changes in Ca availability, but blue tits have also modified aspects of their breeding biology (i.e. clutch size, incubation initiation date) to compensate for changes in demands of eggshell formation. Great tits and blue tits are able to adjust to variations in habitat by advancing or delaying lay dates or by changing their reproductive success (Dhondt et al. 1984, Dhondt 1987) and blue tits are considered to have a wider foraging niche in terms of the range of prey species consumed (Dhondt 1977, Török and Tóth 1999). This difference between the two parid species in their response to environmental variability may indicate a difference in magnitude or even a diverging of life-history strategies or selection pressures or an inter-specific divergence in them.

The Ca content estimations for the whole clutch indicated that blue tits invest more Ca into their clutches than great tits, regardless of Ca availability. This is surprising due to their smaller body size. Small passerines cannot store Ca so females must ingest Ca required for eggshell formation prior to and during egg-laying, most of which is obtained from snail shells (Graveland and van Gijzen 1994). The greater Ca requirement of blue tits might be met by their smaller body sizes, enabling them to access a wider range of Ca-rich foods. Therefore, having greater access to natural Ca dietary sources might explain why blue tits have a decreased sensitivity to changes in natural Ca availability.

3.6 Conclusions

Ca supplementation had a greater effect on eggshell characteristics of both species than local soil Ca concentration, suggesting that females in this population are not suffering from severe Ca limitation but have adapted to these conditions using different strategies without displaying obvious reproductive problems. However, Ca supplements probably provide females of both species with more time to invest in other activities (e.g. foraging for foods rich in other nutrients, incubation). Future Ca supplementation studies should therefore include considerations of the activity budgets of breeding females.

This chapter demonstrated that Ca availability influences eggshell parameters of both blue tits and great tits. The next chapter investigates whether protoporphyrin pigment spots can be used as a proxy for the total protoporphyrin content of eggshells. It compares results of two different eggshell scoring methods, visual pigment scoring, a widely used method, and a more recently introduced method, the pixel pigment scoring method, with measures of total protoporphyrin content of eggshells from chemical analysis.

CHAPTER 4

EGGSHELL SPOT SCORING METHODS CANNOT BE USED AS A RELIABLE PROXY TO DETERMINE PIGMENT QUANTITY

4.1 Abstract

Eggshell maculation of most passerines is due to the deposition of the pigment protoporphyrin which is produced during biosynthesis of blood haem. Its functional significance has only received empirical attention in recent years. This interest has generated a number of hypotheses, some of which remain untested, partly because the quantification of protoporphyrin is analytically challenging and can be prohibitively expensive. Many studies have therefore used the extent of eggshell spotting as a proxy for total eggshell protoporphyrin concentration, although this has not been formally tested. Pigment scoring involves recording visible eggshell pigment attributes, such as spot intensity, distribution and size. Since even immaculate eggs can contain some protoporphyrin, there remains doubt over the degree to which visible pigment correlates with total pigment content of the shell. In this study, we tested whether visible pigment scoring can be used as a proxy for protoporphyrin concentration of an eggshell. We used pigmented eggshells of two common British passerine species to compare eggshell spot intensity, distribution and spot size (as used by the visual pigment scoring method) with direct measures of their protoporphyrin concentration. In addition, we compared an alternative method of pigment scoring, the pixel pigment scoring method, using a computer programme to quantify the number of pixels exceeding a specified colour threshold. We demonstrate that although results from both scoring methods were positively correlated with eggshell protoporphyrin concentrations, the correlations were not sufficiently strong to be used as a proxy when precise determination of pigment content is required. Spotting on great tit eggshells produced stronger correlations with eggshell protoporphyrin concentrations than blue tit eggshells. We encourage comparative methodological studies such as ours to validate eggshell pigment data across different focal species.

4.2 Introduction

The coloration and patterning of avian eggshells is caused by two main types of pigments. These are protoporphyrin IX (brownish hues) and biliverdin (blue and green hues) (Kennedy and Vevers 1973, Gorchein et al. 2009). Protoporphyrin, produced during the biosynthesis of blood haem (Burley and Vadhera 1989), occurs in both the calcite and cuticular layers of the eggshell (Roberts 2004), and is often localized as maculation (i.e. pigment spots) either in distinct layers within or upon the eggshell (Kennedy and Vevers 1976, Kilner 2006). Maculated eggs are represented in all of the 22 passerine families of the Holarctic (Sibley and Monroe 1990).

As the direct measurement of protoporphyrin concentration can be analytically challenging and financially expensive, most studies have used alternative methods to quantify the amount of pigment in eggshells. Visual pigment scoring is such a method, recording for example eggshell pigment intensity, distribution and spot size. Scoring can be carried out on eggs *in situ* (e.g. Gosler et al. 2000) or retrospectively from photographs (e.g. Mägi et al. 2012). One such method was described by Gosler and colleagues (2000, 2005) for the eggs of great tits, and has been used in studies of eggshell thickness (e.g. Gosler et al. 2005, Mägi et al. 2012), the ability of females to counter anaemia during egg-laying (De Coster et al. 2012), and the inheritance of eggshell patterning (Gosler et al. 2000). This method has further been applied to the eggs of other species including blue tits (e.g. Sanz and García-Navas 2009, García-Navas et al. 2011, Holveck et al. 2012), house sparrows (*Passer domesticus*) (López de Hierro and De Neve 2010), and northern lapwings (Bulla et al. 2012). The original method of Gosler (2000, 2005) was designed to quantify the appearance of eggshells, and was never intended to replace direct measurement of protoporphyrin concentration. However, it has subsequently been widely inferred to reflect the amount of pigment in eggshells (reviewed in

Reynolds et al. 2009). It has long been known that even apparently immaculate eggshells contain some protoporphyrin (Kennedy and Vevers 1973), so while it seems reasonable to assume that eggshells with larger, darker, and/or more spots contain more total protoporphyrin (e.g. Cassey et al. 2012a), the strength and linearity of this relationship has never been determined empirically.

Pigment scoring has provided additional useful information and is not necessarily related to the amount of protoporphyrin in eggshells. In great tits, protoporphyrin-pigmented spots have been found to demarcate thinner areas of the shell, with darker spots covering thinner areas than paler spots, and both spot darkness and spread were negatively correlated with local soil Ca content (Gosler et al. 2005). These maculation traits were further found to be heritable along the female line (Gosler et al. 2000). In blue tits, females laying more maculated eggs were found to be in lower body condition (Martínez-de la Puente et al. 2007), and eggs with larger and less evenly distributed spots had higher antibody concentrations (Holveck et al. 2012).

Bird eggs provide an effective bio-monitoring tool (e.g. Ormerod and Tyler 1990, Van den Steen et al. 2010) due to their high lipid contents which concentrate hydrophobic contaminants (Van den Steen et al. 2006). Eggshells in particular are sensitive to persistent organic pollutants, either directly, by blocking Ca uptake to the shell gland (Ratcliffe 1970, Lundholm 1997, Jagannath et al. 2008) or indirectly, by disrupting the haem biosynthesis pathway and consequently pigment concentrations (Casini et al. 2003). Because of the role of protoporphyrins as indicators of environmental pollution (Wayland et al. 1998, Casini et al. 2001), resident passerine species such as blue tits and great tits are particularly effective in monitoring local environmental contamination because of their small territories and foraging areas (Moore 1966, Dauwe et al. 2006).

In this study we investigated whether visible pigment scoring is a reliable proxy for the measurement of the amount of protoporphyrin in eggshells. Using eggshells of two common British species of tit, we compared eggshell spot intensity, distribution and spot size (as used by the visual pigment scoring method) with direct measures of protoporphyrin. Furthermore, we examined an additional method of pigment scoring, the pixel pigment scoring method (PPSM), using a specially designed computer programme which quantified the number of pixels exceeding a specified threshold colour gradient. This method has already been used to quantify eggshell pigment spotting(e.g. Stoddard and Stevens 2010, Cassey et al. 2012a) and has shown that increased maculation on the eggshell corresponds to an associated increase in eggshell protoporphyrin content (Cassey et al. 2012a).

4.3 Materials and methods

4.3.1 Study site, field methods, egg sampling and pigment analysis

Please refer to sections 2.3.1 to 2.3.4 of Chapter Two.

4.3.2 Photographing eggs

Please refer to section 3.2.4 of Chapter Three.

4.3.3 Visual pigment scoring

Eggshell pigmentation was recorded from photographic images. Only one image, per egg, chosen at random was used. Following Gosler et al. (2000, 2005), we scored the eggshell

pigmentation pattern on the basis of three categories: pigment intensity (I, scored in 0.5 increments, from 1 [palest] to 5 [darkest]), distribution (D, scored in 0.5 increments from 1 [> 90% of spots concentrated at a single end] to 5 [spots evenly distributed]), and spot size (S, scored in 0.5 increments from 1 [small spots] to 3 [large spots]). All eggs were scored blind by a single observer (AGG).

4.3.4 Pixel pigment scoring

Eggshell images were used to quantify eggshell coverage by, and intensity of, pigment spots. Spot cover was defined as the amount of spotting in the foreground compared to the background, based on the number of pixels (different from spot distribution of the visual pigment scoring method which refers to whether spots were concentrated at one end of the eggshell or evenly distributed). Spot pigment intensity was defined as the darkness of the spotting based on greyscale intensity (on a scale of 0 [black] to 1 [white]). The mean pigment scores were calculated for two square sections (square 1 at the B region and square 2 at the E region – please refer to Fig. 3.2 of Chapter Three) from multiple images per eggshell. Please refer to section 3.3.4 of Chapter Three for more details.

The PPSM makes two assumptions: 1. that pigment concentration is uniform over the entire eggshell; and 2. that the distribution and intensity of the spots in the analysed squares represent pigment spotting over the entire eggshell. By using discrete segments centrally located rather than the entire eggshell to quantify pigment spotting, we overcome errors caused by peripheral spotting, such as spots blending in with the background, and repeated quantification of spotting of certain areas of the egg. Squares were located in areas of the eggshell (i.e. the B and E regions) where spotting tends to be concentrated in the two focal

species (Gosler et al. 2005). As mean pigment scores were calculated from multiple images per eggshell (i.e. with the eggshell being rotated 90º between images) which covered a large proportion of the eggshell surface area, scores are assumed to be representative of overall eggshell pigment spotting.

4.3.5 Statistical analysis

Fourth-laid eggs were removed from a total of 72 clutches of the two species, 45 from great tits and 27 from blue tits. Pixel pigment scoring data were collected from all 72 eggs. Visual pigment scoring data were collected from a subset of these, totalling 55 eggs, 28 from great tits and 27 from blue tits. Previous studies (Gosler et al. 2005, Higham and Gosler 2006) state that the three components I, D and S are less informative than their first and second principal components, which combine information from all three variables. The principal components PC1 (darkness) and PC2 (spread) were determined from the correlation matrix of I, D and S for the visual scores only.

Statistical analyses were performed in R 2.14.0 (R Development Core Team 2011) using Pearson's product-moment correlation. Scores produced by both methods were correlated against eggshell pigment content, both as total (half shell) protoporphyrin content (μg) and as concentration of eggshell mass (μg g^{-1}). Species were analysed separately. The distributions of the protoporphyrin content and concentration data were normalised by square-root transformation.

4.4 Results

4.4.1 Scoring principal components of visual pigment

In great tits, increasing PC1 explained 67.4% of the variance (Table 4.1) and was negatively related to spot intensity ($r = -0.93$, $df = 26$, $P < 0.0001$) and positively related to spot distribution ($r = 0.71$, $df = 26$, $P < 0.0001$). PC2 explained 24.6% of the variance (Table 4.1) and was positively related to spot distribution ($r = 0.70$, $df = 26$, $P < 0.0001$) and intensity ($r = 0.35$, $df = 26$, $P = 0.065$).

Table 4.1. Eigenvector loadings of principal components 1 and 2 (PC1 and PC2) (and percentage of variance they explain) from a principal components analysis (PCA) on a correlation matrix of three components of eggshell maculation (I: spot intensity, D: spot distribution; and S: spot size) for great and blue tits breeding at Chaddesley Woods NNR, Worcs., UK in 2010.

Maculation measure	**Great tits**	**Blue tits**
	PCA loadings	
PC1	(67.4%)	(51.1%)
I	-0.84	-0.13
D	0.52	0.96
S	-0.17	-0.24
PC2	(24.6%)	(38.6%)
I	0.52	-0.98
D	0.85	-0.16
S	n/a	-0.12

In blue tits, PC1 explained 51.1% of the variance (Table 4.1) and was positively related to spot distribution ($r = 0.99$, $df = 25$, $P < 0.0001$), but negatively related to spot size ($r = -0.47$, $df = 25$, $P = 0.014$). PC2 explained 38.6% of the variance (Table 4.1) and was negatively related to spot intensity ($r = -0.97$, $df = 25$, $P < 0.0001$).

4.4.2 Visual pigment scoring

Both measures of protoporphyrin (total [μg] and as concentration of eggshell mass [μg g^{-1}]) produced similar results (Table 4.2). Therefore, total protoporphyrin content is not discussed hereafter.

In great tits, eggshells which contained more protoporphyrin had pigment spots of higher intensity (i.e. darker; Fig. 4.1a) and larger size (Fig. 4.1b) but with no difference in spot distribution (Table 4.2, Fig. 4.1c). In blue tits, no correlations were found with eggshell protoporphyrin content and pigment spot intensity (Fig. 4.1a), size (Fig. 4.1b) or distribution (Table 4.2, Fig. 4.1c).

Table 4.2. Pearson's product-moment correlations (*r* and associated *P* values) between two methods of pigment scoring and eggshell protoporphyrin concentration (total and per g of eggshell), of great and blue tits at Chaddesley Woods NNR, Worcs., UK. Visible spot scoring methods are given in bold in the column on the left with the explanatory variable tested given below. Highlighted rows indicate a term that is significant ($\alpha = 0.05$).

	Great tit					Blue tit				
		µg (total)		µg g^{-1}			µg (total)		µg g^{-1}	
	df	***r***	***P***	***r***	***P***	**df**	***r***	***P***	***r***	***P***
Visual Pigment scoring										
I	26	**0.60**	**0.002**	**0.58**	**0.001**	25	-0.15	0.47	-0.11	0.57
D	26	-0.20	0.32	-0.19	0.33	25	0.08	0.69	0.05	0.80
S	26	**0.45**	**0.02**	**0.47**	**0.01**	25	0.02	0.92	-0.04	0.86
PC1	26	**-0.53**	**0.004**	**-0.54**	**0.003**	25	0.09	0.66	0.056	0.78
PC2	26	0.25	0.20	0.26	0.18	25	0.13	0.53	0.10	0.62
Pixel Pigment scoring										
Intensity (B region)	43	**-0.62**	**< 0.001**	**-0.61**	**< 0.001**	25	-0.27	0.18	-0.30	0.13
Intensity (E region)	43	**-0.48**	**0.001**	**0.47**	**0.001**	25	0.07	0.72	0.04	0.84
% Spot cover (B region)	43	0.29	0.05	**0.35**	**0.02**	25	0.31	0.12	**0.41**	**0.04**
% Spot cover (E region)	43	0.058	0.71	0.15	0.32	25	0.14	0.47	0.14	0.50

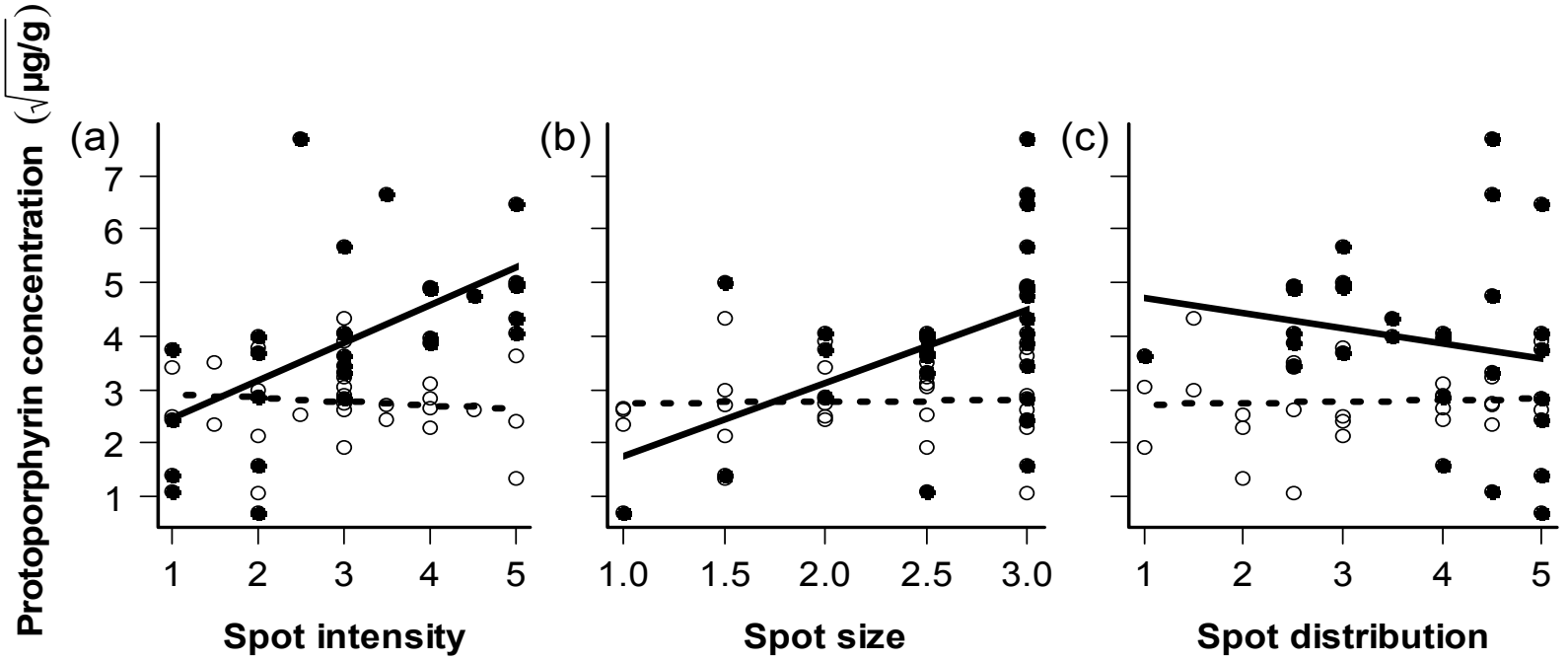


Figure 4.1. The relationship between eggshell protoporphyrin concentration and pigment spot (a) intensity, (b) size, and (c) distribution, as determined from visual pigment scoring of eggshells produced by great tits (filled circles and solid line) and blue tits (open circles and dashed line) breeding in Chaddesley Woods NNR, Worcs., UK in 2010. Lines of best fit are estimated by ordinary least squares regression.

