IS EGGSHELL PIGMENTATION A CONDITION-DEPENDANT STRATEGY?

IMPLICATIONS FOR EGG CRYPSIS IN JAPANESE QUAIL

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Abstract

Avian eggshell colouration fulfills multiple adaptive functions, including egg camouflage. The potential role of the two main eggshell pigments in oxidative stress, biliverdin and protoporphyrin, may be behind a relationship between female immunocompetence and eggshell pigment investment strategies. In this study, environmental conditions were manipulated during different life cycle stages, via a variety of methods, including food-restriction and stress hormone exposure in female Japanese quails (*Coturnix coturnix japonica*), in order to experimentally test the condition-dependence of eggshell pigmentation, and to give first insights into the possible implications for egg crypsis. I demonstrated that eggshell pigmentation strategy is not only affected by female current body condition, but is also shaped by its early life experience such as exposure to stress, and that eggshell colouration is a key factor involved in egg crypsis in Japanese quail. Eggshell colour and maculation were both independently affected by breeding conditions; which stresses the complexity of the relationship between eggshell pigment concentrations and its appearance. My findings imply that eggshell appearance is a female extended phenotypic trait, and that trade-offs between eggshell pigmentation and immune-functions may lead to inter-females differences in their ability to maximise egg crypsis.

For my grandads, pépé Albi and pépé René who have always been supportive and who have always been proud of me whatever my choices were.

A mes grands-pères, pépé Albi et pépé René qui m'ont toujours supportée et qui ont toujours été fiers de moi, quelque soient mes choix.

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I would like to thank Carole Chestnut and Malcolm McColl from Cochno farm (Glasgow) for their help in setting up my experiments, providing care to the birds and making the farm such a nice atmosphere to work in. None of my work would have been achieved without my collaborators who provided me some of the data that I analysed in this thesis: Dr George Lovell and Dr Ivan Mikšík. I also thank Dr Daniel Hanley who provided useful advices that improved some of my manuscripts.

Not feeling home was the main worry I had when I left France and moved to the UK. Luckily, I met amazing people in Birmingham, who became my friends, and I would like to thank them for sharing with me unforgettable evenings in Staff House and elsewhere (and also lab meetings): Abi, Emma T., Emma R., Jolyon, Julia, Kaat (my egg-mate who I have missed in the last two years of my PhD), Katy, Kirsty, Shivani, Simone, Zoe. I would also like to thank Dr Steve Portugal and Dr Golo Maurer for their kind help during the first stages of my PhD. A special thank you to my non-lab friends who started their PhD with me 4 years ago or joined us later, they all moved on now and I am proud of them: Manu and Guillaume, Giulio, Cristian, Sylwia, Kat, Steph and Tom, Kiran and Apo, Paul (I will never forget your driving skills!) and Megan who I will hopefully graduate with next summer.

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List of publications

Chapter Two

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Duval, C., P. Cassey, I. Mikšík, S. J. Reynolds & K. A. Spencer (2013). Condition-dependent strategies of eggshell pigmentation: an experimental study of Japanese quail (*Coturnix coturnix japonica*). *The Journal of experimental biology* ***216***(4): 700-708.

Duval, C., P. Cassey, P. G. Lovell, I. Mikšík, S. J. Reynolds & K. A. Spencer (under review in the Auk: Ornithological Advances). Maternal influence on eggshell maculation: implications for cryptic camouflaged eggs.

Chapter Three

Duval, C., P. Cassey, P. G. Lovell, I. Mikšík, S. J. Reynolds & K. A. Spencer (*in press*). Eggshell appearance does not signal maternal corticosterone exposure in Japanese quail: an experimental study with brown-spotted eggs. *Plos One*.

Declaration of author's contribution

Chapter One

Entirely my own work

Chapter Two

CD designed the experiments with an input from KAS, collected and analysed the data. IM quantified eggshell pigments. PGL wrote the Matlab script to analyse eggshell maculation. CD wrote the manuscripts with an input from PC, SJR and KAS

Chapter Three

CD designed the experiment 1 (section 3.2) with an input from KAS, collected and analysed the data. CZ and KAS designed and conducted the experiment 2, CD collected and analysed the data, with an input from KAS and CZ. IM quantified eggshell pigments. PGL wrote the Matlab script to analyse eggshell maculation. CD wrote the manuscripts with an input from PC, SJR, CZ and KAS

Chapter Four

CD designed the experiment with an input from KAS and PGL, collected and analysed the data. PGL performed the photos analyses in Matlab. CD wrote the manuscript with an input from PC, SJR, KAS and PGL

Chapter Five

Entirely my own work

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Chapter One

GENERAL INTRODUCTION

1.1. Life history theory and the cost of reproduction

Life-history theory postulates that organisms have to face a trade-off between current and future reproduction and consequently, life history traits often vary in an opposite way (Clutton-Brock *et al.* 1982, Reznick 1985, Roff 1992, Stearns 1992, Rose *et al.* 1998). A classical illustration of this is in lines of Drosophila (*Drosophila melanogaster*) selected for a greater longevity, which showed a declined early fecundity (Rose 1984).

In birds, the cost of reproduction has been experimentally investigated as a constraint on both survival (Linden and Møller 1989, Graves 1991, Roff 1992), and reproductive success at the next breeding attempt (Gustafsson and Sutherland 1988). Indeed, resources may be preferentially allocated to reproduction by reducing investment in resources allocated towards somatic protection and maintenance (Stearns 1992). Thus, it is possible that physiological trade-offs arise between traits expressed during different stages of life (Stevens *et al.* 1999, Zera *et al.* 2001), and the optimal situation balances the costs and benefits of such trade-offs against any factor which could alter an individual's quality (Reznick 1985).

1.1.1. Role of the environment in reproduction

1.1.1.1. Concept of "maternal effects"

The environment can have profound long-term effects, both direct (via influencing offspring phenotype) or indirect (through maternal condition) on a developing organism. Indirect effects, also referred to as "maternal effects" (Mousseau & Fox 1998), have been the focus of a large body of literature (Badyaev & Uller 2009, Meylan *et al.* 2012, Hoyle & Ezard 2012, Sheriff & Love 2013). In many species, maternal condition can have a strong influence on offspring physiology, morphology and behaviour (reviewed in Mousseau & Fox 1998). Birds are frequently used as models to study the effects of environmental changes during different

developmental stages (Price 1998). Contrary to mammalian species, the avian embryo does not develop inside the mother's body. Thus, once the egg is laid, the mother cannot directly influence offspring development, except by modifications of her incubation behaviour (Groothuis *et al.* 2005). This allows studies to disentangle environmental effects from maternal effects on embryo physiology, unlike in mammals because of the strong link between the mother and her young during lactation.

Several parameters of reproductive success such as hatchability and chick survival are related to maternal investment. To maximise their reproductive success, female birds are able to modulate their investment not only through adjustments in their clutch size but also through their egg quality (Bernardo 1996), according to parameters such as their own body condition (Hanssen *et al*. 2003) or male attractiveness (Loyau *et al.* 2007a). It has been demonstrated that larger eggs contain more nutrients and are more likely to produce structurally larger chicks at hatching (Ricklefs *et al.* 1978, Williams 1994, Finkler *et al.* 1998). However, variation in the total nutrient content is only one way a female can manipulate the developing environment of her young. The quantity of specific egg yolk/albumen components can also be modulated, including hormones (e.g., testosterone) (Petrie *et al*. 2001, Mazuc *et al.* 2003, Loyau *et al*. 2007a), antibacterial agents (e.g., lysozyme) (Saino *et al.* 2002) or antioxidant molecules (carotenoids, vitamins) (McGraw & Ardia 2003, Blount *et al.* 2004, Biard *et al.* 2005, Costantini 2010). In particular, these egg components determine chick quality by influencing embryonic development, hatching success, sex ratio, chick growth, survival and immunity (Birkhead & Nettleship 1982, Amundsen & Stokland 1990, Arnold *et al*. 1991, Hill 1993, Williams 1994, Amat *et al.* 2001).