In eggs laid by great tits, pigment spots (i.e. PC1) were darker on eggshells containing higher levels of protoporphyrin (Table 4.2, Fig. 4.2a), but not on eggs laid by blue tits. Pigment spot spread (i.e. PC2) was not correlated with eggshell protoporphyrin concentration in either great tits (Fig. 4.2b) or blue tits (Table 4.2).

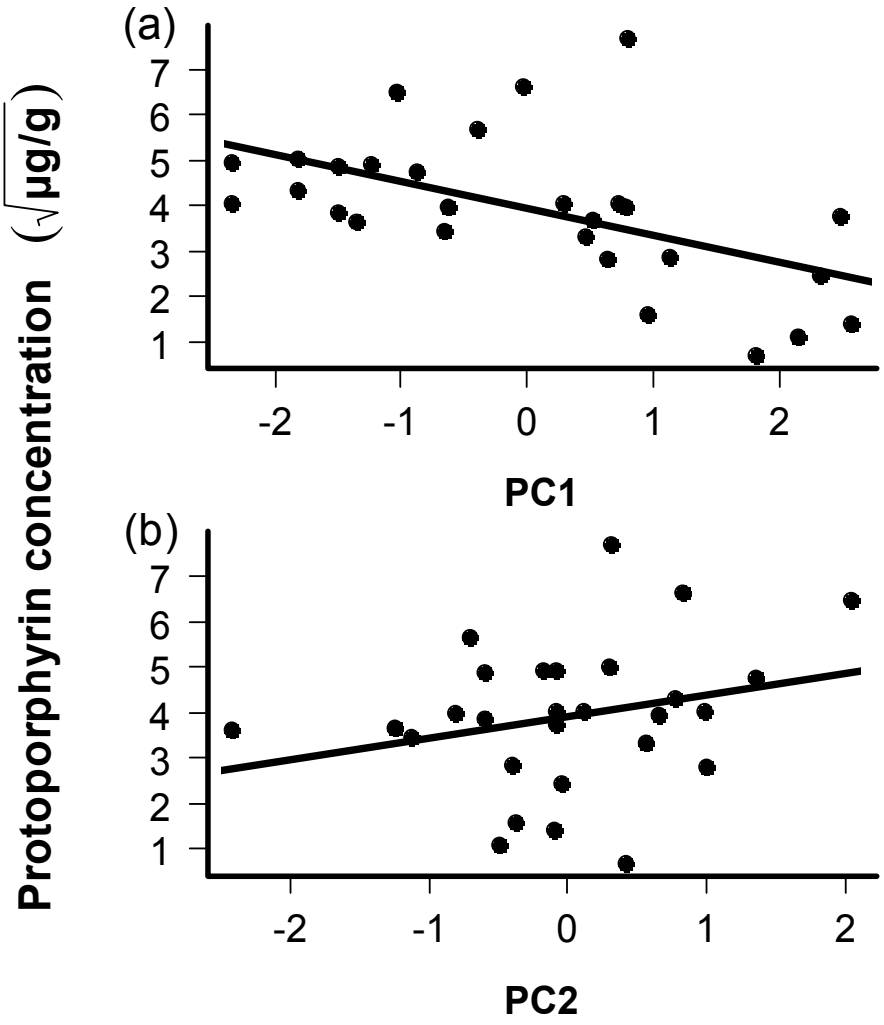


Figure 4.2. The relationship between eggshell protoporphyrin concentration and pigment (a) darkness (PC1) and (b) spread (PC2), as determined from the visual pigment scoring of eggshells produced by great tits breeding in Chaddesley Woods NNR, Worcs., UK in 2010. Lines of best fit are estimated by ordinary least squares regression.

4.4.3 Pixel pigment scoring

Pigment spot characteristics on the B region (i.e. square 1) and the E region (i.e. square 2) of the eggshells were strongly correlated for both species, both for spot intensity and percentage spot cover. Eggshells of both species containing more protoporphyrin had pigment spots covering a greater percentage at the B region (Fig. 4.3a), but not the E region (Table 4.2,

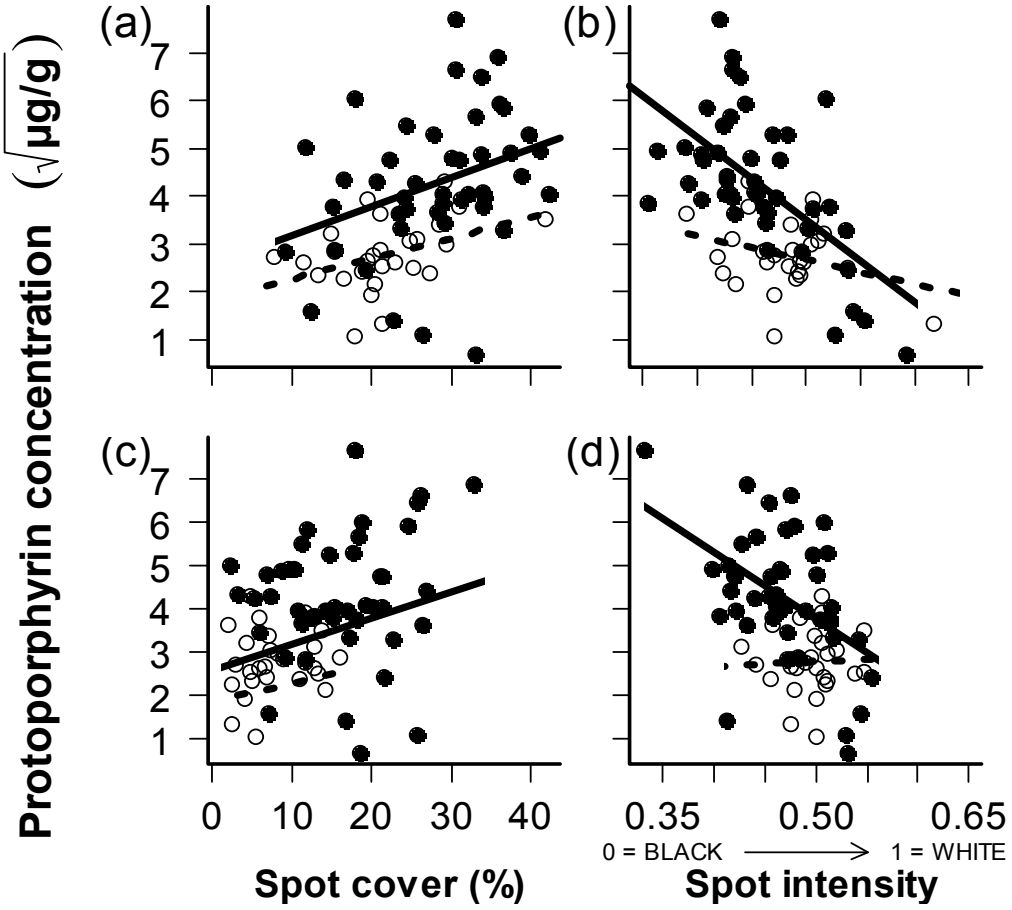


Figure 4.3. The relationship between protoporphyrin concentration and pigment spotting determined from pixel pigment scoring of eggshells for pigment spot (a) cover and (b) intensity for square 1 and for pigment spot (c) cover and (d) intensity for square 2. Eggshells were laid by great tits (filled circles and solid line) and blue tits (open circles and dashed line) breeding in Chaddesley Woods NNR, Worcs., UK in 2010. Lines of best fit are estimated by ordinary least squares regression.

Fig. 4.3c). In great tits, these spots were darker at both the B region (Fig. 4.3b) and at the E region (Table 4.2, Fig. 4.3d). In eggs laid by blue tits, eggshell protoporphyrin concentration and pigment spot intensity were not correlated at either the B region (Fig. 4.3b) or the E region (Table 4.2, Fig. 4.3d).

4.5 Discussion

We investigated whether visible pigment scoring can be used as a proxy for measuring the absolute amount of protoporphyrin within an eggshell. We compared the results of two pigment scoring methods, visual pigment scoring and PPSM, with protoporphyrin in the eggshell using established techniques in analytical chemistry. Results from both scoring methods were correlated with eggshell protoporphyrin concentration. However, the PPSM produced stronger correlations with protoporphyrin concentration (Fig. 4.3) than the visual pigment scoring method (Figs. 4.1 and 4.2). Furthermore, while results from both methods were correlated with eggshell protoporphyrin concentration of great tit eggs, in blue tits visual pigment scoring of eggshells detected no strong correlations between any eggshell spotting parameter and protoporphyrin concentration.

Although results from both scoring methods were correlated with eggshell protoporphyrin concentration, we observe that these correlations are not sufficiently strong to be used as a reliable proxy for protoporphyrin concentration. Correlation coefficients measure the strength of a single linear relationship between two variables and are calculated by comparing how closely the data points are located to the line of best fit (Hazewinkel 2001). This means that even moderately strong correlations, such as were found in this study, have a large dispersion of spot scores, and therefore for each fixed protoporphyrin concentration

there will be a variety of corresponding spot scores. The coefficient of determination (R^2) for the strongest correlation (r = -0.62) with eggshell protoporphyrin (i.e. PPSM on square 1 for spot intensity of great tit eggs; Fig. 4.3b) is 0.38. This means that 62% of the variance in spot intensity was not explained by protoporphyrin concentration. This is likely due, at least in part, to the fact that protoporphyrin is not just represented on the outer (visible) layer of the eggshell but also throughout the shell matrix (Kennedy and Vevers 1976, Roberts 2004, Gosler et al. 2011).

We must stress that we are not criticising previous studies that have used visible pigment scoring of eggs in species whose eggshell spottiness and protoporphyrin concentration have not been corroborated. Significant findings relating eggshell pigmentation patterns to other variables such as female body condition (Martínez-de la Puente et al. 2007), and parental investment (Walters and Getty 2010), are important in their own right. Pigment concentration is not necessarily a more instructive measure of adaptive function than visible maculation because pigment concentration is not simply a function of how much is present (Higham and Gosler 2006), but can be dependent on where and how it is deposited on the shell. However, as eggshells often contain protoporphyrin within the eggshell matrix (Roberts 2004) which is not accounted for by visible pigment scoring methods, we argue that caution must be exercised when relating pigment scores directly to eggshell protoporphyrin concentration.

The visual pigment scoring method was originally described for the eggs of great tits, but has been applied to other species. Therefore, the present study compared methods using the eggs of great tits as well as those of blue tits. Eggshell colour has been found to vary greatly, even between closely related species (Cassey et al. 2010a). As the two focal species exhibit different foraging niches preceding and during egg-laying (Gibb 1954), and blue tits

lay smaller, but more, eggs, it is possible that blue tits deposit most of their protoporphyrin within the shell matrix rather than on the eggshell's surface explaining why no strong correlations were found between eggshell pigment spot intensity and its corresponding protoporphyrin concentration. Most of the hypotheses proposed for the evolution of eggshell colour in species, such as brood parasitism, nest site selection and predation pressure, can themselves cause considerable intra-specific contemporary variation in eggshell colour (Kilner 2006), suggesting that differences in eggshell colour between populations may exist and must be considered when making comparisons. Even the relationship between spot characteristics shown by the PCA found in the present study differed subtly from those found on eggshells laid by great tits at Wytham Woods in Oxford, UK (Gosler et al. 2005).

4.6 Conclusions

We conclude that while visible pigment scoring may give an indication of protoporphyrin concentration, it cannot be used as a reliable proxy for it, at least not in these two tit species. We encourage comparative methodological studies such as ours to validate eggshell pigment data across different focal species. The examined techniques here are still effective for studies aimed at quantifying the appearance of maculated eggshells and may even be valuable for studies on species which are 'endangered' or 'at risk' whose eggs cannot be removed from the wild for destructive use. For these species, we suggest using museum eggshells (Cassey et al. 2010a,b, De Coster et al. 2013) as an alternative to obtain initial information on eggshell protoporphyrin concentration.

In this chapter, it was established that pigment spot scoring cannot be used as a proxy for eggshell protoporphyrin concentration. The following chapter uses Ca supplementation to test the flexibility of females in their expression of eggshell traits in both species. Within-female repeatability of eggshell traits and how these traits are affected by Ca supplementation will be examined as well as whether these eggshell traits are heritable down the female line.

CHAPTER 5

WITHIN-FEMALE VARIATION AND INHERITANCE OF EGGSHELL PIGMENTATION IN TWO SMALL PASSERINES

5.1 Abstract

Eggs have been extensively studied but the causes and consequences of variation in egg traits, especially the relationship between genotypic and phenotypic pressures, remain poorly understood. Phenotypic flexibility, both morphological and behavioural, can vary within an individual in response to environmental cues. Repeatability of a trait could be due to genes inherited down the female line or caused by the consistency of environmental conditions experienced by the individual, such as food supply, or both. Egg size is a highly repeatable trait in passerines, but other traits such as eggshell thickness, Ca concentration, pigment concentration and maculation (extend of spotting on eggshells) have not yet been tested to date. Here, we use Ca supplementation to test the flexibility of females in their expression of such eggshell traits in two passerine species. We examine within-female repeatability of eggshell Ca concentration, protoporphyrin concentration, spot cover, spot intensity and eggshell thickness, and investigate how these traits respond to changes in Ca availability, and whether they are heritable down the female line. We found that egg volume was highly repeatable in both species. In great tits, only eggshell thickness was moderately repeatable and in blue tits, only eggshell Ca concentration and spot cover were moderately repeatable. Ca supplementation negatively influenced eggshell Ca concentration and thickness within females in great tits. No effects of Ca supplementation were found within female blue tits. None of the within-individual repeatable traits was found to be heritable between mothers and their daughters, suggesting that either the environmentally determined (i.e. non-heritable) component of the phenotypes are under selection or that the environment experienced by laying females is too changeable for a heritable trait to have evolved. We conclude that egg traits may have retained phenotypic flexibility to cope with changing environments.

5.2 Introduction

Egg traits have been extensively studied but the causes and consequences of variation in these traits remain poorly understood (Christians 2002). Variation within traits originates both at the genotypic and phenotypic levels, and is necessary for evolutionary change (Stearns 1989). Phenotypic plasticity, the ability of a genotype to alter its phenotype, is required in order for an organism to adapt to a changing environment (Stearns 1989). Phenotypic flexibility, both morphological (e.g. egg size) and behavioural (e.g. timing of reproduction), can vary within an individual in response to environmental cues (Ricklefs 1991, Piersma and van Gils 2011), allowing individuals, and consequently populations, to adapt to variation in ecological conditions such as nutrient availability (Piersma and van Gils 2011).

Some traits, such as egg size, have been shown to be repeatable (e.g. van Noordwijk et al. 1981) and heritable (e.g. Christians 2002) but many other eggshell traits remain poorly studied. One such trait is eggshell pigmentation, the function of which is still largely unknown but relationships have been found with female body condition at laying (Moreno et al. 2006a), immunocompetence during the nestling period (Moreno et al. 2005) and plasma antioxidant levels (Hanley et al. 2008).

Protoporphyrin, responsible for red-brown maculation, has been suggested to have strengthening purposes when dietary Ca is scarce (Gosler et al. 2005), and has been shown to be heritable along the female line (Gosler et al. 2000). The relationship between visible pigmentation and total pigment content is variable (Chapter Four), raising the question whether pigment content is fixed within a female or whether females are able to adjust total pigment content without varying visible spot patterns. Pigment patterning could be repeatable and heritable independently of total pigment content as the patterning is dependent on the size

and position of the pigmenting epithelial cells found within the shell gland wall (Gosler et al. 2000).

Avian eggs provide a useful tool to investigate the relationship between genotypic and phenotypic plasticity. Eggs are routinely produced on a 24-hour cycle (Romanoff and Romanoff 1949) in most small passerines, and when laid they must contain all the nutrients for embryonic development and be able to buffer the embryo against *ex ovo* environmental changes until hatching occurs. Furthermore, the egg contributes components (e.g. yolk) to early post-hatch growth and development of the chick (Deeming 2011). Many eggshell traits influence the hatchability, and the growth and development of the chick post-hatch (e.g. larger eggs produce larger chicks; Schifferli 1973).

Repeatability of an egg trait could be due to genes inherited down the female line (e.g. Gosler et al. 2000), and/or caused by the consistency of environmental conditions experienced by the breeding individual, such as food supply (Christians 2002). Egg size, a highly repeatable trait in most passerine species (van Noordwijk et al. 1981, Christians 2002), can be artificially increased through food supplementation (e.g. Ramsay and Houston 1997; but see Christians 2002), but only when females breed in habitats of low quality (Nager et al. 1997).

Although large variation in egg size exists within populations, individual repeatability is generally above 0.6 and tends to be higher than for other life-history traits such as clutch size or lay date (Christians 2002). Other egg traits which exhibit limited within-female variation are eggshell thickness (e.g. Thompson et al. 1983), and eggshell pigmentation colour and patterning (e.g. Gosler et al. 2000). In species parasitized by common cuckoos, uniformity of eggshell colour and patterns has evolved within clutches as a defence mechanism against brood parasites (Øien et al. 1995, Soler and Møller 1996). Lack of

variation within a trait might reflect persistent but non-genetic variation due to positive relationships between female body condition and some egg traits (Styrsky et al. 2002).

The heritability of a trait can be defined as the ratio of additive genetic and total phenotypic variance (Falconer 1964). According to evolutionary theory, fitness-related attributes, such as many egg traits, should have low heritability. However, traits showing little within-female variation are often deemed heritable (Casini et al. 2001). Egg size is believed to be highly heritable (Ojanen et al. 1979, Christians 2002), as are various components of eggshell patterning (Gosler et al. 2000), however how much of this is due to female quality rather than genetics is still unknown.

Like other passerine species, eggs laid by great tits and blue tits show little variation in traits within a clutch and between years. These egg traits include egg volume (great tits: van Noordwijk et al. 1981; blue tits: Griffith et al. 2009), and eggshell pigmentation (great tits: Gosler et al. 2000; blue tits: Griffith et al. 2009). Here, we use Ca supplementation to test the flexibility of females in their expression of eggshell traits in two passerine species. We examine within-female repeatability of Ca and protoporphyrin concentrations, spot cover, spot intensity and eggshell thickness, and investigate the responses of these traits to changes in Ca availability, and whether they are heritable down the female line. We predict females to show little variation in eggshell thickness and Ca concentration as egg volume is highly dependent on these two traits. However, if changes in eggshell Ca concentration occur, we predict a concomitant negative relationship with protoporphyrin concentration, spot cover and spot intensity.

5.3 Materials and methods

5.3.1 Study site, field methods, egg sampling and pigment analysis

Egg data were derived from the 2009-2012 breeding seasons with the 2009 data preceding the start of the Ca supplementation study. Please refer to sections 2.3.1 to 2.3.4 of Chapter Two for further details on material and methods.

Nestlings were ringed with a unique BTO metal ring in the nest on day 10. Adults were caught on the same day using nestbox spring traps and ringed/identified. If un-ringed, adults were ringed with a unique BTO metal ring on the right leg (under ringing license C5707). If already ringed, the BTO ring number was recorded. These uniquely numbered rings allowed us to identify mother-daughter relationships.

5.3.2 Pixel pigment scoring

Please refer to section 3.3.4 of Chapter Three for full details of methods. Pigment spot cover and intensity were calculated from the B region (crown; see Fig. 3.2 of Chapter Three) of the eggshell only, as this area is the most heavily spotted with the strongest correlation with eggshell total protoporphyrin content (see Table 4.4 and Fig. 4.4 of Chapter 4).

5.3.3 Statistical analysis

All statistical analyses were performed in R 2.15.3 (R Development Core Team 2011). Repeatabilities (intra-class correlations) were calculated following Lessells and Boag (1987). When studying the effects of Ca supplementation on within-female expression of egg traits,

GLMMs, using likelihood ratio chi-squared tests, were performed using the LME4 package (R Development Core Team 2011). Model simplification was performed using backward stepwise regression to find the minimal adequate model using Chi-squared (GLMMs) tests to compare the residual deviance of models including and excluding explanatory variables. Chi-squared values are derived from Wald tests which examine the relative contribution of each explanatory variable in the model. After term deletion using ANOVA, *P* values were calculated for the remaining significant variables in the minimal adequate model using MCMC simulations by means of the pvals.fnc from the languageR package (R Development Core Team 2011). Eggshell traits included in the model as explanatory variables were: Ca concentration (μg mm^{-2}), protoporphyrin concentration (μg g^{-1}), spot cover (%), spot intensity (0-1), mean eggshell thickness (μm), and egg volume (mm^3). GLMMs included 'year' and 'woodland area' as random effects. If multiple eggs were removed per female from either of the Ca-treatment areas, within-clutch means were used in the analyses. The distribution of the protoporphyrin concentration data was normalised by square-root transformation.

As no cross-fostering experiments were undertaken to control for shared environments we were unable to study heritability directly. Instead, we look at similarities between the eggs of mothers and their daughters. Pearson's product-moment correlation coefficients were calculated to examine correlations between mother and daughter egg traits. Eggs were removed from 25 mother-daughter couplets of great tits and 18 couplets of blue tits. When eggs were removed from multiple daughters within a 'family' then within-family means were used for all egg traits in analyses.

5.4 Results

5.4.1 Within-female variation of egg traits

Repeated egg samples were removed from a total of 23 great tit females (2 years = 15; 3 years = 5 and 4 years = 3) and 19 blue tit females (2 years = 18 and 3 years = 1). Egg volume was shown to be highly repeatable for both species, although other egg traits were (Table 5.1).

Table 5.1. Intra-class correlation (r, repeatability, and its associated se) of egg attributes within female tits at Chaddesley Woods NNR, Worcs., UK, between 2009 and 2012.

Egg trait	**Great tit**		**Blue tit**	
	r	**se**	**r**	**se**
Egg volume (mm^3)	0.79	0.071	0.76	0.099
Ca ($g\ mm^{-2}$)	-0.23	0.16	0.38	0.21
Protoporphyrin ($\mu g\ g^{-1}$)	0.30	0.17	0.12	0.24
Spot cover (%)	0.20	0.16	0.38	0.23
Spot intensity	0.16	0.16	0.41	0.22
Thickness (μm)	0.42	0.15	0.18	0.22

Out of the aforementioned sample sizes, at least one egg was removed from a control and one egg from a Ca-supplemented area for 14 great tits and 10 blue tits. Whilst controlling for year and woodland area effects, Ca supplementation was negatively correlated with eggshell Ca concentration and thickness of eggs laid by the same female great tit compared to when un-supplemented (Table 5.2, Fig. 5.1), but no such effect was found for blue tit females (Table 5.2, Fig. 5.2). Although not significant (P = 0.07), Ca supplementation was positively correlated with eggshell protoporphyrin concentration.

Table 5.2. Statistical outputs from models (*Chisq* and associated *P* values [two sets of *P* values are given: *P* – calculated using ANOVA, and *P* mcmc – calculated using MCMC simulations. 'N/A' signifies that MCMC simulations were not run]) of egg traits from repeated eggshell samples from either Ca-supplemented or un-supplemented (control) great tit and blue tit females. GLMMs control for year and woodland area. Explanatory variables are given in in the column on the left. Bold text indicates a term, verified through MCMC simulations, that is significant ($\alpha = 0.05$).

Egg trait	**Great tits**					**Blue tits**				
	Model effect estimate ± 1 SE	**df**	***Chisq***	***P***	***P* mcmc**	**Model effect estimate ± 1 SE**	**df**	***Chisq***	***P***	***P* mcmc**
Egg volume (mm^3)	8.88 ± 17.96	6	0.24	0.62	N/A	-13.58 ± 15.45	6	0.71	0.40	N/A
Ca ($g\ mm^{-2}$)	**4.43 ± 2.00**	**6**	**4.48**	**0.034**	**0.04**	3.36 ± 2.06	6	2.40	0.12	N/A
Thickness (µm)	**3.62 ± 1.25**	**6**	**6.47**	**0.01**	**0.04**	1.07 ± 1.37	6	0.59	0.44	N/A
Protoporphyrin ($\mu g\ g^{-1}$)	-0.90 ± 0.33	6	5.67	0.02	0.07	-1.15 ± 0.57	6	3.52	0.06	0.10
Spot cover (%)	1.31 ± 2.68	6	0.17	0.68	N/A	-5.64 ± 4.56	6	1.45	0.23	N/A
Spot intensity	-0.001 ± 0.02	6	0.01	0.92	N/A	-0.02 ± 0.02	6	0.55	0.46	N/A

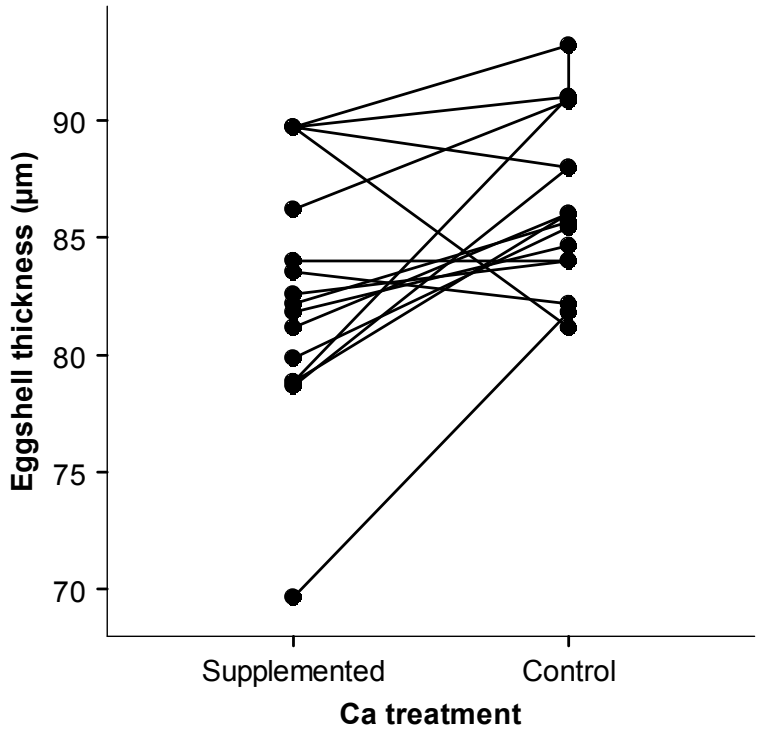


Figure 5.1. Mean thickness of un-pigmented eggshell of great tits laid by the same female when Ca-supplemented and un-supplemented (control) during the breeding seasons between 2009 and 2012 at Chaddesley Woods NNR, Worcs., UK.

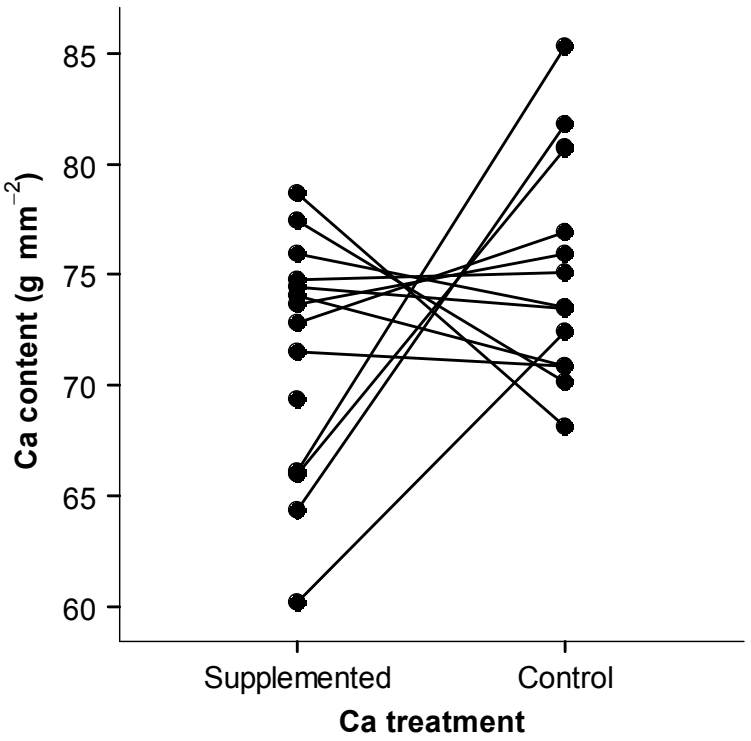


Figure 5.2. Mean Ca concentration (ash mass) of eggshells of great tits when either Ca-supplemented and un-supplemented (control) during the breeding seasons between 2009 and 2012 at Chaddesley Woods NNR, Worcs., UK.

5.4.2 Correlation of egg traits between mothers and their daughters

Eggs were removed from 25 mother-daughter couplets of great tits and 18 couplets of blue tits. The spread of years of egg collection of mothers' and daughters' eggs varied between 0 (collected in the same year) and 4 years. Sample sizes were too small to control for 'year' effects. Focal eggs traits were un-correlated between eggs laid by mothers and their daughters (Table 5.3).

Table 5.3. Pearson's product-moment correlation coefficients and linear regression slope (± 1 SE) of traits of eggs collected from mothers and their daughters of great tits and blue tits breeding at Chaddesley Woods NNR, Worcs., UK, between 2009 and 2012.

Egg trait	**Great tit**				**Blue tit**			
				Model effect				**Model effect**
	df	***P***	**r**	**estimate ± SE**	**df**	***P***	**r**	**estimate ± SE**
Egg volume (mm^3)	23	0.23	0.25	0.17 ± 0.14	15	0.40	0.22	0.19 ± 0.22
Ca ($g\ mm^{-2}$)	18	0.34	0.23	0.12 ± 0.13	9	0.42	0.27	0.29 ± 0.35
Protoporphyrin ($\mu g\ g^{-1}$)	18	0.25	0.27	0.24 ± 0.20	9	0.67	0.14	0.088 ± 0.20
Spot cover (%)	19	0.28	-0.25	-0.25 ± 0.23	12	0.55	0.18	0.21 ± 0.33
Spot intensity	19	0.51	0.15	0.11 ± 0.17	12	0.21	0.35	0.45 ± 0.34
Thickness (μm)	22	0.46	0.16	0.16 ± 0.22	12	0.09	0.47	0.65 ± 0.35

5.5 Discussion

We used Ca supplementation to test female flexibility in their expression of egg traits in two passerine species by looking at within-female repeatability, and how traits were affected by Ca supplementation. Furthermore, we examined whether egg traits are heritable down the female line. Contrary to what was predicted, eggshell traits, apart from egg volume, were not deemed repeatable. Ca supplementation was negatively correlated to eggshell thickness and Ca concentration and positively correlated with protoporphyrin concentration.

5.5.1 Within-female variation of egg traits

Out of the six egg traits studied, only egg volume was found to be highly repeatable (Table 5.1), with repeatability values comparable with those from other studies ($0.58 < rs < 0.72$; reviewed in Christians 2002). In addition, in eggs laid by blue tits, eggshell Ca concentration was moderately repeatable ($r = 0.38$). Although blue tits have a higher mean clutch Ca content than great tits (see section 3.4 in Chapter Four), blue tits tend to lay smaller eggs limiting the possible variation in shell Ca content. As a result females may have lower daily Ca-specific foraging demands for eggshell formation, causing this trait to become less plastic.

Surprisingly, eggshell Ca concentration was not repeatable within great tits suggesting that females are capable of laying consistently sized eggs regardless of Ca availability. Repeatability values for both egg length ($r = 0.69$) and breadth ($r = 0.65$) were smaller than those for egg volume ($r = 0.79$) suggesting that females might be adjusting egg shape in order to lay consistently sized eggs. A spherical egg provides the highest resistance against external forces (Bain 1991), and is the most conservative in the use of shell materials (Gosler et al.

2005). Changes in egg shape are not related to hatching and breeding success in these passerines species (Encabo et al. 2001) and therefore changing egg shape (r = 0.60) could be the most effective way to produce eggs in environments with variable Ca availability. Unfortunately, variation in egg shape was not considered in our study. Eggshell thickness in eggs laid by great tits was moderately repeatable, which is not altogether surprising given the high repeatability of egg volume, as larger eggs tend to have thicker shells (Olsson 1936). Eggshell thickness is positively related to Ca availability (Graveland et al. 1994), and therefore it is beneficial for birds to demonstrate some plasticity in the expression of this trait.

Females mobilise Ca for eggshell formation via two means, from the labile medullary bone (De Coster et al. 2013, Hargitai et al. 2013) or from the digestive tract which is traced to the Ca-specific foraging behaviour and extrinsic availability of Ca (Whitehead 2004). The skeletons of small passerines only contain *c*. 20% of the Ca required to form one eggshell (Graveland and van Gijzen 1994) which is likely depleted and replenished between each egg that is laid (Reynolds 2001). Females must therefore invest heavily (in terms of both time and energy) in Ca-specific foraging (Graveland 1996). Energetic constraints on the laying female during eggshell formation could therefore be more influential on the egg trait than it being an adaptive response to change in food (including Ca) availability (Nilsson and Svensson 1993).