1.1.1.2. Eggshell characteristics as maternally-derived traits

Maternal investment in the eggshell has received less attention. It protects the embryo from mechanical damage; controls water loss (Board & Halls 1973, Carey *et al.* 1983, Handrich 1989) and regulates gas exchange (Tullet 1984) between the embryo and the external environment. It also prevents contamination by bacteria (Board 1980) and other pathogens; and provides a source of nutrients, primarily calcium, to the developing embryo (Burley 1989, Reynolds & Perrins 2010). Environmental factors can strongly affect eggshell structure as gas exchange through the shell allows sufficient water loss and while preventing the embryo from been dehydrated (Board & Scott 1980, Tullett 1984). Ambient humidity seems to have been the strongest selection pressure on eggshell structure (Kern & Cowie 2000, Deeming 2002): low relative humidity can lead to embryo desiccation, while high humidity increases the risk of mechanical restriction of the embryo. Most of the avian eggshell dry mass consists of a crystalline form of calcium carbonate, and laying females have a high demand for calcium, especially for eggshell formation (Reynolds *et al.* 2004). Calcium can be mobilised from the skeleton but mostly comes from the mother's diet (Pahl *et al.* 1997, Larison *et al.* 2001, Reynolds 2001). Thus, calcium availability in the environment is an important limiting factor that can influence the quality and structure of the eggshell (Gosler *et al.* 2005).

In addition, among the compounds deposited by females into the eggshell are pigments. Eggshell pigmentation is due to the presence of two main pigments: biliverdin and protoporphyrin that are not found in the environment. Instead, both biliverdin and protoporphyrin are part of haem biosynthesis pathway (Thiel 1968) and possess opposite physiological properties (see section 1.2.2). Thus, their deposition in the eggshell only depends on their synthesis by the female. Eggshell biliverdin and protoporphyrin concentrations could be maternally-derived traits of which the deposition is modulated by

female according to her body condition during reproduction, as is the case for yolk and albumen components.

1.1.1.3. Maternally-derived stress

Environmental variation during reproduction, such as increased predation risk, decreased food availability, increased social stressors or a decline in habitat integrity (reviewed in Sheriff & Love 2013) can act as pressures and increase maternal glucocorticoids (GCs). GCs are referred to as 'stress' hormones, namely corticosterone (CORT) in birds, and play a role in physiological and behavioural responses to stress (Sapolsky *et al.* 2000, Wingfield 2005). Indeed, any stressful stimulus can lead to the activation of the Hypothalamic-Pituitary-Adrenal (HPA) axis, eventually resulting in the release of CORT (Wingfield 1994, Romero 2004). CORT induces an increase in glucose released to maximise the energy available for the optimization of life-saving behavioural strategies (Munck *et al.* 1984, Wingfield 1998). Elevated CORT levels can be sustained for a long period of time when individuals are faced with chronic stress and unable to bring their concentration back to a basal level (Angelier & Chastel 2009). Chronic stress exposure can have negative effects on the nervous system and cause deficiencies of some immune and physiological functions such as the capacity of eliminating free radicals that increase oxidative stress (McEwen & Stellar 1993, Sapolsky 2000, de Kloet *et al.* 2005, Costantini *et al.* 2011). A recent hypothesis proposed that early life conditions may shape physiology and behaviour in order to enhance fitness if early environmental conditions match those experienced across life stages. According to this 'environmental matching hypothesis', the mismatch between environmental conditions at different stages of life may be responsible for the negative effects of developmental stress (Bateson *et al.* 2004, Gluckman *et al.* 2005, Monaghan 2008).

Mothers can expose their offspring to the stress they face (Maternally-Derived Stress, MDS) during reproduction directly via modification of their maternal care, or indirectly via GCs transferred into the egg (Almasi *et al.* 2012). Some studies in birds have shown that an experimental increase in maternal GCs during the laying period can induce an increase in GC concentration in both egg yolk and albumen (Hayward & Wingfield 2004, Love *et al.* 2005, Saino *et al.* 2005, Almasi *et al.* 2012). Offspring respond to MDS via physiological and behavioural changes that can affect neural development such as the HPA axis and amygdala function (Hayward & Wingfield 2004, Love & Williams 2008a, Sheriff *et al.* 2013). Moreover, MDS induces morphological changes such as a low hatching weight and small structural size (Saino *et al.* 2005, Love & Williams 2008b, Sheriff *et al.* 2009). Thus, egg characteristics strongly depend on maternal stress, however less is known about maternally deposited eggshell components, and this is especially true in the case of eggshell pigments.

1.2. Eggshell pigmentation

Eggshell pigmentation is responsible for the high diversity of egg colours and patterns observed across the class Aves (Kennedy & Vevers 1976, Kilner 2006, Walters 2006, Cassey *et al.* 2010a, Fig. 1.1), and has received poor attention in the context of maternal effects in birds.

Figure 1.1. Photograph of eggshells showing the diversity of colours and patterns in the class Aves (Photo credit: Golo Maurer).

1.2.1. Eggshell pigments: composition, synthesis and deposition

The two main pigments found in avian eggshells are Porphyrins: protoporphyrin and biliverdin (Kennedy & Vevers 1976, Gorchein *et al.* 2009), and they are both derivatives of haem from erythrocytes (Thiel 1968). Protoporphyrin consists of four pyrrole rings (tetrapyrroles) and its formation starts with the synthesis of porphobilinogen from glycine and active succinate (Solomon 1987) (Fig. 1.2). Porphobilinogen is converted to urporphyrin which gets decarboxylated to protoporphyrinogen. The molecule becomes colourful when the protoporphyrinogen auto-oxidises to protoporphyrin (Sparks 2011).

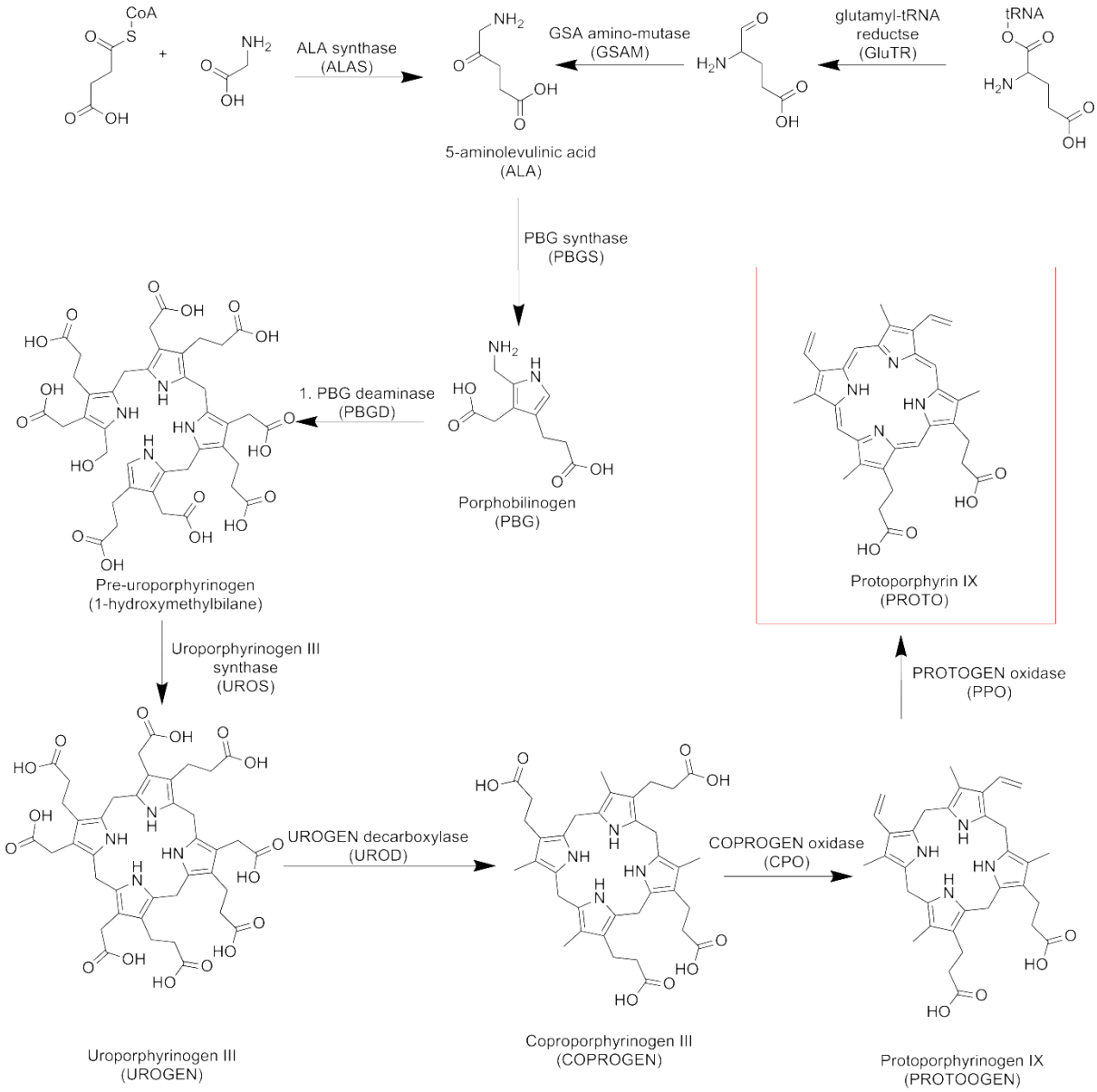

Figure 1.2. Chemical schematic of the biosynthesis of protoporphyrin IX (http://commons.wikimedia.org/wiki/File:Protoporphyrin_IX_Biosynthetic_pathway.png)

Similarly, Biliverdin is an open chain, tetrapyrrolic pigment. Biliverdin comes from the cleavage of haem by haem oxygenase-1 (Ryter *et al.* 2006) (Fig. 1.3) and is a major pigment found in the bile of vertebrates.