Repeatability estimates for eggshell pigment values in eggs laid by blue tits (percentage spot cover: r = 0.38; spot intensity: r = 0.41), are similar to those found in a previous study looking at spot intensity (r = 0.43), distribution (r = 0.49) and size (r = 0.46) (Sanz and García-Navas 2009). However, repeatability estimates for eggshell pigment values in eggs laid by great tits (percentage spot cover: r = 0.20; spot intensity: r = 0.16), are dis-similar to those found in a previous study investigating spot intensity (r = 0.67), distribution (r = 0.46) and size (r = 0.54) (Gosler et al. 2000). Gosler *et al.* (2000) argued that the

repeatability of eggshell pigmentation patterns found in eggs laid by great tits could be due to the mechanisms of pigment deposition. Pigment is deposited by static epithelial cells in the shell gland wall while the egg is spiralling within the shell gland (Solomon 1991).

Despite the moderate repeatability estimates for visible pigment, eggshell protoporphyrin concentration was not repeatable within eggs laid by blue tits (Table 5.1). Previous results show that eggshell pigmentation patterns and protoporphyrin concentration are not correlated in blue tits (see Table 4.4 and Fig. 4.4 of Chapter Four). If, as Gosler et al. (2000) suggest, the amount of visible spotting is fixed due to the positioning of the epithelial cells depositing pigment onto the shells, females may not have much flexibility in the distribution and intensity of outer visible spotting. However, females may have more control over the deposition of protoporphyrin within the shell matrix, and adjust quantities according to the demands of egg formation as determined by the female's nutritional needs or by natural food availability (e.g. low soil Ca concentration). In contrast to previous findings (e.g. Mägi et al. 2012), spotting on the eggs of great tits in our study population were not repeatable. Pigment 'darkness' can be dependent on a female's constitutive innate immunity and can cause significant variation within clutches (De Coster et al. 2013) and possibly also variation between clutches of the same female. However, this inconsistency in findings of recent studies could be due to a difference in spot scoring methods. Many studies (e.g. Sanz and García-Navas 2009, De Coster et al. 2012) quantifying pigmentation use the visual pigment scoring method in which observers visually score eggshell pigment intensity, distribution and spot size (see section 4.3.3 of Chapter Four for more details). Our study uses the PPSM (described in section 4.3.4 of Chapter Four), which quantifies the number of pixels exceeding a specified threshold colour gradient, and therefore is more sensitive in quantifying eggshell maculation.

The relationship between Ca supplementation and expression of egg traits within females is complex and contradicts previous results found in this study population (Chapters Two and Three). When Ca-supplemented, great tits laid eggs with thinner shells and less Ca concentration compared to those laid when un-supplemented. Thinner eggshells could be as a consequence to the shells containing less Ca. These conflicting results could be due to several reasons. Firstly, the low repeatabilities of some of eggshell traits tested (Table 5.1) may not provide a good reference to compare the Ca-supplemented results with. Secondly, the results are based on a small sample size (i.e. 14 great tits and 10 blue tits), but this was beyond our control because eggs were removed from clutches prior to female identification. While handling and identification of females during egg-laying would have been desirable, it often causes the female to desert (Kania 1992). Due to small sample sizes we were further unable to test whether the order in which females were first Ca-supplemented or un-supplemented influenced the effects of the Ca-supplementation.

5.5.2 Correlation of egg traits between mothers and their daughters

None of the focal egg traits were correlated between those laid by mothers and their daughters. Although egg volume was found to be highly repeatable within females of both species (Table 5.1), no significant correlation was found between eggs laid by mothers and their daughters, despite this trait having been shown to be highly heritable in previous studies (e.g. Jones 1973, Ojanen et al. 1979). We must bear in mind that our results are based on single eggs which may not be representative of the whole clutch. However, all data are based on the fourth laid egg, which, for both species, is in the middle of the laying sequence and

therefore would be laid after the female has acquired sufficient nutrients to start egg-laying but not before these nutrient pools have been markedly depleted.

The results of the within-female repeatability and the heritability of eggshell pigmentation conflict with those of Gosler et al. (2000). They analysed mean pigment scores per clutch whereas we used pigment scores of a single egg per clutch. It is possible that females have a total capacity for pigmenting eggs per breeding attempt which is spread across all eggs within a clutch but that this is not reflected in individual eggs. Pigment spotting can decrease with laying order indicating that pigment may be limited in the short-term (López de Hierro and De Neve 2010; but see Gosler et al. 2005). This would mean that the pool of protoporphyrin is established before clutch initiation and that it is depleted during egg-laying. Alternatively, it is possible that such discrepancies between studies are due to different selection pressures on these egg traits between the study populations. Significant variation in lay date, a repeatable (Nager and van Noordwijk 1995) and heritable (van der Jeugd and McCleery 2002) trait, between two populations of great tits has been shown. Both populations showed significant variation in phenotypic plasticity but not in genotype-environment interactions (Husby et al. 2010), and showed a population-level response to increasing spring temperatures by advancing lay date. However, at the individual-level, responses were markedly different. One population showed genetically influenced variation between individuals in response to temperature (Nussey et al. 2005) whilst the other population showed no variation between individuals at all (Charmantier et al. 2008).

Any phenotypic trait will be influenced by both genetic and environmental components. However, a heritable trait will not evolve if only the environmentally determined (i.e. non-heritable) component of the phenotype is under selection (Price et al. 1988, Rausher 1992). For example, the heritability of body size in great tits is higher in environments which

are of better quality (e.g. ancient deciduous woodland; Riddington and Gosler 1995). Furthermore, genetic variation of a trait might be maintained by environmental diversity. Temporal variation may favour discrete genotypes at different times, resulting in varied expression maintained in the population (Roff 1997, Kruuk et al. 2001). It is possible that the quality (i.e. food abundance) of our study site, a mixture of ancient (e.g. oak [*Quercus* spp.] and ash [*Fraxinus excelsior*]) and planted (e.g. Scots pine [*Pinus sylvestris*] and European Larch [*Larix deciduas*]) woodland, is not as good as those in which these egg traits were found to be heritable or that the micro-environment is more varied promoting the preservation of a variety of genotypes.

5.6 Conclusions

We found that some egg traits are repeatable within females but that most were not. It remains unclear whether these results are due to small sample sizes or low repeatability of assessment methods or whether these traits have remained flexible in changing environments. Energetic constraints on the laying female during eggshell formation could be more influential on the individual egg traits than them being an adaptive response to the environment (Nilsson and Svensson 1993). None of the within-individual repeatable traits were found to be heritable between mothers and their daughters, suggesting that either the environmentally determined (i.e. non-heritable) component of the phenotypes are under selection or that the environment experienced by the laying females is too changeable for a heritable trait to have evolved.

Although blue tits and great tits are closely related and visually it appears that their eggs may be quite similar, data from the past four chapters have illustrated that these two species show different relationships between egg traits and exhibit different strategies in acquiring and mobilising Ca. The next chapter will investigate how pigment concentrations and colour diversity co-vary with phylogenetic affiliations among British passerine species.

CHAPTER 6

DO EGGSHELL PIGMENTS CO-VARY WITH LIFE-HISTORY AND NESTING ECOLOGY TRAITS AMONG BRITISH PASSERINES?

6.1 Abstract

Ancestral eggs are believed to have been white and immaculate. It is highly unlikely that any one hypothesis can explain the diversity and evolutionary distribution of eggshell coloration. Indeed some hypotheses are more plausible for some species than for others, suggesting that eggshell coloration may have many functions. Little is known about how eggshell pigments vary amongst closely related species and how such variation is related to eggshell appearance and life-history and nesting ecology traits. To assess the apparent diversity of avian eggshell coloration, we compared quantified concentrations of two key eggshell pigments, protoporphyrin and biliverdin, using museum eggshells of 73 British passerine species to explore whether phylogenetic relationships between species explain patterns of eggshell pigments. Furthermore, we examined how eggshell pigments and appearance, quantified from photographs, vary with life-history (i.e. incubation time, egg volume, eggshell thickness, clutch size, calcium diet) and nesting ecology traits (i.e. cavity type, nest location) amongst species. This study shows that eggshell pigment concentrations (standardised per mass of eggshell) are highly phylogenetically conserved. Controlling for phylogeny, protoporphyrin concentration was associated with species laying larger clutch sizes, thicker eggshells and those nesting on the ground or in scrubs. Biliverdin concentrations were associated with species laying thicker eggshells and consuming low-calcium diets but not with any nesting-ecology traits. We encourage future studies that test these key hypotheses to compare eggshell pigmentation of closely related species as this phylogenetic association may be essential to explain the functional significance of diversity in eggshell coloration among avian species.

6.2 Introduction

No single hypothesis explains the variance of eggshell coloration, and some hypotheses seem more probable for some species than for other species (e.g. crypsis is more likely for ground-nesting than hole-nesting species), suggesting that eggshell coloration may have many functions. Therefore, understanding the evolution of eggshell pigmentation is useful in explaining the variation found throughout the class. The diversity of visible eggshell coloration among birds can largely be explained by the nesting ecology and life-history dependence of individual species (Kilner 2006). These traits have also been shown to explain partly the diversity of eggshell pigment concentrations of non-passerines (Cassey et al. 2012a). However, it has not yet been ascertained how eggshell pigments vary amongst closely related passerine species.

The two main eggshell pigments, protoporphyrin, responsible for brown hues, and biliverdin, responsible for blue-green hues, are abundant in all avian eggshells (Kennedy and Vevers 1973, 1976, Gorchein et al. 2009). A survey examining eggshell appearance and pigment content of 108 species found that nearly half of species had eggshells containing protoporphyrin only, and just over a third had eggshells containing both protoporphyrin and biliverdin IXα (Kennedy and Vevers 1976). Another comparative study found that in maculated eggshells (i.e. where pigment was present as a pattern) pigment was either restricted to the shell surface or found in various layers of the eggshell (Harrison 1966).

Although ancestral eggs are believed to be white and immaculate, immaculate they are not necessarily devoid of pigments (Kilner 2006), as even some white, immaculate eggs contain traces of eggshell pigments (Kennedy and Vevers 1976, De Coster et al. 2013). The presence of eggshell pigments throughout avian evolution is feasible as both pigments are

produced during the biosynthesis of haem (Wang et al. 2009). The eggs of extant paleognath species (e.g. *Megalapteryx didinus*) have been shown to contain either protoporphyrin or biliverdin or both (Igic et al. 2010). Therefore, it is highly probable that these eggshell pigments are ancient in origin and highly conserved throughout the diverse radiation of the class Aves (Igic et al. 2010).

To assess the apparent diversity of passerine eggshell coloration, we used museum eggshells of 73 British species. First, we examined whether eggshell pigments are phylogenetically related across species. Second, we examined how eggshell pigments and appearance vary with life-history and nesting ecology traits across species while controlling for phylogenetic relationships. These traits were chosen to examine three hypotheses for the functional significance of eggshell coloration, which have received considerable attention in the past decade: crypsis to avoid predation and brood parasitism, the sexual-selection for the evolution of eggshell coloration (SSEC), and the structural-function hypothesis.

We predicted that open-nesting species would lay eggs with higher concentrations of both pigments than those nesting in cavities, and that ground- and scrub-nesting species would lay eggs with higher concentrations of both pigments than those nesting in trees. 'Crypsis' hypotheses (Wallace 1889, Götmark 1993) suggest that eggs are pigmented to match their nesting environment better and are therefore less visible to predators. Species nesting in cavities are less prone to predation and therefore would not require camouflage (Westmoreland and Kiltie 2007). We also predicted that eggshell biliverdin concentration would be positively related to maternal reproductive investment. The SSEC hypothesis of Moreno and Osorno (2003) proposed that egg colour acts as a sexually selected trait in females, to display their genetic and phenotypic qualities to males as a post-mating selection mechanism. Although we were not able to test this hypothesis directly (due to a lack of

detailed data on parental care for the 73 focal species), we were able investigate whether eggshell colour and pigment concentrations are related to indices of maternal reproductive investment (i.e. incubation duration and clutch size). Increased egg volume, clutch size and incubation length are energetically expensive and can affect future reproductive effort and adult mortality (Royama 1970, Visser and Lessells 2001, Deeming 2011). Species with longer incubation lengths tend to spend less time incubating in order to invest in themselves and therefore their lifetime reproductive effort (Martin 2002). Finally, we predicted that species on low-Ca diets would lay eggs with thinner shells containing higher concentrations of protoporphyrin than those on high-Ca diets as the pigment would strengthen the eggshell. The 'structural-function hypothesis' (Gosler et al. 2005) proposed that pigment spots reinforce the structural integrity of eggshells when dietary Ca is scarce (Solomon 1991), and therefore eggs laid by species consuming low-calcium diets would contain greater amounts of protoporphyrin in order to strengthen the eggshell.

6.3 Materials and methods

6.3.1 Eggshell samples

Eggshells of British breeding bird species were provided for destructive use by the Natural History Museum at Tring, Herts., UK (Russell et al. 2010). These eggs were collected prior to 1954 by private collectors and lack specific data (i.e. collection date and location) required for the use in extensive quantitative analyses (Russell et al. 2010). For each species, three eggs were chosen randomly from different collections to ensure that eggs were not removed from the same clutch or female. Natural History Museum accession numbers are specified for all of the eggshell samples used (Appendix 1).

Data were generated from half-eggshells only with the other half of respective eggshells were used as part of a separate study (refer to Maurer et al. 2012). Methods follow those specified in Cassey et al. (2012a). Half-eggshells were cleaned in de-ionised water and dried at room temperature for 48 hours. Eggshell thickness was measured twice at three distinct areas, the B, E and P regions (see section 2.3.3 of Chapter Two for further details). Average thickness was used in all subsequent analyses. Half-eggshells were photographed (see section 3.2.4 of Chapter Three for further details) against a velvet black background, as well as against a white 2 × 2 mm grid. The surface area of the half-eggshell was estimated from photographs using the 'Egg Area Measurement' plugin in ImageJ (Rasband 1994-2012).

Museum eggshells can differ significantly in colorimetrics from freshly collected eggshells (Cassey et al. 2010b, 2012b). Time in storage (i.e. photo-oxidation) did not affect pigment concentration in the museum samples of non-passerines (Cassey et al. 2012a). To confirm this, we compared pigment concentrations in museum eggs of blue and great tits in three different collections with freshly-laid eggs of 55 blue tits and 67 great tits collected as part of a previous study (see section 2.3.2 of Chapter Two).

6.3.2 Pigment quantification

Please refer to section 2.3.4 of Chapter Two for further details. Pigment concentrations were standardised for mass of the half-eggshell (μg g^{-1} of eggshell) and surface area (μg mm^{-1}).

6.3.3 Colorimetry of sample eggshell colour

Each image was loaded and processed separately. Processing comprised two main phases: selection of the regions of the image to analyse and calculation of the image statistics. In order to select the regions of the image, the image was first partitioned into egg and background regions using a simple binary threshold on a greyscale version of the image. The threshold level was determined using the method of Otsu (1990), to locate where the intra-class variance is minimized, and the inter-class variance is maximized (see section 3.3.4 of Chapter Three).

Having identified the egg region of the image, a circular sub-sample comprising a third of the pixelated areas was identified (Fig. 6.1). This had the effect of excluding pixels found

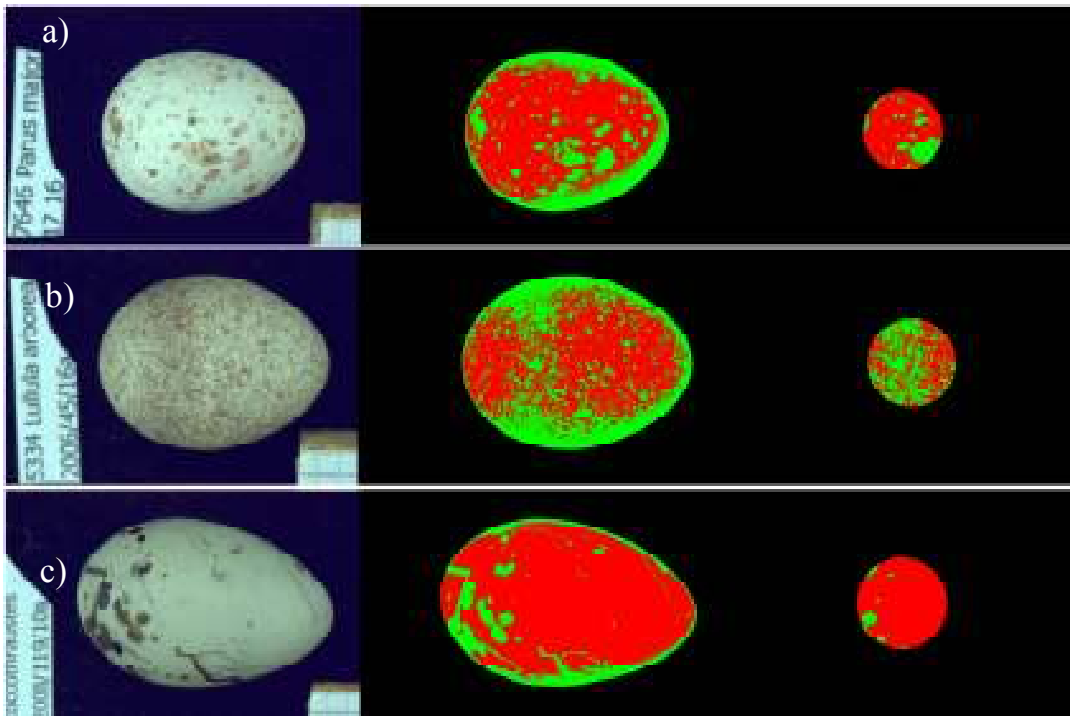


Figure 6.1. Photographs showing how images were partitioned into egg and background regions, and then how the circular sub-sample was subsequently identified, for a sample of eggs: (a) great tit, (b) woodlark (*Lullula arborea*), and (c) hawfinch (*Coccothraustes coccothraustes*). (Photos: G. Maurer, University of Birmingham; P.G. Lovell, University of St Andrews).

near the edge of the egg, thereby avoiding parts of the image where the pigment spots may have been distorted due to eggshell curvature. In order to achieve this, the square-root of the total number of pixels was taken (√pix), creating a rectangular area enclosing all the pixels within the egg. A circular mask with a radius of the size √pix/2 was created, giving a circular sub-sample excluding a third of the outer parts of the egg. The circular sub-sample was located by taking the centroid (i.e. the mean, midX) of the x and y pixels of the whole egg mask. The centroid was then shifted towards the B region of the eggshell using the following equation:

$$midX = midX * 0.88. \quad \text{(Eqn 6.1)}$$

All photographs were taken in the standardised RAW format. Images were converted from RGB to XYZ and consequently to CIELAB space using the Matlab image processing toolbox (The Mathworks, Natick, MA, 2000). The CIELAB colour space (Commission Internationale de l'Eclairage, Paris, France, 1976), commonly used in the measurement of eggshell colour (e.g. Moreno et al. 2004) consists of the L* channel, corresponding to lightness of the colour, the a* channel, corresponding to red(+)/green(-) colour values and the b* channel corresponding to yellow(+)/blue(-) values.

The principal colour within the sub-sample was ascertained using a kmeans clustering method. In this way the mean L*a*b* values and their standard deviations were calculated for the maculated (i.e. foreground) and un-maculated (i.e. background) of the sub-sample. The extent of maculation present was defined as the amount of spotting in the foreground compared to the background (based on number of pixels) using the same greyscale threshold and the method of Otsu (1990) and their ratio calculated.

In addition, the extent to which maculation occurred on whole eggshells (as opposed to half-eggshells) was scored by three independent observers using the three point scoring

system introduced by Kilner (2006): 0 – eggshell was near immaculate; 1 – eggshell has a dominant background colour but some maculation is noticeably present; and 2 – eggshell is extensively maculated. Average scores were calculated across observers.

6.3.4 Comparative life-history and nesting ecology traits

Data on life-history traits were collected from several sources, including *The Birds of the Western Palearctic* (Cramp and Simmons 1978−1994) and *Handbook of the Birds of the World* (del Hoyo et al. 1992-2011). Information on life-history (clutch size, incubation length, Ca diet) and nesting ecology traits (cavity type, nest location) was taken from species-specific monographs (for more details see Cassey et al. 2012a).

Life-history and nesting ecology traits were defined as: incubation length – the duration between the onset of incubation and hatching; clutch size – the mean number of eggs laid per breeding attempt; cavity type – none or cavity (tree/burrow); nest location – ground, scrub, or tree/cliff; Ca-rich diet – predominantly animal (e.g. insects and beetles) or predominantly omnivorous (e.g. berries and seeds as well as invertebrates). This categorisation makes the assumption that those species with predominantly animal diets consume higher concentrations of Ca than those with omnivorous diets (Graveland and van Gijzen 1994, Bureš and Weidinger 2003).

6.3.5 Phylogenetic tests

Phylogenetic relationships between passerine species were constructed using a commonly used (e.g. Dawideit et al. 2009, Cassey et al. 2012a) phylogeny of breeding British birds,

constructed using molecular data from 249 species (Thomas 2008). To assess the magnitude of the phylogenetic signal in protoporphyrin and biliverdin pigment concentrations, we estimated Pagel's lambda (λ) (Pagel 1999, Freckleton et al. 2002), using the R-library motmot (available from: http://www.r-project.org/). Pagel's λ varies between 0, phylogenetic independence, and 1, a trait which co-varies in direct proportion to a species' shared evolutionary history, consistent with a Brownian motion model of trait evolution (Freckleton et al. 2002).

6.3.6 Statistical analysis

All statistical analyses were performed in R 2.15.3 (R Development Core Team 2011). General Linear Models (GLMs), with normal error structures, were run using the mean values per species. Tukey *post-hoc* (95% CIs) tests were implemented for significant factors with multiple levels to compare confidence intervals on the differences (diff) between the observed means, and upper (upr) and lower (lwr) bounds of the confidence intervals. The distribution of protoporphyrin and biliverdin concentrations (standardised for both eggshell mass [μg $g^{-1}$of eggshell] and surface area [μg mm^{-1}]) were normalised by $\log_{10}$ transformation. Due to high numbers of 0s or near-0s of biliverdin concentrations present in eggshells, biliverdin concentration was separated into three levels: those greater than 1, those between 0.1 and 1, and those smaller than 0.1.

Statistical models examining the influence of life-history and nesting ecology traits on eggshell pigment concentrations were constructed controlling for phylogenetic relationships using phylogenetic generalized linear models (pgls) in the package caper (Comparative Analyses of Phylogenetics and Evolution; http://CRAN.R-project.org/package=caper/). Full

models used the following traits as explanatory variables: eggshell thickness (μm), egg volume (mm^3), incubation length (days), clutch size, cavity type, location of nest, and Ca diet. Mean values per species were used.

All statistical analyses were performed on 73 species, except for the pgls models, for which two species (lesser redpoll [*Carduelis cabaret*] and hooded crow [*Corvus cornix*]) were removed due to lack of phylogenetic data. It has been suggested that pigment may be deposited throughout the entire depth of the eggshell (e.g. Jagannath et al. 2008), or, conversely, that the majority of the pigment is deposited on the outermost layer of the eggshell (Wang et al. 2007). We therefore applied two measurements of pigment concentrations derived by standardising for either eggshell mass (μg g^{-1} of eggshell) or eggshell surface area (μg mm^{-2}). Spearman's correlation coefficients were calculated between pigment concentrations when standardised per unit mass and surface area. Both measurements of pigment concentration were included in all subsequent analyses.

6.4 Results

6.4.1 Sample validation

There was no significant difference in the concentration of protoporphyrin (Wilcoxon signed-rank test: W = 133, $P = 0.41$) nor biliverdin (W = 66, $P = 0.31$) between fresh and museum eggshell samples of great tits. The same was true for protoporphyrin (W = 54, $P = 0.33$) and biliverdin (W = 129, $P = 0.10$) of blue tits (Table 6.1).

Table 6.1. A comparison of the mean (± 1 SE) pigment concentrations of fresh great tit and blue tit eggshells collected from Chaddesley Woods NNR, Worcs., UK and those in the NHM Tring egg collection.

Eggshell pigment	**Great tits**		**Blue tits**	
	Control	**Museum**	**Control**	**Museum**
Protoporphyrin (μg g^{-1})	19.85 ± 1.89	12.30 ± 2.42	11.24 ± 1.10	17.26 ± 7.06
Biliverdin (μg g^{-1})	0.079 ± 0.065	0.050 ± 0.04	0.086 ± 0.036	$6.67 \times 10^{-5} \pm 6.67 \times 10^{-5}$

6.4.2 Pigment concentration and eggshell appearance

The mean sample concentrations, standardised either for mass (μg g^{-1}) or surface area (μg mm^{-2}) of eggshell, were highly correlated across species for both protoporphyrin (Spearman's correlation coefficients: r = 0.96, n = 73, $P < 0.0001$), and biliverdin (r = 0.99, $n = 73$, $P < 0.0001$). Across species, total concentration (mean per species) of protoporphyrin and biliverdin was positively correlated (r = 0.64, $n = 73$, $P < 0.0001$; Fig. 6.2), as was the case when standardized for mass (r = 0.41, $n = 73$, $P < 0.001$) and surface area (r = 0.46, $n = 73$, $P < 0.0001$).

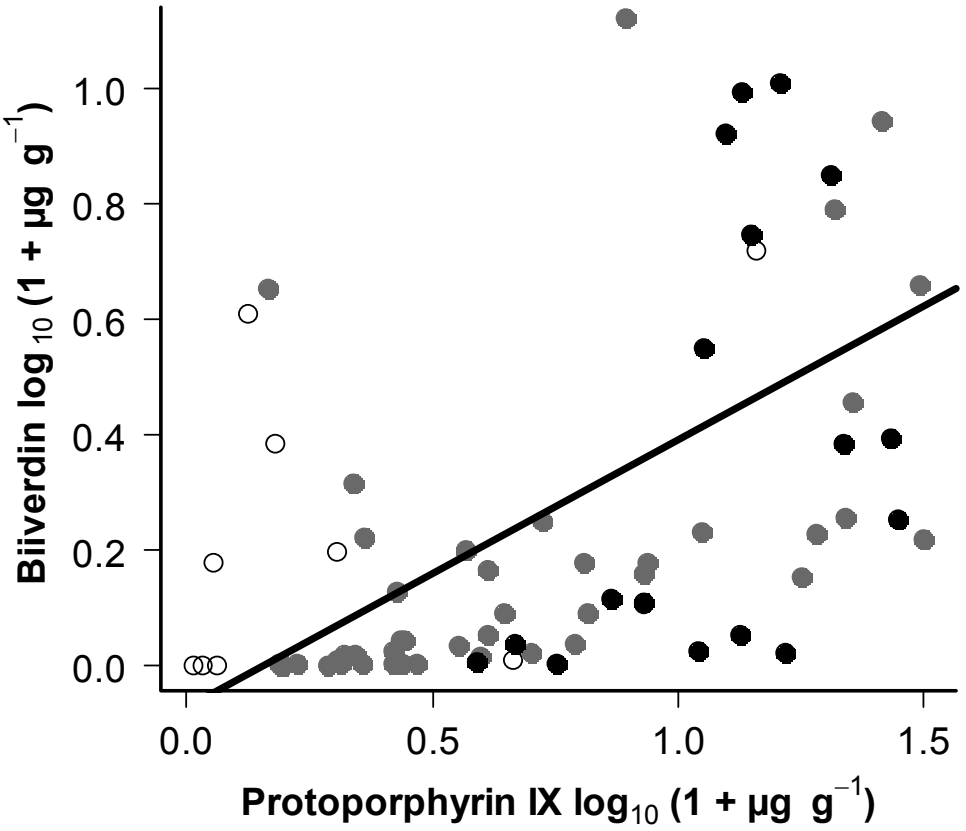


Figure 6.2. Scatterplot of the relationship between the mean concentrations of the two pigments protoporphyrin and biliverdin present in eggshells across species. Species which lay immaculate eggs (open points), maculated eggs but with clear, dominant background colour (grey points) and maculation covering the majority of the egg (black points) are distinguished. Lines of best fit are estimated by ordinary least squares regression.

Variation in the L*a*b* colour space coordinates were significantly correlated with an in increase in concentration (µg g^{-1}) of both protoporphyrin and biliverdin for both background and foreground coloration of the eggshell (Table 6.2). Protoporphyrin was negatively correlated with lightness (L*) of both foreground and background coloration, positively correlated with both red (+a*) and yellow (+b*) background coloration and red (+a*) foreground coloration. In contrast, biliverdin concentration was negatively correlated

with saturation (L*) of background coloration and positively correlated with green (-a*) foreground coloration.

Table 6.2. Results of a multivariate regression model assessing the effect of protoporphyrin and biliverdin concentrations ($\log_{10}$ μg g^{-1}) on measures of the L*a*b* colour space response variables for the non-maculated (background) and maculated (foreground) eggshell coloration taken from mean species (n = 73). The explanatory variables (pigments) are given in bold, while the response (eggshell colour) variables are listed below. Bold text indicates a term that is significant (α = 0.05).

Colour space variables		Estimate (SE)	df	T-value	*P*
Protoporphyrin IX					
Background	L*	**-4.75 (0.69)**	**70**	**-6.86**	**< 0.0001**
	a*	**2.52 (0.31)**	**70**	**8.20**	**< 0.0001**
	b*	**1.05 (0.45)**	**70**	**2.34**	**0.022**
Foreground	L*	**-8.06 (0.86)**	**70**	**-9.32**	**< 0.0001**
	a*	**3.58 (0.41)**	**70**	**8.68**	**< 0.0001**
	b*	-0.10 (0.66)	70	-0.15	0.88
Biliverdin					
Background	L*	**-2.84 (0.67)**	**70**	**-4.25**	**< 0.0001**
	a*	-0.47 (0.30)	70	-1.56	0.12
	b*	-0.42 (0.43)	70	-0.97	0.34
Foreground	L*	0.34 (0.83)	70	0.41	0.68
	a*	**-1.76 (0.40)**	**70**	**-4.44**	**< 0.0001**
	b*	0.61 (0.63)	70	-0.97	0.34

Eggshell maculation was not affected by biliverdin concentration ($F_{2,70}$ = 1.99, P = 0.15) but increased with increasing protoporphyrin concentration ($F_{2,70}$ = 7.84, P < 0.001; Fig. 6.3), with all three maculation scores being significantly different from one another (0−1: P = 0.049, diff = 2.75, lwr = 0.0078, upr = 5.49; 0−2: P < 0.001, diff = 4.92, lwr = 1.89, upr = 7.94; 1−2: P = 0.042, diff = 2.17, lwr = 0.062, upr = 4.29).

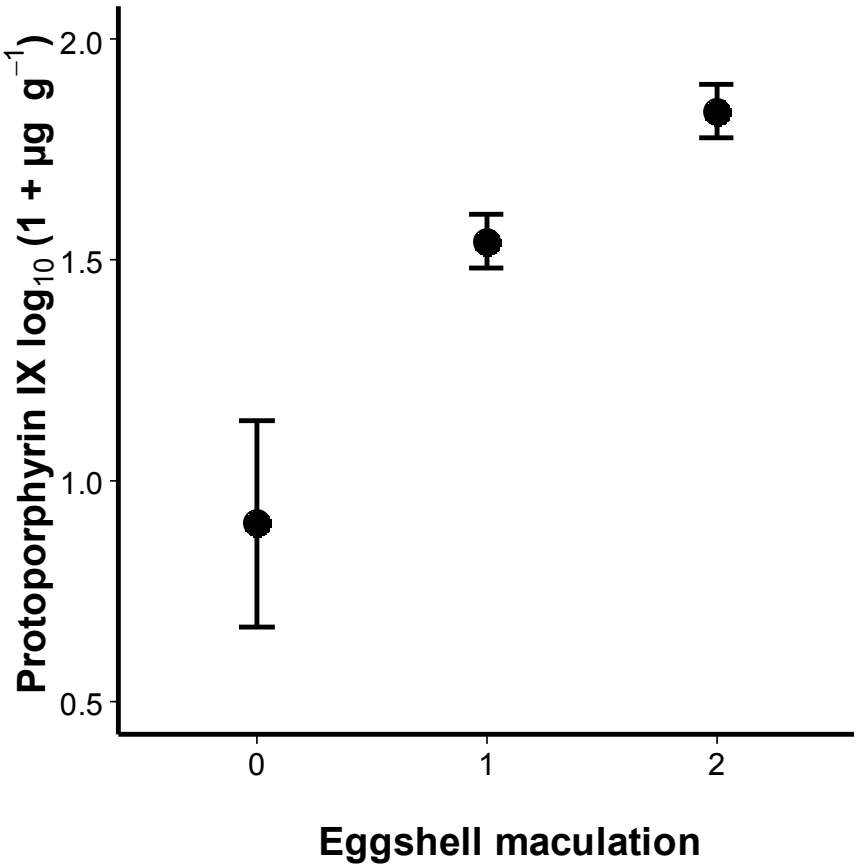


Figure 6.3. The relationship between mean (± 1 SE) protoporphyrin concentration and eggshell maculation (0: immaculate; 1: maculated but with clear, dominant background colour; and 2: maculation covering most of the eggshell) using means of 73 species in the NHM Tring egg collection.