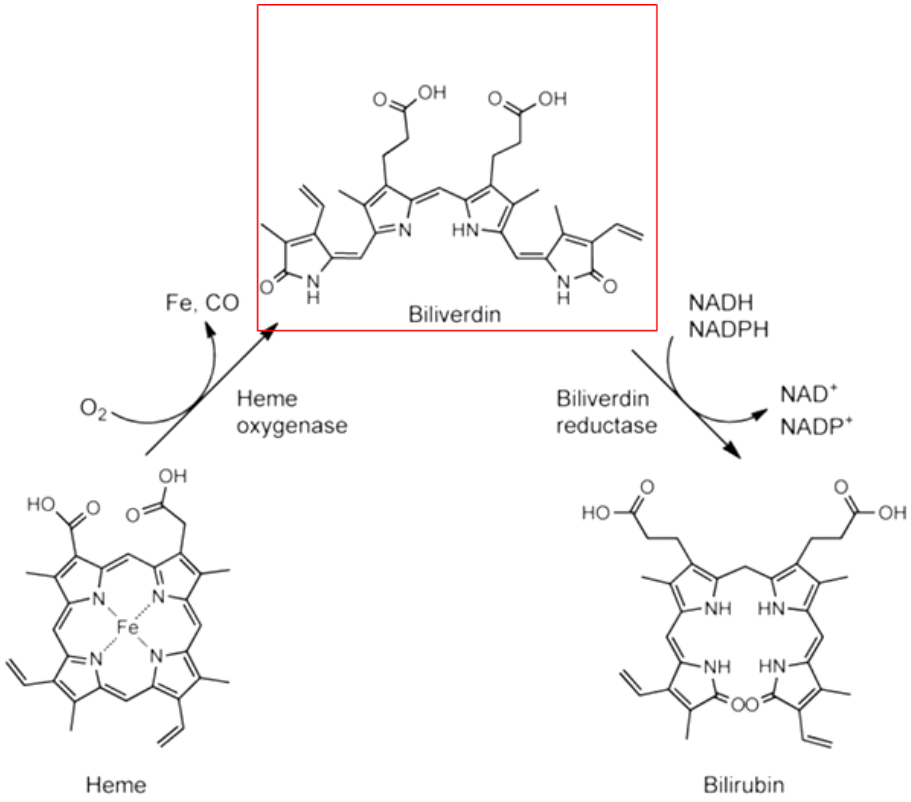

Figure 1.3. Chemical schematic of the enzymes and intermediates involved in the biosynthesis of biliverdin (adapted from http://www.urmc.rochester.edu/labs/Maines-Lab/history/index.cfm).

Both pigments show absorption bands in the visible region of the light spectrum, resulting in intense colouration. Protoporphyrin is responsible for red-brown colours while biliverdin provides shades of blue to green (Kennedy & Vevers 1976, Fig. 1.4).

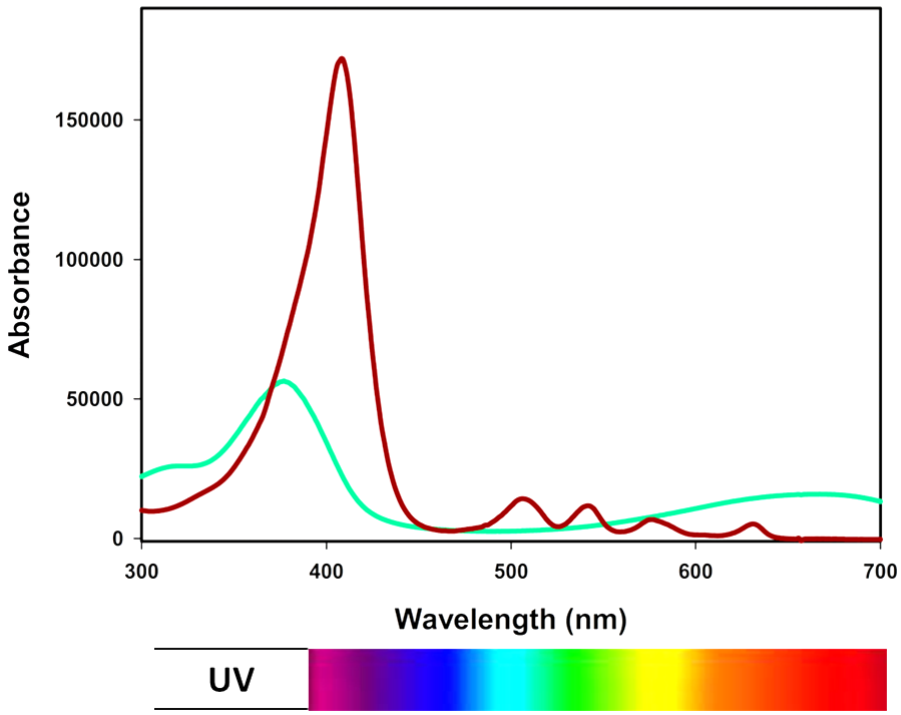

Figure 1.4. The absorbance spectra of protoporphyrin IX (brown line), and biliverdin (green line), and the light spectrum.

Eggshell pigments are deposited into, or onto, the eggshell in either the uterus or shell gland during the last stage of egg formation, just few hours before oviposition. The origin of eggshell pigments is still under debate as while the uterus has been shown to be the site of pigment secretion (Breen & Bruyn 1969); its role in pigment synthesis is still unclear. As protoporphyrin and biliverdin are products of haem degradation, both pigments could either be directly derived from blood cells or be synthesised in the uterus. In chickens (*G. gallus domesticus*) that lay either blue or brown eggs, the concentrations of biliverdin in blood, bile and excreta was similar in both groups, whereas in the uterus and eggshell of blue eggs layers it was higher (Zhao *et al.* 2006). More recently, Wang *et al.* (2010) showed that Haem-Oxigenase-1 (HO-1) expression in the uterus of hens laying blue-green eggs was much higher than in brown-eggs layers. Nevertheless, much less is known about the synthesis of

protoporphyrin. In Japanese quail, protoporphyrin was found to accumulate in the uterus up until 20 hours after ovulation and then decrease rapidly (Soh *et al.* 1993). Similarly, Baird *et al.* (1975) showed an increased concentration of protoporphyrin in the uterus of hens laying white and brown eggs during egg formation. It was only as recently as 2007 that researchers proposed that biliverdin and protoporphyrin were synthesised in the uterus and then deposited into or onto the eggshell (Wang *et al.* 2007). The question of transport for both pigments also remains unclear as it has been proposed that the differences in eggshell pigmentation patterns might be not only due to differences in pigment concentrations but also variation in the speed of pigment transport and deposition onto the eggshell (Baird *et al.* 1975, Liu *et al.* 2010). The deposition of eggshell pigments has been studied primarily in the context of Poultry Science. In Japanese quail, protoporphyrin is deposited throughout the shell integument, from the membrane to the cuticle (Tamura & Fujii 1967), whereas in domestic chicken, higher concentrations are found in the cuticle (Baird *et al.* 1975). In Passeriformes species, pigments are detected in the outer third of the shell or outer half of the shell (Harrison 1966). In species laying spotted eggs, when the cuticle is thin or absent, spots are created by intermixing pigments within the calcium matrix, while the spotting found within the cuticle is known as cuticular pigment (Romanoff & Romanoff 1949) (Fig. 1.5). The diversity of observed eggshell patterns is clearly due to a complex interaction between biliverdin and protoporphyrin during eggshell formation and much remains to be understood about the physical process of pigmentation.