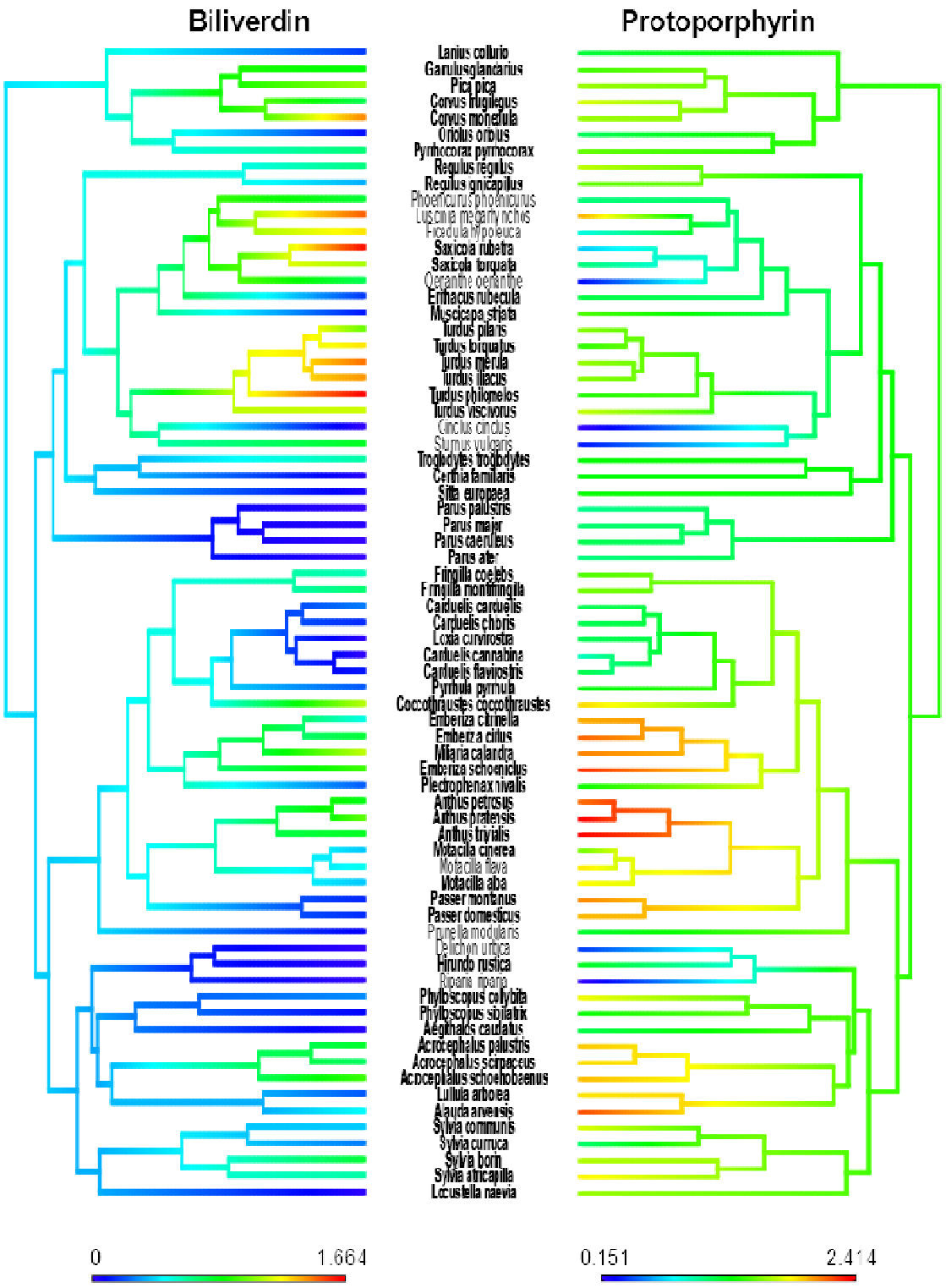
6.4.3 Phylogenetic patterns in eggshell coloration

The percentages of eggshell pigment concentrations attributed to within-species (among the three replicate eggshell samples from different collections) compared to total variance among the 71 British passerine species varied from 17.87 to 26.73 % (Table 6.3).

Table 6.3. Results from a nested Analysis of Variance to establish the amount of variance of eggshell pigment concentrations (standardised for mass and surface area of the eggshell) attributed to the three replicate eggshell samples compared to the total variance among species, for 71 species of British passerine held within the NHM Tring egg collection. The percentages of variance attributed to within-species compared to total variance among species are included in brackets.

Comparison		**Protoporphyrin**	**Biliverdin**
	df	***r***	***r***
Per unit mass (µg g^{-1})			
Within-species	147	17.54	18.81
Total	218	98.18	51.56
		(17.87%)	(26.73%)
Per unit surface area (µg mm^{-2})			
Within-species	147	9.96	22.72
Total	218	46.69	105.18
		(21.33%)	(21.60%)

Both pigments co-vary in direct proportion to their species' shared evolutionary history, with the degree of relatedness (Pagel's λ) being highly significant (Fig. 6.4, Table 6.4).


Biliverdin
Protoporphyrin
Lanius collurio
Garrulus glandarius
Pica pica
Corvus frugilegus
Corvus monedula
Oriolus oriolus
Pyrrhocorax pyrrhocorax
Regulus regulus
Regulus ignicapillus
Phoenicurus phoenicurus
Luscinia megarhynchos
Ficedula hypoleuca
Saxicola rubetra
Saxicola torquata
Oenanthe oenanthe
Erithacus rubecula
Muscicapa striata
Turdus pilaris
Turdus torquatus
Turdus merula
Turdus iliacus
Turdus philomelos
Turdus viscivorus
Cinclus cinclus
Sturnus vulgaris
Troglodytes troglodytes
Certhia familiaris
Sitta europaea
Parus palustris
Parus major
Parus caeruleus
Parus ater
Fringilla coelebs
Fringilla montifringilla
Carduelis carduelis
Carduelis chloris
Loxia curvirostra
Carduelis cannabina
Carduelis flavirostris
Pyrrhula pyrrhula
Coccothraustes coccothraustes
Emberiza citrinella
Emberiza cirlus
Miliaria calandra
Emberiza schoeniclus
Plectrophenax nivalis
Anthus petrosus
Anthus pratensis
Anthus trivialis
Motacilla cinerea
Motacilla flava
Motacilla alba
Passer montanus
Passer domesticus
Prunella modularis
Delichon urbica
Hirundo rustica
Riparia riparia
Phylloscopus collybita
Phylloscopus sibilatrix
Aegithalos caudatus
Acrocephalus palustris
Acrocephalus scirpaceus
Acrocephalus schoenobaenus
Lullula arborea
Alauda arvensis
Sylvia communis
Sylvia curruca
Sylvia borin
Sylvia atricapilla
Locustella naevia
0
1.664
0.151
2.414

(previous page) **Figure 6.4.** Phylogenetic tree for 71 British passerine species used in the comparative analysis investigating the relationship between eggshell pigment concentrations. The coloured branches illustrate the concentration (log_{10}) of the two pigments protoporphyrin IX and biliverdin. Species laying maculated eggshells are labelled in bold (refer to section 6.2.3 for information on how eggshell maculation was scored).

Table 6.4. The extent of phylogenetic association (Pagel's λ) for pigment concentrations standardised for mass (μg g^{-1}) and surface area (μg mm^{-2}) of the eggshell for 71 species of British passerine. Pagel's λ varies between 0, phylogenetic independence, and 1, a trait which co-varies in direct proportion to a species' shared evolutionary history. The Likelihood Ratio (LR) values are presented with Pagel's λ set to 0 and 1.

Pigment concentration	**Pagel's λ**	***P* values**	
		LR test = 0	**LR test = 1**
Per unit mass (μg^{-1})			
Protoporphyrin IX	1.00	< 0.0001	1.00
Biliverdin	1.00	< 0.0001	1.00
Per unit surface area (mm^{-2})			
Protoporphyrin IX	0.92	< 0.0001	0.18
Biliverdin	1.00	< 0.0001	1.00

6.4.4 Comparative life-history and nesting ecology traits

Eggshell protoporphyrin and biliverdin concentrations (standardised by mass only) were both positively influenced by eggshell thickness (Fig. 6.5, Table 6.5). Eggshell protoporphyrin

concentration was further negatively influenced by the interaction between nest location (Table 6.5, Fig. 6.6) and clutch size (Table 6.5, Fig. 6.7). Eggshell biliverdin concentration was positively influenced by the species' Ca diet (Table 6.5, Fig. 6.8).

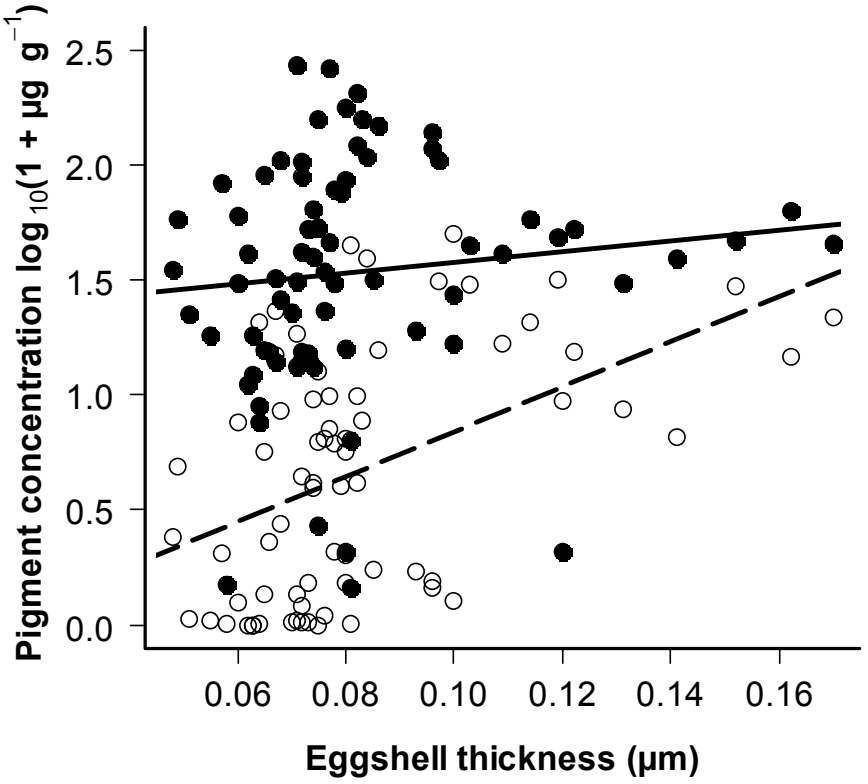


Figure 6.5. The relationship between eggshell pigment concentration per g of shell for both protoporphyrin (filled points, solid line [intercept = 1.34, slope = 2.32]) and biliverdin (open points, dashed line [intercept = -0.14*x*, slope = 9.83]), and eggshell thickness of 71 British passerine species from the NHM Tring egg collection. Points are mean values per species.

Table 6.5. Statistical outputs from models of life-history and nesting ecology traits (*F* and associated *P* values) of 71 British passerines contained within the NHM Tring egg collection in relation to protoporphyrin and biliverdin concentrations in the eggshell. Explanatory variables are provided in the left-hand column. Bold text indicates a term that is significant ($\alpha = 0.05$).

Life-history trait	**Protoporphyrin (µg g^{-1} eggshell)**				**Biliverdin (µg g^{-1} eggshell)**			
	Model effect estimate ± SE	**df**	***F***	***P***	**Model effect estimate ± SE**	**df**	***F***	***P***
Incubation time	0.012 ± 0.044	1,61	0.066	0.80	0.012 ± 0.047	1,61	0.06	0.80
Egg volume	$-5.42 \times 10^{-5} \pm 4.12 \times 10^{-5}$	1, 62	1.72	0.19	$-2.61 \times 10^{-5} \pm 4.31 \times 10^{-5}$	1,63	0.37	0.55
Ca diet (High/Low)	0.22 ± 0.17	1,63	1.70	0.20	**0.39 ± 0.17**	**1,69**	**4.94**	**0.024**
Cavity type (Cavity/None)	0.21 ± 0.11	1,64	3.31	0.074	0.042 ± 0.12	1,62	0.11	0.74
Eggshell thickness	**8.41 ± 3.02**	**1,65**	**7.77**	**< 0.01**	**6.57 ± 2.85**	**1,69**	**5.34**	**0.024**
Clutch size	**- 0.41 ± 0.12**				0.0083 ± 0.042	1,66	0.038	0.85
Nest location	ground: 2.97 ± 0.63				ground: - 0.30 ± 0.31	2,68	1.81	0.17
	scrub: - 0.36 ± 1.04				scrub: - 0.10 ± 0.13			
	tree: - 2.46 ± 0.67				tree: - 0.19 ± 0.10			
Clutch size: nest location	ground: 2.97 ± 0.63	**2, 66**	**6.42**	**< 0.01**	ground: 0.48 ± 0.66	2,65	1.37	0.27
	scrub: 0.057 ± 0.20				scrub: 0.19 ± 0.21			
	tree: 0.44 ± 0.13				tree: 0.22 ± 0.13			

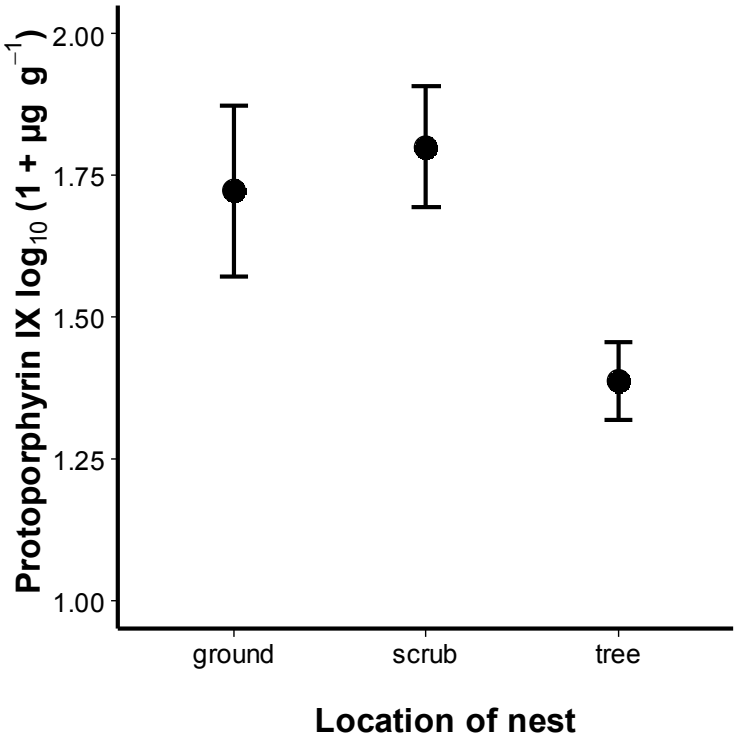


Figure 6.6. The relationship between mean (± 1 SE) eggshell protoporphyrin concentration and nesting location (ground: *n* = 16; scrub: *n* = 13; and tree: *n* = 42) of 71 British passerine species contained within the NHM Tring egg collection.

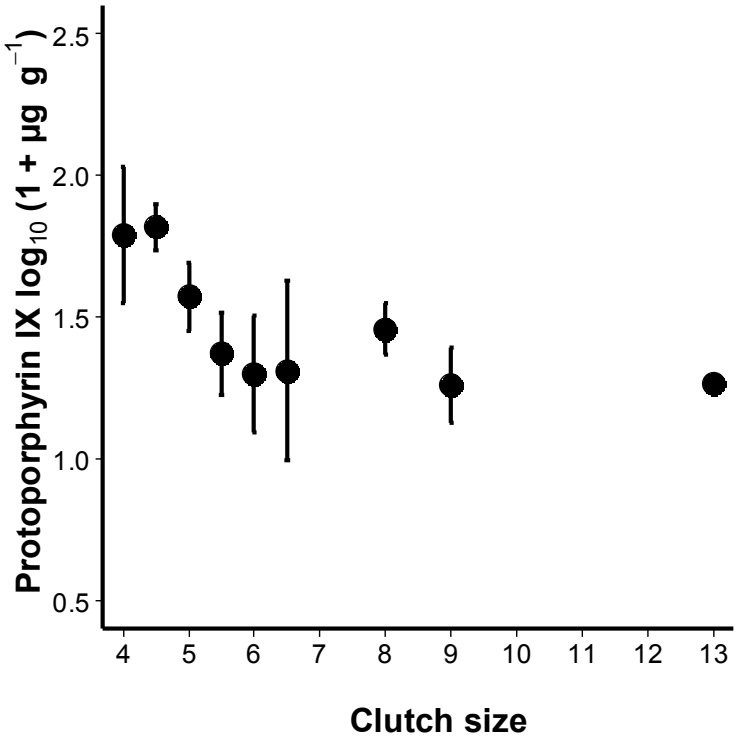


Figure 6.7. The relationship between mean (± 1 SE) eggshell protoporphyrin concentration and mean clutch size of 71 species of British passerine contained within the NHM Tring egg collection.

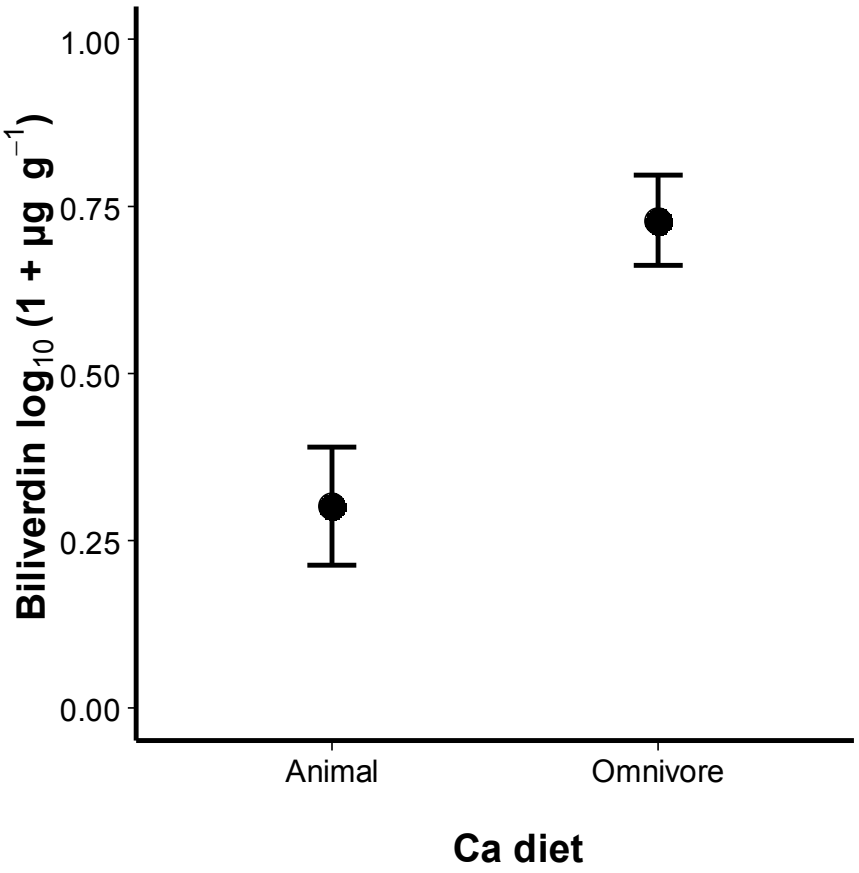


Figure 6.8. The relationship between mean (± 1 SE) eggshell biliverdin concentration and Ca diets for 71 British passerines species held within the NHM Tring egg collection.

6.5 Discussion

Our research shows that across species, total concentration of protoporphyrin and biliverdin were positively correlated. The amount of both pigments within the eggshell was directly proportionate to the evolutionary relatedness between species. Whilst controlling for phylogenetic relatedness, higher concentrations of eggshell protoporphyrin were correlated with species which laid eggs with thicker shells, smaller clutches and were ground/shrub

nesters rather than tree nesters. Higher concentrations of eggshell biliverdin were correlated with species which laid eggs with thicker shells and had high Ca diets.

6.5.1 Pigment concentration and eggshell appearance

Eggshell concentrations standardised either for shell mass or surface area of the eggshell were highly correlated across species suggesting that pigments occurring in the outermost eggshell layer are representative of pigments spread throughout the eggshell. We further found that concentrations of protoporphyrin and biliverdin are positively correlated across species, which substantiates the proposition that the two pigments are derived from the same precursor metabolic pathway (Wang et al. 2009; but see Needham 1974).

As expected, protoporphyrin pigmentation was related to red and yellow coloration of both the background and foreground of the eggshell. Protoporphyrin pigmentation has a reflectance peak (632 and 699 nm) corresponding to the red region of the spectra (Courrol et al. 2007) and was linked to increased maculation (Fig. 6.2). Biliverdin concentration was related to green foreground coloration but not to either green or blue background eggshell coloration. Biliverdin pigmentation has an absorbance peak (632 and 699 nm) corresponding to the blue region of the spectra (Falchuk et al. 2002) and has previously been related to blue eggshell coloration (e.g. Cassey et al. 2012a). However, we may have been dealing with too small a sample in the present study because a large number of species' eggshells contained low concentrations of biliverdin (62.5% of eggshell contained < 5 μg g^{-1} of eggshell) and a significant relationship might be found with a larger sample size. The more pigment (protoporphyrin and/or biliverdin) present in the eggshell, the less light is reflected and therefore the more the colour is saturated. The variability detected in eggshell pigment

concentrations is considerable (e.g. Fig. 6.1) and demonstrates that the visual coloration alone cannot be used to simply determine the presence or absence of eggshell pigments.

6.5.2 Phylogenetic patterns in eggshell coloration

We found that pigment concentrations exhibited strong co-varying phylogenetic patterns. These results are similar to those found by a previous study using equivalent eggs of non-passerines from the NHM collection at Tring (Cassey et al. 2012a). Using comparative analyses of eggshell coloration, Kilner (2006) concluded that the ancestral bird egg was white and without speckling, and that this trait is retained only in those species whose nest sites are safe from predation. Species may therefore be laying pigmented eggs due to having retained this trait rather than it serving a purpose such as for matching nesting environment, displaying a females' genetic or phenotypic qualities or for structural strengthening.

6.5.3 Comparative life-history and nesting ecology traits

Protoporphyrin-based eggshell pigmentation was negatively correlated with the interaction of nest location and clutch size. This interaction between these two traits could be due to the fact that species nesting in trees (e.g. Hooded Crow) lay larger clutches as they are less prone to predation (Slagsvold 1982) and therefore have not evolved cryptic coloration. Our results showed that eggs laid in nests located in scrub or on the ground contained higher concentrations of protoporphyrin than those nests found in tree nests. As eggshells with greater concentrations of protoporphyrin tend to be more maculated, this suggests that those eggs contain more pigment as an adaptation for crypsis. Egg coloration has a significant effect

on predation rates and hence on nestling survival rates (Westmoreland and Kiltie 2007, Westmoreland 2008), and could therefore have had a large evolutionary influence. Surprisingly, eggs laid by open-nesting species were not found to contain higher concentrations of protoporphyrin than those laid by cavity-nesting species. Amongst the family Turdidae, nest site explained some of the variation in colour and patterning on eggshells. Those species which were hole-nesters were more likely to lay immaculate white eggs while 80% of species which had exposed nests laid eggs covered in red or brown speckling (Lack 1958). Species less prone to predation (i.e. those nesting in trees) tend to lay larger clutches (Slagsvold 1982) which would explain the associated negative correlation with eggshell protoporphyrin concentration.

The SSEC hypothesis proposes that biliverdin is associated with costly maternal investment through its antioxidant properties, and therefore predicts that eggshell biliverdin concentration should be positively associated with reproductive investment (Moreno and Osorno 2003, Soler et al. 2005). As all species included in our study showed some aspect of bi-parental care, this trait could not be considered in the present study. Aspects of paternal investment were also not included. We found no significant relationship between biliverdin concentration in eggshells and clutch size, a trait associated with reproductive investment (Perrins and Moss 1975, Monaghan and Nager 1997). Increased clutch size is energetically expensive and can affect future reproductive effort and adult mortality resulting in a trade-off between current and future reproductive investment (Visser and Lessells 2001, Deeming 2011). Furthermore, it has been suggested that as protoporphyrins can induce oxidative stress, females laying eggs containing high protoporphyrin content may signal their high body condition through their oxidative tolerance (i.e. high condition) or, on the contrary, through their physiological stress (i.e. low condition) (Moreno and Osorno 2003, Holveck et al. 2012).

Our results show a negative association between eggshell protoporphyrin concentration and clutch size supporting the hypothesis that females laying less pigmented eggs are in better body condition and hence able to lay larger clutch sizes. However, clutch size may not be the best trait to investigate the association between eggshell pigment content and reproductive investment across species, as it is dependent on a range of environmental factors (Cody 1966, Haywood 2013) and often shows considerable variation within individual species (Christians 2002).

Both pigments were positively associated with eggshell thickness. Protoporphyrin may increase eggshell strength by acting as a shock absorber within the eggshell matrix (Solomon 1991, Gosler et al. 2005). According to the structural-function hypothesis (Gosler et al. 2005), we predict thicker eggshells (i.e. more Ca) to contain lower concentrations of protoporphyrin, as no extra structural-strengthening is required. However, our results indicated that thicker eggshells contained greater protoporphyrin concentrations and no relationship was found between a species' Ca diet and eggshell protoporphyrin concentration. Surprisingly, biliverdin-based eggshell pigmentation was associated with a species' Ca diet. Those species consuming a predominantly animal (i.e. high Ca) diet laid eggs with less biliverdin than those consuming an omnivorous (i.e. low Ca) diet. These results were unexpected and might be due to a difference in sample size (animal diet = 11; omnivorous diet = 62). There are two major flaws with our categorisation of Ca diets. Species on omnivorous diets (e.g. great tit) often consume a mostly animal diet (Royama 1970) when these are abundant (Martin 2002), and therefore possibly consume a similar diet to those species consuming a pure-animal diet. Females of these species also increase Ca consumption immediately prior to and during egg-laying, most of which is obtained from snail shells (Graveland and van Gijzen 1994). Categorising diets can be problematic as foraging niches often vary across species with range

(Snow 1954), habitat quality (Blondel et al. 1991, Wilkin et al. 2009b) and with inter-specific competition (Alatalo et al. 1985). In addition, passerines are not able to store Ca in their skeletons (Pahl et al. 1997) so a high Ca diet all-year around may not be beneficial when it comes to Ca requirements during egg-laying.

6.6 Conclusions

This study shows that eggshell pigment concentrations of British passerine species are highly phylogenetically conserved, complimenting previous studies on non-passerines (Cassey et al. 2010a) and those on visible coloration only (Kilner 2006). Furthermore, both eggshell protoporphyrin and biliverdin concentrations are phylogenetically related to crucial nesting ecology and life-history traits. We have highlighted how results from our phylogenetic study can be interpreted through a number of key hypotheses for the functional significance of eggshell coloration across species. We encourage future studies testing these key hypotheses to compare eggshell pigmentation of closely related species as this phylogenetic association may be essential to explain the functional significance of eggshell coloration of avian species.

CHAPTER 7

GENERAL DISCUSSION

7.1 Aims of the thesis

The great diversity of avian eggshell pigmentation and its possible adaptive significance has fascinated biologists for a long time. Despite the resurgence of academic interest in the functional significance of eggshell colour in the past decade, our understanding of eggshell pigments and coloration is still minimal. The primary aim of this thesis was to contribute to the existing knowledge that attempts to explain a functional and ecological role for eggshell coloration.

Eggs of great tits and blue tits have a dominant white background covered with protoporphyrin pigment spots (Fig. 1.3). As explained in Chapter One, it is unlikely that hole-nesting species, including blue and great tits, have evolved eggshell coloration for the purpose of crypsis, as these species experience low probabilities of predation (Martin 1995, Bennett and Owens 2002), or for a signalling function (following the SSEC hypothesis) as eggshell coloration may not be visible to cavity-nesting passerines (Cassey et al. 2009, Holveck et al. 2010). Three other main hypotheses in the literature may explain eggshell coloration of blue and great tits: eggshell spottiness (i.e. protoporphyrin content) may be related to female health and body condition (e.g. Martínez-de la Puente et al. 2007, De Coster et al. 2013), but whether increased pigmentation suggests a positive or negative health status remains unclear; eggshell pigmentation could have thermoregulatory properties but whether this is a direct (i.e. adaptive) effect or an indirect effect caused by reduced thickness of pigmented shell is uncertain (e.g. Higham and Gosler 2006, Sanz and García-Navas 2009); eggshell spottiness and/or pigment concentration may be used to reinforce the structural integrity of eggshells under conditions when dietary Ca is scarce (Gosler et al. 2005). This 'structural-function' hypothesis may be the most applicable hypothesis to account for the pigmentation of the eggs of hole-nesting species such as blue and great tits. Within the context of this hypothesis, this

thesis documented the use of Ca supplementation having explored the relationships between eggshell thickness, Ca and protoporphyrin concentrations and visible pigment spotting.

7.2 Summary of results

Chapter Two demonstrated that in eggs laid by both species, protoporphyrin-pigmented spots were thinner than the adjacent un-pigmented background eggshell. This was true for eggs laid by both Ca-supplemented and un-supplemented blue tits, but only for un-supplemented great tits. In eggs laid by Ca-supplemented great tits this discrepancy in eggshell thickness disappeared. **Chapter Three** found that females of both species were influenced more by Ca supplementation than by local soil Ca concentration suggesting that females in our study population are not suffering from severe Ca limitation. **Chapter Four** documented the ineffectiveness of eggshell spot scoring methods as a proxy for pigment quantity. Although results from the two focal spot scoring methods were positively correlated with eggshell protoporphyrin concentrations, the correlations were not sufficiently strong when precise determination of pigment content is required. **Chapter Five** found that only a few egg traits were repeatable within females, but none of these traits was found to be heritable between mothers and their daughters, concluding that egg traits that have retained phenotypic plasticity may be highly conserved in the focal species to accommodate marked potential fluctuations in the breeding habitat, including food availability, and microclimate. **Chapter Six** demonstrated that eggshell pigment concentrations were highly phylogenetically conserved. Furthermore, both eggshell protoporphyrin and biliverdin concentrations are phylogenetically related to crucial nesting ecology and life-history traits.

7.2.1 Were the focal populations Ca-deficient?

The soil Ca survey of 2009 found that Chaddesley Woods NNR is an acidic woodland with corresponding low levels of soil Ca found in some areas with some even lower than sites having documented severe Ca-limited reproduction (Table 3.1). However, despite these low levels of soil Ca at the study site, females of both species did not show signs of Ca limitation (e.g. defective eggshells – Drent and Woldendorp 1989) (Chapter Three).

For Ca supplementation studies to be effective, the focal study species must show signs of being Ca-deficient (Reynolds et al. 2004). Ca supplementation had a greater effect on eggshell characteristics of both species than local soil Ca concentration, suggesting that females in this population are not suffering from severe Ca limitation but have adapted to these conditions using different strategies without displaying obvious reproductive problems (e.g. declines in breeding productivity − Tilgar et al. 2002). Foraging for Ca may incur energetic (Walsberg 1983) and temporal costs, with females spending over 30% of their evening activity in Ca-specific foraging (Graveland and Berends 1997). Ca supplements, therefore, likely provide females of both species with more time to invest in other activities, such as foraging for foods rich in energy required for egg production (e.g. Nager et al. 1997).

Requirements for dietary Ca remain high after eggshell formation because Ca is required in the diet of nestlings for mineralisation of their skeletons (Starck 1998). In great tits, the required Ca intake to form one eggshell is estimated to be 58 mg (Graveland and Berends 1997).Total Ca intake required for successful skeletal formation of meadow pipit (*Anthus pratensis*) nestlings, a bird similar in size to great tits. is estimated to be 139.8 mg, mainly though the consumption of snailshells (Bureš and Weidinger 2000). Ca supplementation of great tit females during brood-rearing increased nestling growth and

fledgling numbers (Mänd et al. 2000a, Tilgar et al. 2004, Tilgar et al. 2005), although this was not found in blue tits (Ramsay and Houston 1999). Therefore, despite females of both species showing no signs of Ca limitation during egg-laying, they may still have been Ca-limited during the nestling phase. Adult females are potentially faced with a trade-off between re-paying their Ca deficit following egg formation versus supplying nestling with Ca-rich foods (Graveland and Berends 1997). My study simply addressed the egg-laying period and no 'downstream' breeding parameters were examined in this regard.

7.2.2 Does the structural-function hypothesis explain pigmentation of great tit and blue tit eggs?

Chapter One introduces the primary hypotheses explaining eggshell coloration and highlights those applicable to hole-nesting species such as great tits and blue tits. The structural-function hypothesis was initially considered to most likely apply to eggs laid by hole-nesting species, with it proposing that protoporphyrin is deposited onto the eggshell for structural strengthening when exogenous Ca is scarce (Gosler et al. 2005). However, results from this study provided mixed support for this hypothesis. Ca supplementation had subtle positive effects on the populations of blue tits and great tits breeding in Chaddesley Woods NNR. Protoporphyrin spots were thinner than the adjacent un-pigmented background eggshell of eggs laid by un-supplemented females of both species (Chapter Two). Ca supplementation restored parity in respective eggshell thicknesses only in eggs laid by great tits (Chapter Two) but increased overall eggshell thickness in eggs laid by both species (Chapter Three). In eggs laid by great tits, Ca supplementation did not affect eggshell protoporphyrin concentrations but females laid eggs with shells which had reduced spot coverage (Chapter Three). In eggs

laid by blue tits, Ca supplementation increased eggshell protoporphyrin concentration but did not influence eggshell spot cover or intensity (Chapter Three).

Support for the structural-function hypothesis from this study is inconclusive. Both species provided some evidence of compensatory use of protoporphyrin for structural strengthening of the eggshell, through the use of spotting, but not through increased total protoporphyrin concentration. However, there were no direct links between eggshell protoporphyrin content (concentration and visual) and Ca concentration. Changes in eggshell traits, including but not limited to protoporphyrin concentration, may not be direct consequences of Ca supplementation. For example, females may have more time and/or energy to invest in foraging for nutrients other than Ca as a result of its supplementation.

The structural-function hypothesis was proposed as a results of work on the great tit population at Wytham Woods, Oxfordshire, UK (Gosler et al. 2005), but its applicability has been studied with mixed success in both passerines (e.g. support: northern lapwing – Bulla et al. 2012; dispute: blue tits – Sanz and García-Navas 2009) and non-passerines (e.g. support: Eurasian sparrowhawk – Jagannath et al. 2008; dispute: black-headed gull – Maurer et al. 2011b). However, as documented in Chapter Six, eggshell pigments co-vary in direct proportion to species' shared evolutionary traits and are phylogenetically associated with nesting and life-history strategies. This means that eggshell pigments of closely related species likely have similar functions or have been retained from a common evolutionary ancestor. Therefore, if the structural-function hypothesis explains eggshell pigmentation of great tits than it will likely explain eggshell pigmentation of other closely-related species. Nevertheless, we cannot exclude the possibility that for some species this trait may have become redundant or, conversely, gained additional functions, whilst not for another closely-

related species. It is possible that pigmentation on the eggs of blue and great tits may simply be due to a shared evolutionary ancestor and that this trait has become functionally redundant.

7.2.3 Are results from the Chaddesley Woods NNR population applicable to other tit populations?

Some of our findings are inconsistent with those from other study populations. For example, the relationship found between spot characteristics (i.e. I, D and S) (Table 4.1) differed from those found on eggshells laid by blue tits from the Cabañeros National Park population (Ciudad Real, Spain); Sanz and Garcia-Navas (2009) found that spot intensity (I) had little influence on PC2 (0.086) but much on PC1 (0.83). The same is the case in great tits (Table 4.1) where we found that spot size (S) had little influence on either PC1 or PC2 which was in marked contrast to findings of Gosler et al. (2005) in Wytham Woods. Furthermore, our study showed that the P region of great tit eggshells were thickest (Fig. 2.4), whilst in great tits at Wytham Woods it was the B region (Gosler et al. 2005). What could account for these inconsistencies between populations?