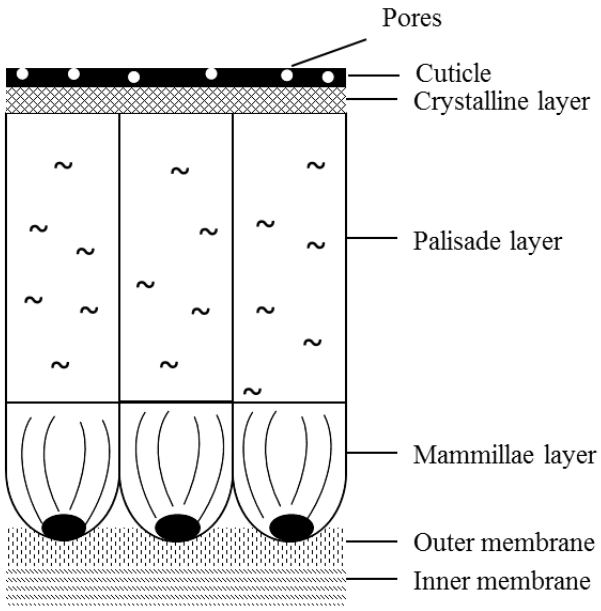

Figure 1.5. Diagram of the side view of the egg shell.

1.2.2. Physiological properties of eggshell pigments

Porphyrins have been extensively studied for their role in oxidative stress (Bonkovsky *et al.* 2013), particularly protoporphyrin and biliverdin which may possess opposing antioxidant properties (Kachadourian *et al.* 2003). Protoporphyrin, thought to be responsible for the brown spots on maculated eggshells, may possess pro-oxidant properties and induce an oxidative stress response when accumulated in the liver (Afonso *et al.* 1999). *In vitro*, protoporphyrin can directly stimulate the synthesis of haem oxygenases (HOs) such as HO-1 or heat shock proteins (HSPs) (Shan *et al.* 2000), which are synthesised after cellular stress and prevent proteins from misfolding (Åkerfelt *et al.* 2010). In contrast, bilirubin and biliverdin are thought to possess the opposite (i.e. antioxidant) properties (McDonagh 2001). In human blood plasma, both bile pigments would be involved in the oxidation of lipids (Frei *et al.* 1988) and proteins (Neuzil *et al.* 1993). In addition, they possess anti-inflammatory (Nakamura *et al.* 1987), anti-viral (Mori *et al.* 1991), anti-apoptotic (Dudnik *et al.* 2001) and

anti-mutagenic functions (reviewed in Bulmer *et al.* 2008). In mammals, the biliverdin/bilirubin combination is involved in immune responses (Otterbein *et al.* 2003, Sedlak *et al.* 2009), and biliverdin for instance may accelerate the development of the embryo in amphibians (Falchuk *et al.* 2002) which do not produce bilirubin.

1.2.3. Adaptive roles of eggshell pigmentation

The diversity and functionality of avian eggshells patterns have attracted the attentions of evolutionary ecologists for many years. Until the 1920s, predation and brood parasitism were the main selection pressures proposed to explain eggshell colouration patterns (Kilner 2006). Here, I explore the evidence for each of these hypotheses in turn: egg crypsis, egg recognition, structural function and post-mating sexual signalling.

1.2.3.1. Egg crypsis

Crypsis is defined as the resemblance in colouration between an animal or an object to its background (Edmunds 1990). In birds, the ancestral eggshell is hypothesised to have been white without any apparent pigmentation (Kilner 2006) and colourful eggshells are thought to have evolved due to environmental pressures that are specific to each nesting area, helping to enhance egg camouflage and avoid predation (Wallace 1889). Blue-green and immaculate white eggshells may not be cryptic because of their visual contrast with the nest (Westmoreland & Best 1976, Blanco & Bertellotti 2002, Magige *et al.* 2008); whereas brown eggshells may be less predated as their detectability is decreased (Tinbergen *et al.* 1962, Götmark 1992, Solís & De Lope 1995, Yahner & Mahan 1996, Castilla *et al.* 2007, Westmoreland 2008). For example, Lack showed that hole-nesters lay mostly white eggs, whereas 80% of birds that nest in open areas lay spotted eggs which may enhance their concealment (Lack 1958). Two strategies have evolved in birds in order to hide the eggs from

predators: 1) building a nest with a dome and hide the eggs with nest material, and 2) in ground-laying species, matching egg colour with the colour of the nest background to enhance egg crypsis and make it visually undetectable (Wallace 1889). Brown and spotted eggshells are thought to particularly enhance egg crypsis and may be less predated than non-spotted and brighter eggshells, but both experimental and natural studies have found mixed support for this hypothesis (Ricklefs 1969, Collias 1984, Götmark 1992, Weidinger 2001, Underwood & Sealy 2002). For instance, in stone curlews (*Burhinus oedicnemus*), laying eggs of which the colouration matched the ground decreased predation rate (Solís & De Lope 1995). Similarly, Yahner and Mahan (1996) showed that nests with brown chicken eggs were less likely to be disturbed by predators than white chicken eggs using artificial nests. The cryptic colour of eggs in semi-palmated Plovers (*Charadrius semipalmatus*) could make eggs less conspicuous (Nguyen *et al.* 2003). In addition, cryptic colouration had a survival advantage depending on the predator species in red-legged partridges (*Alectoris rufa*) (Castilla *et al.* 2007). Lee and colleagues suggested that eggs that matched nest background colour were more likely to hatch. Particularly, in sites where nest concealment was low, eggs matching the colour of the nest showed a better survival (Lee *et al.* 2010).

Yet, most experimental studies using painted eggs actually found no differences in predation rates between natural and artificial painted eggs (e.g. Montevecchi 1976, Götmark 1992, Weidinger 2001). One major explanation for the range of conflicting results may lie in the use of artificially painted eggs or artificial nests that can never match natural levels of crypsis. For instance, Ortega *et al.* (1998) found that artificial nests were predated significantly more often than American robin (*Turdus migratorius*) nests tested in their natural environment, even when Japanese quail eggs were placed in the nest. Consequently, while the egg crypsis hypothesis is valid for most ground-laying species, biases in the experimental design as well as inconsistency in the method for measurement of egg

colouration and maculation can lead to contradictory findings (see review Cherry & Gosler, 2010).

1.2.3.2. Egg recognition

Variation in eggshell colour and patterning, facilitating eggshell recognition, may be an adaptive strategy in colonially nesting species or any species where egg dumping or parasitism is common (Gaston *et al.* 1993) but the majority of studies have found little conclusive evidence for individual clutch recognition in these species (Tschanz 1959, Shugart 1987, Schaffner 1990). Similarly, Hanley and colleagues (2013) tested whether eggshell conspicuousness in ratites served an intraspecific signalling function to advertise nest location to females in communally nesting species, but did not find any support for this hypothesis.

Alternatively, eggshell colouration may facilitate the recognition of brood-parasite eggs laid in the host species nest. Eggshell appearance may be involved in an arms race between brood parasites and host species (Øien *et al.* 1995, Langmore *et al.* 2009, Stoddard & Stevens 2010). Mimicking the appearance of the host species eggs may be the best strategy to ensure host parents will raise the brood-parasite chicks (Davies 2000). The common cuckoo (*Cuculus canorus*) represents a classical example of an evolutionary arms race and drew the early interest of Alfred Russell Wallace (Wallace 1889) because of the degree of achieved egg mimicry. Numerous studies have compared the degree of mimicry between hosts and parasites eggs (Brooke & Davies 1988, Davies & Brooke 1989, Moksnes & Røskaft 1995), however, it is only recently, with advances in predictive models of avian perceptual vision that the behaviour of the signal receiver (avian viewer) has been included in the analyses (Stoddard & Stevens 2011).

One strategy adopted by birds to counteract brood parasitism is to lay a clutch of highly similar eggs in terms of colour and patterns, so that they are visually different from other nests (Swynnerton 1918, Victoria 1972). Thus, brood parasites cannot mimic the colour of the host eggs and its eggs will be likely rejected (Øien *et al.* 1995, Soler & Møller 1996, Moskát *et al.* 2002, Stokke *et al.* 2002a, Avilés & Møller 2003, Avilés *et al.* 2006, Kilner 2006). In great reed warblers (*Acrocephalus arundinaceus*), decreasing egg pattern/colour uniformity within a clutch by painting additional spots on the eggshells increased birds tolerance to parasite eggs (Moskát *et al.* 2008). The authors concluded that great reed warbler hosts may lay a clutch of homogeneously coloured/patterned eggs to facilitate cuckoo egg discrimination (Moskát *et al.* 2008). In chaffinches (*Fringilla coelebs*), increasing the perceived chromatic contrasts between natural parasite and host eggs enhanced the discrimination of parasite eggs (Avilés *et al.* 2010).

Yet, most studies have focused on eggshell background colour (Cherry & Gosler 2010) rather than eggshell patterning such as maculation (brown spots) and recently, it has been shown in the house sparrow (*Passer domesticus*) that egg rejection increased significantly when spot patterns, rather than eggshell colour, were experimentally modified (López-de-Hierro & Moreno-Rueda 2010). Thus, both eggshell colour and pattern may play a crucial role in egg recognition in the context of brood parasitism.

1.2.3.3. Structural function

Other hypotheses to explain the evolution of eggshell pigmentation have focused on the physical properties of the eggshell and authors have proposed that the deposition of pigments could either help to directly strengthen the eggshell ("structural function hypothesis", Gosler *et al.* 2005) or protect the embryo from environmental insults (reviewed in Maurer *et al.* 2011a).

A number of observational studies have proposed that eggshell pigments (particularly eggshell pattern or maculation) in the great tit (*Parus major*) may relate to female calcium availability and eggshell thickness (Gosler *et al.* 2005, Higham & Gosler 2006). According to Gosler *et al.* (2005), the eggshell is thinner where the brown spots are present, and changes in eggshell thickness are associated with both the distribution and the intensity of the spots. The authors suggested that protoporphyrin, which is responsible for the brown maculation in spotted eggs (Kennedy & Vevers 1976), could counterbalance a lack of calcium and therefore directly reinforce eggshell strength (Solomon 1987, Gosler *et al.* 2005). Subsequent studies, which have supported the "structural function hypothesis", proposed that maculation may reduce eggshell permeability during incubation (Higham & Gosler 2006), and that thin-shelled egg would indicate that female suffered from a lack of calcium during breeding (Ar *et al.* 1974, Graveland *et al.* 1994). Nevertheless, recent studies that have experimentally tested the structural function of eggshell pigmentation have found only mixed support. In blue tits (*Cyanistes caeruleus*), females supplemented with calcium laid eggs with spots that were more widely distributed over the eggshell than the control, and laid less defective eggshells, suggesting that spots distribution might indicate calcium deficiency in that species (García-Navas *et al.* 2011). In contrast, in great tits, there was no correlation between eggshell thickness and eggshell pigmentation, and the authors did not find any evidence of an effect of calcium supplementation on eggshell pigmentation (Mägi *et al.* 2012). In a similar experiment, great tits supplemented with calcium laid thinner eggshells with darker spots, but the effect was only significant for one year of study (Hargitai *et al.* 2013). Thus, it seems that the relationship between eggshell pigmentation, eggshell strength and its calcium content remain to be experimentally tested, in particular with a quantification of eggshell protoporphyrin concentration.

Another physical function of eggshell pigmentation might be to protect the embryo from environmental factors such as solar radiation (Lahti 2008, Magige *et al.* 2008) or microbial contamination (Bulmer *et al.* 2008, Ishikawa *et al.* 2010). Eggshell pigmentation could serve to maintain a viable temperature for embryonic development as protoporphyrin and biliverdin provide a near infra-red reflectance (Fig. 1.2), avoiding the risk of overheating (Bakken *et al.* 1978, but see Westmorland *et al.* 2007). Moreover, solar radiation (UV-B, 290-320 nm) could also harm the embryo and cause DNA damage (de Gruijl *et al.* 2001) with detrimental effects during development. The spectral properties of protoporphyrin and biliverdin may help to absorb UV-B radiation and avoid potentially lethal damage in embryos. In addition to its role in light filtration, eggshell pigmentation may prevent the egg from microbial infection through the eggshell surface as UV-radiation induces bacteria and fungi death (Fargues *et al.* 1997, Davies-Colley *et al.* 1999, Chavez *et al.* 2002). Porphyrins are also known to possess photodynamic antimicrobial properties and are used in medical research to kill cancer cells or pathogens when combined with visible light (Dolmans *et al.* 2003), thus conferring potential antimicrobial properties to pigmented eggshells in birds (Ishikawa *et al.* 2010).

1.2.3.4. Post-mating sexual signalling

Moreno and Osorno (2003) outlined the Sexually Selected Eggshell Colouration (SSEC) hypothesis and proposed that investment, particularly in blue-green coloured eggshells, is costly for females because of the antioxidant properties of biliverdin (Moreno *et al.* 2006). Accordingly, only high quality females would be able to balance the trade-off between fighting against their own oxidative stress and allocating high amounts of biliverdin in their eggshells. Consequently, eggshell colouration could signal female quality and be utilised by the males to inform their investment strategies with respect to their offspring (reviewed in Riehl 2011). Similarly, the "blackmail hypothesis" proposed that conspicuous egg

colouration may help female persuading the male to increase their paternal effort in nesting areas where the risk of nest predation is high or brood parasitism is frequent (Hanley *et al.* 2010).

Some descriptive and experimental studies have investigated the signalling role of eggshell colour towards males and provided mixed support (reviewed in Reynolds *et al.* 2009, Cherry & Gosler 2010, Riehl 2011). Some studies have found a relationship between blue-green egg chroma and paternal investment (Moreno *et al.* 2004, Soler *et al.* 2008), but others have not (López-Rull *et al.* 2007). Using cross-fostering manipulations in pied flycatchers (*Ficedula hypoleuca*), Moreno *et al.* (2006) showed that males provide more investment to clutches with bluer eggs. In contrast, in the collared flycatcher (*Ficedula albicollis*), there was no effect of eggshell blue-green colouration on paternal effort (i.e. chicks feeding rate and nest defence behaviour) (Krist & Grim 2007). Similar results in ring-billed gulls (*Larus delawarensis*) showed no significant relationship between eggshell colouration and paternal care (Hanley & Doucet 2009).

Most studies to date on eggshell colouration have focused on blue-green eggs (Moreno *et al*. 2006, Siefferman *et al*. 2006, Krist & Grim 2007, López-Rull *et al*. 2007, Polačiková *et al.* 2007, Soler *et al*. 2008, Cassey *et al*. 2008a, Hanley & Doucet 2009, Polačiková & Grim 2010, English & Montgomerie 2011). However, spotted eggs of many small passerines (e.g. blue tits, great tits, house sparrows) have recently attracted researchers (Morales *et al.* 2006, Sanz *et al.* 2009, Holveck *et al.* 2010, López-de-Hierro & Moreno-Rueda 2010) because they are mainly coloured by protoporphyrin which possesses pro-oxidant properties, contrary to biliverdin (Moreno & Osorno 2003, and see 1.2.2). In that context, the SSEC hypothesis proposes that low quality females with inefficient antioxidant capacities may be incapable of eliminating protoporphyrin but, instead, may passively deposit high amounts into eggshells. However, the use of eggshell patterning in spotted eggs as a signalling function for the male

has had very little support to date (Sanz & García-Navas 2009, Walters & Getty 2010, López-de-Hierro & De Neve 2010, Stoddard *et al.* 2012).

1.2.4. Eggshell pigmentation as a maternally-derived trait?

Given the physiological properties of biliverdin and protoporphyrin (see 1.2.2), their deposition in the eggshell might be closely related to female body condition and physiological state, such as their antioxidant capacity, during egg production which is a costly process (Alonso-Alvarez *et al.* 2004). To date, mostly correlative studies have investigated the relationship between eggshell colouration and female condition or egg quality parameters (reviewed in Cherry & Gosler 2010).

1.2.4.1. Female physiology and blue-green egg pigmentation

In species laying blue-green eggs such as the pied flycatcher, eggshell brightness is negatively associated to female immuno-competence (Moreno *et al.* 2005) and blue-green colouration is positively associated with female body condition (i.e. mass/tarsus length^3 or residuals of a regression of body mass on tarsus length) (Morales *et al.* 2006, Siefferman *et al.* 2006). In contrast, Hanley & Doucet (2009) did not find any association between eggshell colouration and female body condition (i.e. mass/ (tarsus length + bill length) in ring-billed gulls. Eggshell blue-green colouration also indicates female antioxidant capacity in the gray catbird (*Dumetella carolinensis*) (Hanley *et al.* 2008). One experimental study in the pied flycatcher found a small increase in blue-green colouration after supplementation of the females with mealworms (Moreno *et al.* 2006). In the same species, an increased reproductive effort through nest removal led to a negative association between eggshell colouration and female plasma antioxidant levels, compared to control birds, suggesting a trade-off between allocating biliverdin to oxidative stress responses and laying highly

colourful eggshells (Morales *et al.* 2008). In the spotless starling (*Sturnus unicolor*), an experimental decrease of female body condition via feather removal induced a decrease in eggshell blue-green colouration (Soler *et al.* 2008). Another study in blue-footed boobies (*Sula nebouxii*) revealed an increase in blue-green colouration of the second egg laid after supplementation with carotenoids, suggesting that biliverdin colouration is costly to produce in term of antioxidant allocation (Morales *et al.* 2011). In contrast, reproducing under low or high antioxidant-diet did not influence eggshell colouration in Araucana chicken (Dearborn *et al.* 2012).