When gene flow is not homogenous, evolutionary change can be rapid and occur over small spatial scales (Garant et al. 2005). A large-scale (ranging in latitude from 41º N to 91º N and in longitude from 4º E to 60º E) study of 15 populations of pied flycatchers found that variation in eggshell traits was not caused by geographical location or habitat type (Morales et al. 2013). However, differences between geographically isolated populations of blue tits were caused by variation in environmental conditions (e.g. parasite load and caterpillar abundance) rather than in genetic divergence between populations (Simon et al. 2005). Egg traits such as size and composition have been shown to be variable due to environmental rather than genetic

differences (e.g. Bourgault et al. 2007, Schaper and Visser 2013). Therefore, inconsistencies in findings between the Chaddesley Woods NNR population and those of others are most likely due to variation in environmental conditions rather than genetic divergence. This means that results from this study population can be applied to other populations of blue and great tits, but variation in environmental conditions experienced by these populations must be considered in their interpretation.

We must not ignore that inconsistencies between populations could be due to methodological inconsistencies and/or human error. The visual spot scoring method, designed to quantify the degree of eggshell maculation, has not been tested under standardized conditions. Results produced by this method may not be reproducible, especially the comparison of results between observers from different studies. The differences found in regional eggshell thickness between our study and that of Wytham Woods could be due to a discrepancy of methods. Contrary to the Wytham Woods study (Gosler et al. 2005) we measured eggshell thickness with membranes intact. Thicker membranes are associated with thinner eggshells (Castilla et al. 2009) and so could be compensating for reduced eggshell thickness in specific regions of the eggshell.

7.3 Recommendations for future research

Before being able to fully understand the functional significance of eggshell pigments, the synthesis, deposition and microstructure of pigmentation within the eggshell matrix must first be investigated. Although much research has been conducted on eggshells and their pigments, the majority is derived from poultry science and, therefore, the focus has been driven by commercial pressures imposed by the human food market (e.g. resistance to breakage during

transportation, taste, visual perception). There are two main shortcomings often associated with studies of the functional significance of protoporphyrin-pigmented eggs: the lack of understanding of the synthesis and mobilisation of protoporphyrin, and the failure to recognise the distinction between protoporphyrin deposition (i.e. maculation parameters) and concentration in eggshells. We encourage future studies to consider these carefully before carrying out studies that focus on the modes of synthesis and deposition of protoporphyrin onto eggshells.

7.3.1 Understanding pigment synthesis, mobilisation and deposition

While the synthesis pathway of protoporphyrin is well documented, the site(s) where synthesis may occur is still not fully understood (Sparks 2011). Protoporphyrin is a product of haem catabolism but evidence for this pigment being synthesised within the uterus, or even elsewhere, is circumstantial (Sparks 2011). Furthermore, whether the synthesis and deposition of protoporphyrin are detrimental or beneficial to the laying female or embryo remains unclear. The accumulation of protoporphyrin within the liver can cause oxidative stress (Afonso et al. 1999). The deposition of large amounts of protoporphyrin onto the eggshell may indicate a female's ability to cope with intense oxidative stress, or alternatively, may indicate that protoporphyrins have been successfully removed (Moreno and Osorno 2003, Holveck et al. 2010). Pigment spotting could further affect eggshell permeability (but see Higham and Gosler 2006, Sanz and García-Navas 2009, Deeming 2011), insufficient or excessive water loss being detrimental for normal embryonic development (Lundy 1969, Webb 1987). Like protoporphyrin, biliverdin is also a product of haem and may be derived from red blood cells but it has recently been discovered that biliverdin may be produced *de*

novo in the uterus (Zhao et al. 2006, Wang et al. 2011). Biliverdin may have some anti-oxidant properties (Kaur et al. 2003) leading to the hypothesis that it may be costly for females to deposit this pigment onto their eggs (Moreno and Osorno 2003). Some studies have demonstrated positive relationships between biliverdin-based eggshell coloration and egg characteristics, female and/or chick body condition and immune capacity (e.g. López-Rull et al. 2008, Moreno et al. 2008), but other studies have failed to find significant relationships (e.g. Cassey et al. 2008, Krištofík et al. 2013). Before we can truly understand the function of protoporphyrin and biliverdin in eggshells, we must understand their related physiology.

The deposition of pigments, and when this occurs during the process of eggshell formation, is also not well understood. Eggshell pigmentation patterns vary widely across species, from distinct spots on the eggs of blue and great tits (Fig. 1.3) to a mixture of spots and streaks on the eggs of hawfinches (Fig. 6.1). It is believed that distinct spots on eggshells is resultant from pigment secretion into the eggshell cuticle, while larger patches and streaks result from larger quantities of pigment secreted during the final stages of eggshell formation, possibly whilst the egg is in rotation (Solomon 1987, Sparks 2011).

7.3.2 Distinguishing between pigment concentration and eggshell colour

Eggshells often contain pigment within the eggshell matrix (Roberts 2004) which is not accounted for by most current methods which quantify eggshell colour only. The comparison of methods such as visual and pixel-based spot scoring with direct measures of protoporphyrin concentration was a crucial part of my thesis work as it was found that the former scoring methods cannot be used as a reliable proxy for the latter (Chapter Four). Nor does quantifying eggshell background coloration accurately reflect eggshell protoporphyrin or

biliverdin content (Chapter Six). Therefore, we argue that caution must be exercised when relating eggshell colour (i.e. background colour or spotting) directly to eggshell pigment concentration.

Significant findings relating eggshell pigmentation patterns to other variables such as female body condition (Martínez-de la Puente et al. 2007), and parental investment (Walters and Getty 2010) are fundamental. Pigment concentration is not necessarily a more instructive measure of adaptive function than visible maculation because it is not simply a function of how much is present (Higham and Gosler 2006). It can depend on where and how it is deposited on or within the shell. However, future studies using eggshell pigmentation patterns must be clear in interpretation of findings and not simply extrapolate visual spotting to protoporphyrin concentrations.

It has been suggested that pigment may be deposited throughout the entire depth of the eggshell (e.g. Jagannath et al. 2008), or, conversely, that the majority of the pigment is deposited on the outermost layer of the eggshell (Wang et al. 2007). Therefore, future investigations should examine the function of eggshell pigmentation, considering both the visible pigment spots on the eggshell surface and pigment throughout the eggshell as quantified for example, by HPLC analysis.

7.3.3 Eggshell pigmentation as an environmental monitoring tool

Avian eggs can act as an effective bio-monitoring tool (e.g. Ormerod and Tyler 1990, Van den Steen et al. 2010) due to their high lipid contents, which concentrate hydrophobic contaminants (Van den Steen et al. 2006). Eggshells in particular are sensitive to persistent organic pollutants, either directly, by blocking Ca uptake to the shell gland (Ratcliffe 1970,

Lundholm 1997) or indirectly, by disrupting the haem biosynthesis pathway and consequently pigment concentrations (Casini et al. 2003). Resident passerine species such as blue tits and great tits are particularly effective in monitoring local environmental contamination because of their small territories and foraging areas (Moore 1966, Dauwe et al. 2006).

Understanding eggshell protoporphyrin pigmentation offers the possibility of a non-destructive bio-assay of the health of the egg, the laying female and/or the environment. For instance, spotting on eggs has been linked to eggshell thickness (Gosler et al. 2005, Chapters Two and Three) which is sensitive to environmental Ca availability (Gosler et al. 2005) and pollutants (Eeva and Lehikoinen 1995), and has been identified as an indicator of egg quality (Sanz and García-Navas 2009). Eggshells of Eurasian sparrowhawks with protoporphyrin spots as an internalised layer showed a strong correlation between eggshell DDE (dichlorodiphenyldichloroethylene) content and shell thickness (Jagannath et al. 2008). Egg colour has further been found to be a good predictor of environmental contamination in herring gulls (*Larus argentatus*), although this correlation was found only with blue-green chroma (i.e. biliverdin) and not brown chroma (i.e. protoporphyrin) of eggs (Hanley and Doucet 2012). Establishing eggshell pigmentation as an environmental monitoring tool would allow us to monitor contaminants, such as heavy metals (e.g. lead, cadmium) and their effects by simply finding nests of free-living birds and documenting pigmentation on their eggs, creating a powerful tool in the monitoring of populations and the effectiveness of conservation actions.

7.4 Conclusions

One of the major assumptions of the structural-function hypothesis is that eggshells should contain more protoporphyrin (either through more extensive coverage of pigment spots or through higher concentrations within the eggshell or both) when dietary Ca availability is limited. This thesis is novel in investigating the relationship between eggshell protoporphyrin and Ca concentrations of both eggshells and breeding diet. Although results are equivocal due to females in the focal population possibly not being Ca-limited, several of the findings contribute greatly to the existing knowledge that attempts to explain a functional and ecological role for eggshell coloration. First, we showed that despite low local soil Ca concentration, female blue tits and great tits are not necessarily Ca-limited but that Ca supplements may still influence eggshell traits and breeding behaviour, possibly by providing females with more time to invest in other activities (e.g. foraging for foods rich in other nutrients, incubation). Secondly, we demonstrated the importance of quantifying eggshell pigment concentrations directly rather than using a proxy, such as pigment scoring, as visible eggshell pigmentation is not necessarily related to total eggshell pigment content. Finally, we showed that passerine eggshell pigment concentrations are highly phylogenetically conserved. We encourage future studies testing these key hypotheses to compare eggshell pigmentation of closely related species as this phylogenetic association may be essential to explain the functional and ecological significance of eggshell coloration of avian species.

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APPENDIX 1

Table A1.1. Eggshells were made available for chemical analyses through a destructive loan of British breeding bird species stored at the Natural History Museum, Tring, UK. These eggs were collected prior to 1954 by private collectors and are lacking in the quality of data required to be added to the main collection (Russell et al. 2010). For each species, three eggs were randomly chosen from different collections to ensure that eggs were not removed from the same clutch or female. Natural History Museum (NHM) accession numbers are provided for each of the samples.

Sample number	NHM accession numbers	Species
4, 5, 204	2008/32/8, 2008/55/68, 2008/138/22	*Acrocephalus palustris*
7, 244, 245	2008/221/37, 2008/32/5, 2008/119/37	*Acrocephalus schoenobaenus*
8, 9, 10	2008/80/26, 2005/61/31, 2008/122/77	*Acrocephalus scirpaceus*
205, 206, 11	2008/80/38, 2008/12/6, 2008/257/6	*Aegithalos caudatus*
12, 207, 208	2008/47/2, 2008/199/15, 2008/72/312	*Alauda arvensis*
16, 17, 18	2008/140/52, 2007/109/308, 2008/133/12	*Anthus petrosus*
13, 14, 15	2008/120/5, 2000/17/20, 2008/85/4	*Anthus pratensis*
19, 20, 21	2008/133/11, 2008/140/55, 2008/119/30	*Anthus trivialis*
23, 24, 25	2008/80/3, 1987/27/8, 2008/257/43	*Carduelis cabaret*
1, 202, 203	2005/61/26, 2008/119/40, 2008/32/19	*Carduelis cannabina*
26, 27, 209	2008/221/29, 2008/134/16, 2008/98/47	*Carduelis carduelis*
29, 210, 211	2008/221/17, 2008/120/8, 2008/72/396	*Carduelis chloris*
2, 3, 201	2008/50/143, 2008/80/13, 2008/122/67	*Carduelis flavirostris*
30, 212, 213	2003/25/54, 2008/32/21, 2008/50/55	*Certhia familiaris*
33, 34, 35	2008/169/33, 2008/12/20, 2004/91/9	*Cinclus cinclus*
36, 37, 38	2008/122/82, 2008/140/4, 2008/119/10	*Coccothraustes coccothraustes*
44, 45, 46	2008/22/49, 2008/165/6, 2008/50/84	*Corvus cornix*
47, 48, 214	2008/221/3, 2008/47/24, 2008/119/24	*Corvus frugilegus*
49, 50, 51	2003/13/2, 2008/177/32, 2008/199/76	*Corvus monedula*
52, 53, 54	1987/27/4, 2008/119/33, 2008/122/74	*Delichon urbica*
55, 56, 57	2006/45/28, 2008/98/5, 2008/12/12	*Emberiza calandra*
61, 62, 63	2008/72/370, 2007/31/11, 2008/50/141	*Emberiza cintrinella*
58,59, 60	2008/80/2, 2008/140/3, 2008/221/74	*Emberiza cirlus*
64, 65, 66	2008/199/51, 2008/72/354, 2000/17/11	*Emberiza schoeniclus*
67, 68, 69	2005/61/18, 2008/133/30, 2008/221/45	*Erithacus megarhynchos*
70, 71, 72	2007/31/4, 2008/123/4, 2008/90/7	*Erithacus rubecula*
74, 75, 76	2008/34/3, 2008/122/65, 2008/80/19	*Ficedula hypoleuca*
77, 215, 216	2008/126/4, 2007/111/7, 2008/36/7	*Fringilla coelebs*
78, 79,80	2008/44/1, 2008/122/88, 2008/50/140	*Fringilla montifringilla*
81, 217, 218	2008/169/9, 2008/169/10, 2008/72/148	*Garrulus glandarius*
82, 219, 220	2008/34/1, 2005/61/28, 2004/91/22	*Hirunda rustica*
83, 84, 221	2000/17/18, 2008/59/6, 2008/13/49	*Lanius collurio*

85, 86, 87	2008/122/84, 1987/27/18, 2008/140/49	*Locustella naevia*
88, 89, 90	2008/138/3, 2008/50/63, 2008/140/46	*Loxia curvirostra*
91, 92,93	2008/95/12, 2006/45/16, 1987/27/7	*Lullula arborea*
94, 95, 96	2008/72/415, 2008/177/56, 2008/199/16	*Motacilla alba*
97, 98, 222	2008/221/63, 2008/199/41, 2008/50/154	*Motacilla cinerea*
99, 100, 101	200/119/38, 2008/123/8, 2008/80/31	*Motacilla flava*
102, 103, 104	2004/91/23, 2008/36/5, 2000/17/9	*Muscicapa striata*
105, 106, 107	2008/50/127, 2008/177/47, 2008/72/301	*Oenanthe oenanthe*
108, 109, 223	2004/91/1, 2008/138/12, 1987/27/1	*Oriolus oriolus*
113, 114, 224	2008/199/4, 2008/221/69, 2008/257/30	*Parus ater*
115, 116, 225	2008/12/8, 2008/221/68, 2008/257/4	*Parus caeruleus*
119, 120, 121	2008/12/10a, 2007/109/165, 2000/17/16	*Parus major*
122, 123, 124	2008/13/36b, 2008/221/65,	*Parus palustris*
227, 228, 229	2001/102/11, 2008/90/3, 2005/61/9	*Passer domesticus*
125, 126, 230	2008/80/27a, 2008/59/19a, 2008/119/36	*Passer montanus*
132, 133, 231	2007/31/17, 2008/177/50, 2008/12/15	*Phoenicurus phoenicurus*
134, 135, 232	2007/31/12, 2008/122/79, 2008/12/4	*Phylloscopus collybita*
136, 137, 233	2008/55/67, 2008/50/69, 2005/61/7	*Phylloscopus sibilatrix*
127, 128, 235	2005/61/36, 2008/98/14, 2008/257/19	*Pica pica*
143, 144, 145	2007/109/103, 2008/140/5, 2008/50/47	*Plectrophena nivalis*
140, 141, 142	2008/98/33, 2008/27/6, 2005/61/17	*Prunella modularis*
146, 148, 236	2008/122/30, 2007/31/22, 2008/139/2	*Pyrrhocorax pyrrhocorax*
149, 150, 246	2000/17/12, 2007/31/2, 2008/59/16	*Pyrrhula pyrrhula*
151, 152, 237	2008/122/57, 2008/257/10c, 2007/109/128	*Regulus ignicapilla*
153, 154, 155	2003/25/57, 2000/17/13, 2008/177/51	*Regulus regulus*
156, 157, 158	2005/61/24, 2008/32/17, 2008/122/54	*Riparia riparia*
159, 160, 161	2008/257/14, 200/199/9, 2008/80/11	*Saxicola rubetra*
162, 163, 164	2008/221/42, 2008/133/38, 2000/17/5	*Saxicola torquata*
166, 167, 168	2008/50/40, 2008/221/70, 2008/120/7	*Sitta europaea*
180, 241, 242	2008/12/19, 2008/221/18, 2008/127/8	*Sturnus vulgaris*
169, 170, 243	2008/221/76, 2008/120/6, 2008/80/2	*Sylvia atricapilla*
171, 172, 173	2008/257/29, 2007/31/5, 2000/17/8	*Sylvia borin*
174, 175, 176	1987/27/19, 2008/80/20, 2005/61/10	*Sylvia communis*
177, 178, 179	2008/133/41, 2008/140/57, 2008/80/22	*Sylvia curruca*
181, 182, 238	2008/221/72, 2005/61/12, 2008/257/5	*Troglodytes troglodytes*
187, 188, 239	2008/162/3, 2008/138/10, 2007/109/301	*Turdus iliacus*
183, 184, 185	2008/98/49, CS3, CS4	*Turdus merula*
189, 190, 191	2008/126/3, 2008/91/2, 2008/90/6	*Turdus philomelos*
192, 193, 194	2008/50/157, 2007/109/157, 2008/162/2	*Turdus pilaris*
195, 196, 197	2008/72/133, 2008/138/3, 1987/27/5	*Turdus torquatus*
198, 199, 200	2008/199/47, 2008/32/29, 2008/27/3	*Turdus viscivorus*

PUBLICATIONS FROM THESIS

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