If eggshell pigmentation is a maternally-derived trait, eggshell colouration may also be associated with traits that reflect egg quality such as egg mass or internal compounds (e.g. hormones, immune factors), which are also related to maternal condition during egg formation. For example, in pied flycatchers, blue-green colouration was positively associated with egg Immunoglobulin (IgY) levels (Morales *et al.* 2006), a major component of offspring humoral immunity, transferred by mothers to enhance offspring performance and survival (reviewed in Dias da Silva & Tambourgi 2010). Eggshell blue-green colouration was also positively correlated to egg yolk lutein concentration (e.g. collared flycatcher, Hargitai *et al.* 2008; but see Cassey *et al.* 2008b, common blackbird [*Turdus merula*] and song thrush [*Turdus philomelos*]), or eggshell biliverdin concentration (e.g. spotless starling, López-Rull *et al.* 2008).

1.2.4.2. Female physiology and brown-egg pigmentation

In species laying brown-spotted eggs, mainly pigmented by protoporphyrin, the relationship between eggshell pigmentation and female or egg characteristics is more complex due to the mixed predictions of the SSEC hypothesis, the lack of information related to protoporphyrin deposition into the eggshell, and the diversity in the methods used to analyse maculation

patterns. For instance, in blue tits, brown-spot colour intensity was positively associated with female tarsus length (Sanz & García-Navas 2009) and with yolk antibodies (Holveck *et al.* 2012) and females in lower body condition laid more maculated eggs and showed a higher stress level (i.e. heat shock proteins concentration) (Martínez-de la Puente *et al.* 2007). In contrast, in great tits, heavier females laid paler eggs with less brown spots (Stoddard *et al.* 2012). In house sparrows, older females laid eggs with darker spots (López-de-Hierro & De Neve 2010), and in common kestrels (*Falco tinnunculus*) better quality females laid more maculated eggs (Martínez-Padilla *et al.* 2010). In reed warblers (*Acrocephalus scirpaceus*), eggshell colouration was independent of female condition but indicated both egg testosterone and lysozyme content (Krištofík *et al.* 2013).

In the poultry science literature some authors have suggested that stress can influence the deposition of eggshell pigments during egg formation in brown egg layers (Whittow & Naughton 1999). For example, stress induces a premature termination of eggshell pigmentation, thus leading to whitened eggs in chicken (Mills *et al.* 1991, Nys *et al.* 1991). In commercial laying hens, viral infections that affect the reproductive tract may also induce lighter eggs. In addition, different stressors (environmental, social, etc.) could potentially also increase eggshell spottiness (Butcher & Miles 2003) in the same species.

1.2.4.3. Limits of studies on eggshell pigmentation

The common thread to most of these previous studies is that they were correlative and all measured different parameters related to female physiology or egg quality, which might explain the inconsistency of the results. Nevertheless, a few experimental studies have attempted to investigate the effect of female condition on eggshell colouration. However, only few of them provided data on eggshell pigments concentrations. Indeed, most of these studies have focused on indices of eggshell appearance to test whether eggshell colouration

could be influenced by maternal condition; assuming that eggshell appearance is a reliable proxy of its pigment content. In immaculate blue-green eggshells, mainly pigmented with biliverdin, very few studies have found a positive correlation between eggshell colouration and biliverdin content (Moreno *et al.* 2006, López-Rull *et al.* 2008). In brown-spotted eggshells, maculation (defined as the presence of colourful spots, Cherry & Gosler 2010) is thought to be mainly due to variation in protoporphyrin (brown pigment) concentration as this pigment is found in much higher concentrations in such eggshells (Gorchein 2012). This highlights the limit of studies that do not quantify eggshell pigment content (Cassey *et al.* 2012a) and suggests that eggshell colouration might result from a complex interaction between biliverdin and protoporphyrin.

Consequently, eggshell pigmentation might be influenced by maternal condition; however, it is crucial to quantify eggshell pigments concentrations to be able to directly assess maternal investment into the eggshell. Moreover, the adaptive significance of eggshell colouration is more likely to be a combination of several of the hypotheses mentioned earlier. Indeed, if eggshell pigmentation is dependent on female condition during egg formation and in particular her stress exposure, any environmental variation during egg laying might have a significant impact on some properties of eggshell appearance such as egg camouflage in ground-laying species. Thus, being able to camouflage the eggs might be a trait of female quality, which may vary with female condition, and the combination of these hypotheses remains to be tested in either a correlative or an experimental manner.

1.3. Aims of the thesis

The main aim of my thesis was to investigate experimentally the relationship between eggshell appearance and female condition, and investigate the importance of eggshell

appearance on egg crypsis. I used the Japanese quail (Fig. 1.6) that was first described as a research model for the study of avian development in 1959 (Padgett & Ivey 1959).

Figure 1.6. Photograph of a female Japanese quail (Photo credit: Camille Duval).

The Japanese quail is an excellent study species because it is relatively easy to expose to variation in experimental conditions, and its physiology is well known. They have a short reproductive cycle: sexual maturity is reached after 6-7 weeks. Females lay clutch sizes of approximately 8 eggs and incubate for 16-18 days. Moreover, birds exhibit overt sexual behaviour that persists throughout the year and this makes studies of reproduction relatively straightforward under experimental and control conditions. Another characteristic that makes the Japanese quail an interesting model for scientists is the aspect of their eggs. Quail eggshell colour varies from white to blue-green, has variable red-brown spots, and protoporphyrin and biliverdin both contribute to eggshell pigmentation (Gorchein 2012, Fig. 1.7). This makes the species highly suitable for studies of the eggshell pigment deposition under different environmental conditions, and its relationship with female physiology.

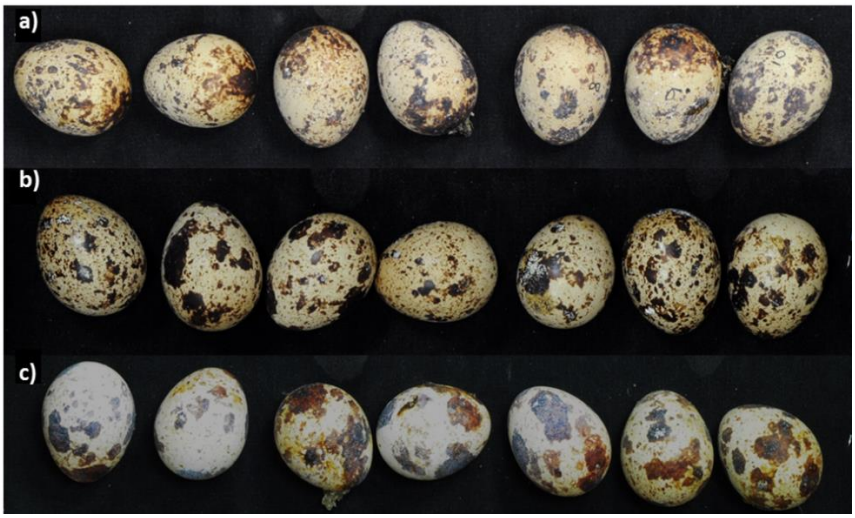

Figure 1.7. Photographs on a black velvet background of Japanese quail eggs laid by 3 different females (a), (b), (c) (Photo credit: Camille Duval).

To address the aims of this study, I used various experimental designs to manipulate female environmental conditions during reproduction through diverse modifications at adult stage such as food quantity restriction and short term stress exposure. I also experimentally induced pre and /or post natal stress exposure at early stage in life to investigate the effect on eggshell pigmentation in the long-term. I then assessed the effect of such manipulations on eggshell reflectance (spectrophotometry), maculation (image analyses) and pigment content (High Performance Liquid Chromatography) as well as on female condition parameters such as body condition (calculated as the residuals from a regression of body mass on tarsus length), basal stress level (Plasma CORT) and antioxidant factors. In addition, following a recent study on laying substrate choice in Japanese quail (Lovell *et al.* 2013), I performed a similar behavioural experiment to assess whether the colour but also the texture (patterning) of laying substrates had a significant importance in females choice.

1.4. Structure of the thesis

In Chapter Two, I first present a preliminary study that aimed at determining the daily food requirement of the population of experimental quails. Then, I examine the effect of food restriction on female body condition and egg mass, eggshell reflectance, eggshell pigment concentrations and eggshell maculation at both inter and intra-individual levels. In Chapter Three, I examine how stress can influence female body condition, plasma CORT concentrations and plasma antioxidant factors, as well as eggshell reflectance, eggshell pigments concentrations and eggshell maculation, both on short and long-term scales. In Chapter Four, I explore how laying substrate choice is driven by the patterning of both egg and substrate. Finally, in Chapter Five I summarise my main results and provide directions for future research.

Chapter Two

EGGSHELL PIGMENTATION AS A CONDITION-DEPENDENT TRAIT

2.1. Abstract

Biliverdin (antioxidant) and protoporphyrin (pro-oxidant) are two key eggshell pigments and their concentration may be related to female body condition. Here, I investigated whether female body condition influences eggshell pigmentation in the Japanese quail, using food restriction as an environmental manipulation. I determined a female-specific daily food requirement in a pilot study and then conducted a food-restriction experiment. Twenty four females were either food-restricted or receiving *ad libitum* food (i.e. controls), and two eggs at the beginning and the end of the food manipulation were collected. Food restriction should reduce female body condition and hence their antioxidant status. Given the physiological properties of each eggshell pigment, I predicted that food-restricted females would deposit more protoporphyrin and less biliverdin, resulting in eggshells of reduced brightness but increased brown-red colour intensity, and increased maculation degree, predominantly due to protoporphyrin. I found no significant effect of food restriction on eggshell reflectance. However, food-restricted females were in lower body condition, as predicted, and they increased the deposition of protoporphyrin and decreased the amount of biliverdin invested in their eggshells. Control females decreased the percentage of maculation compared with food-restricted females which maintained constant maculation across the trial period. Thus, manipulating eggshell maculation may be a strategy adopted by better females to keep constant eggshell reflectance. This suggests that in a species laying brown-spotted eggshells, females are maximising the cryptic nature of their eggs as they limit visible changes that could be detected by predators, despite variations in eggshell pigments deposition. Females may face a trade-off between maintaining their eggs cryptic towards predators while undergoing variation in their body condition.

2.2. Pilot study - On the use of commercial quails as study organisms: lessons about food intake from individual variation in body mass

2.2.1. Introduction

The Japanese quail is a galliform species in the phasianid family, which was domesticated many centuries ago and today meets some of our demands for meat and egg production (Prabakaran 2003). Several aspects of the biology of Japanese quail make them an interesting model species for poultry science research programmes and studies. They are economically important for agriculture with eggs consumed in large numbers in Asia (ca. 9 billion Japanese quails produced in China, Hong-Kong and Japan per year), while meat is consumed extensively in Europe (105 million quails produced in France and Spain per year) (Kayang *et al.* 2004, Minvielle 2004). They also serve as an excellent laboratory animal species as costs of maintenance are low, resistance to disease and egg production are high, sexual maturity is reached after 6 weeks, and, thus, three to four generations can be produced per year (Wilson 1972, Vali 2008). Consequently, this species has been extensively studied: 414 research articles are listed on the PubMed database since 2010 alone (http://www.ncbi.nlm.nih.gov/pubmed), last date accessed 02/10/2013, and 1025 are listed on the Web of Knowledge database since 2010 (http://apps.webofknowledge.com), last date accessed 02/10/2013), mainly in the poultry sciences within the context of gaining a better understanding of the species' biology to improve economic outputs.

Nutrition is one aspect of the species' biology that has attracted much study in the last 50 years as it is an important factor that determines both egg and meat quality (reviewed by Shim & Vohra 1984, Shrivastav & Panda 1999). There are many parameters that have been

examined to assess individual nutritional requirements (reviewed by Shim & Vohra 1984). The Sub-Committee on Poultry Nutrition of the National Research Council (NRC) published the 9th revised edition of the Nutrient Requirements of Poultry (NRC, 1994) in which it included details of the nutrient requirements of the species as percentages or units per kg of diet. Their guidelines suggest that the metabolisable energy (ME) (i.e. the amount of energy available from food once the energy lost in the faeces, urine, and combustible gases have been subtracted) accessible to adults should be 2,900 kcal ME kg^{-1} (equivalent to 12,134 kJ kg^{-1}). This recommendation is sometimes exceeded by commercial producers who supplement the birds with more nutrients to produce higher quality eggs and meat (Bou *et al.* 2009).

Food intake measurement in the poultry sciences has long been studied (Van Hemel & Myer 1969), and a mean value per group of birds is usually used as an index of dietary requirements. For instance, Pinto *et al.* (2002) provided a diet containing 12,760 kJ kg^{-1} of food (equivalent to 3,050 kcal kg^{-1}) to 45 day-old Japanese quails and found that their mean daily food intake was 25.8 g with birds initially weighing a mean of 138 g (N = 600). Mean daily ME intake per quail was calculated as 331.5 kJ, which is substantially higher than the 260 kJ per day proposed by Shivastav *et al.* (1980) as the daily ME requirement of Japanese quails. However, no value of body mass was provided in this study. The variation in prescribed food intake reported in the literature for this species reveals that experimental conditions and characteristics of different strains of experimental animals may markedly influence their daily food requirements. Ignoring the variance in the structural size and body mass that exists between individuals by calculating a 'population' mean for daily food requirements may have severe negative consequences for animal welfare with some birds severely under-fed while others risk adverse effects from over-feeding. It is noteworthy that recommendations for daily food intake in the literature such as those provided by

Wolfensohn and Lloyd (2003) (i.e. 100 g of food per day per adult female weighing between 120 g and 300 g) are based on a mean requirement, lacking a measure of standard error. Thus, they do not acknowledge variance in nutritional requirements of commercial and/or experimental animals. Studies risk working with results from animals that have not undergone identical manipulations, undermining conclusions that could be biased or simply incorrect, adding significant variation to the experiment and potentially resulting in a larger sample size of animals being required to attain acceptable statistical power.

2.2.2 Materials and methods

Experiments were conducted using a captive population of outbred Japanese quail at the University of Glasgow (Cochno Research Centre and Farm, Scotland). All experimental procedures were carried out under UK Home Office Project Licence 60/4068 (Karen Spencer), and Personal Licences 30/8939 (Camille Duval).

All the birds came from eggs artificially hatched at the farm. At the beginning of the experiment, all females were 6-weeks old and of the same reproductive status (i.e. this was their first reproductive attempt). I used 26 females individually housed in cages that were 51 cm high × 46 cm wide × 61 cm long, with deep litter on the floor. Ambient temperature was maintained at 18.0–18.9°C and the light regime was 14L:10D (hours). Birds were fed with a standard commercial diet (BOCM Ltd, Suffolk, England) (Table 2.1) that had an energy content of 12,750 kJ kg^{-1}. Birds were weighed to the nearest 1 g on an electronic balance (Fisher Scientific, Fisherbrand, SG-2001, Loughborough, UK) just prior to the feeding trial.

Table 2.1. The nutrient content of the BOCM Pauls Farmgate Layer Pellet for Poultry diet that was fed to Japanese quails in this study of the daily food requirements for laying birds[a].

Nutrient	**Content**
ME (kJ/kg)	12,750
Oil (%)	4.0
Protein (%)	16.0
Fibre (%)	6.5
Ash (%)	13.50
Methionine (%)	0.30
Moisture (%)	13.80
Vitamin A-retinol (iu / kg)	7,000
Vitamin D3- cholecalciferol (iu / kg)	3,000
Vitamin E, alpha-tocopherol acetate (iu / kg)	15
Sodium selenite-selenium (mg / kg)	0.25
Copper sulphate-copper (mg / kg)	20
Inclusions	
Wheat (%)	40–25
Wheatfeed, sunflower extract? (%)	25–10
Calcium carbonate, bakery by-product, soya bean extract, distillers (%) dark grains, soya bean heat treated, mono-calcium phosphate, vitamins / minerals, salt, lysine, methionine, sodium bicarbonate	10–0
Natuphos	5,000G[b]
Additive (Na-K salts)	
Roxazyme	
Luthein, Zeaxanthin, Capsanthin	

Note: [a]Reproduced by kind permission of BOCM Pauls Farmgate. [b]G: Granulate.

2.2.2.1. Preliminary calculations

In order to provide birds with an excess of food per day during the feeding trials I needed to calculate a mass of food that would provide sufficient energy for all females in the experiment. Females were significantly heavier than NRC guidelines (NRC, 1994) at the start

of the feeding trial and they also showed high inter-individual variability in body mass (mean: 312.5 g, range: 245–380 g, variance = 1,346.6, SD = 36.7 g, N = 26; Fig. 2.1).

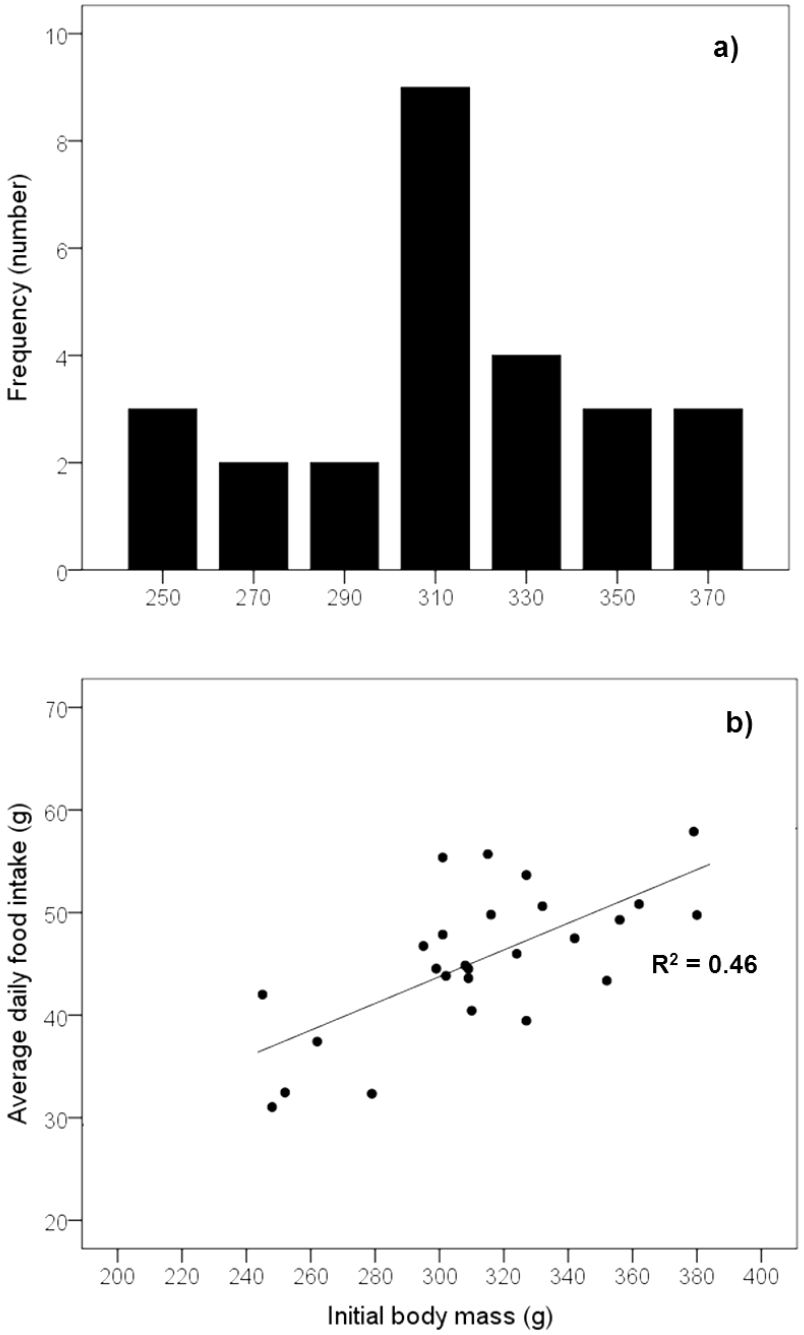

Figure 2.1. (a) Frequency distribution of the initial body masses of 26 experimental Japanese quails. Note that although there is a discernible mode between 300 and 320 g, the majority (65%) are either lighter (N = 7) or heavier (N = 10) than this range. (b) The relationship between their initial body mass and their average daily food intake.

The heaviest female in the feeding trial was 380 g, more than twice the body mass upon which dietary recommendations are based (NRC 1994). According to theoretical calculations based on the total energy content of the food (i.e. 12,750 kJ kg^{-1}) and on the theoretical daily energy requirement of 331.5 kJ of a group of females with a mean mass of 138 g (Pinto *et al.* 2002), the mean daily food requirement for the group was 58.8 g of food per day for a quail weighing 312.5 g (equivalent to 750 kJ day^{-1}). The feeding trial was repeated for four consecutive days in order to determine the repeatability of food intake rates.

2.2.2.2. Food intake measurement

On day 1 of the feeding trial, all cages were cleaned thoroughly using a wire brush to remove every piece of food and cage substrate before each feeder was weighed empty to the nearest 0.01 g using a portable electronic balance (Fisher Scientific, Fisherbrand, SG-2001, Loughborough, UK). To avoid food-restricting the heaviest female in this experiment, I provided all quails with 70 g of food. At 9 am (GMT) on days 2–4 of the feeding trial, all food was carefully collected from each cage and from each feeder using tweezers, and was then weighed to determine the quantity of food eaten by each subject in the previous 24 h (Boswell *et al.* 2002, Hull *et al.* 2007).

2.2.2.3. Statistical analyses

I used the method of Lessells and Boag (1987) in STATISTICA version 6.0 (Statsoft) to calculate the within-female repeatability of food intake over the trial. I performed a linear regression of female body mass on daily food intake to test if the relationship could be used in an applied way to predict dietary requirements from body mass dynamics. All residuals were normally distributed.

2.2.3. Results

I found that food intake was significantly repeatable within females across the 3 days of measurement ($r = 0.8$, $P < 0.001$), revealing that the inter-female variance in food intake was much higher than that within females, and that the food intake was consistent across the days of the feeding trial. There was considerable variability in mean daily food intake (calculated for each female over the feeding trial) (mean = 45.4 g, range: 31.1–57.9 g, variance = 49.7, SD = 7.1 g, N = 26) and mean daily energy intake (mean = 579.1 kJ, range: 395.8–738.1 kJ, variance = 8,083.2, SD = 89.9 kJ, N = 26) across females in this study (Fig. 2.1, Table 2.2). Female body mass was also positively correlated with mean daily food intake ($R^2 = 0.5$, $F_{1,24} = 20.5$, $P < 0.1$; Fig. 2.1), revealing that the heaviest females were also the ones that ate the most.

2.2.4. Discussion

Food intake rates depend upon the ME content of the ration but also the bird's age, body mass and reproductive status as well as ambient temperature (Shim & Vohra 1984). Previous studies have found that adult Japanese quails require a daily ME intake of 218 kJ (Farrell *et al.* 1982), 228 kJ (Thompson & Boag 1976), 260 kJ (Yamane *et al.* 1980) and 324 kJ (Boon *et al.* 1999) with the variation being due to different energy contents of diets (ranging from 12,300 to 17,000 kJ kg^{-1} of diet). This corresponds to a daily food intake ranging from 17 to 19 g of food. The birds in my feeding trial were larger than those in previous experimental studies, explaining why the daily food intake rates I measured were relatively high (mean = 45.4 g, range: 31.1–57.9 g). There is a large amount of variability in recommended daily food intakes found in animal welfare guidelines (from 20 to 100 g) (Cooper 1987, Wolfensohn & Lloyd 2003) and I suggest that calculating food intake rates specific to an experimental

Table 2.2. The initial body mass and daily food intakes of 26 female Japanese quails on days 2–4 of a feeding trial to determine the variability of their food intake requirements. Females are ordered by ascending initial body masses.

Initial body mass (g)	Food intake (g) on day:			Mean daily food intake (g)	Mean daily energy intake (kJ)
	1	2	3		
245	41.1	43.6	41.4	42.0	535.6
248	29.7	31.9	31.5	31.0	395.8
252	28.2	33.4	35.8	32.5	413.8
262	38.7	40.6	33.0	37.4	477.2
279	33.3	29.7	34.0	32.3	412.3
295	46.0	44.3	50.0	46.7	595.9
299	44.1	44.9	44.6	44.5	567.8
301	47.4	50.2	46.0	47.9	610.1
301	51.3	56.4	58.5	55.4	705.9
302	41.9	47.5	42.1	43.8	558.8
308	41.7	44.8	48.0	44.9	571.8
309	39.8	49.5	44.3	44.5	567.6
309	44.9	45.3	40.7	43.6	555.9
310	42.5	40.6	38.3	40.4	515.7
315	55.6	54.9	56.5	55.7	710.1
316	44.7	54.1	50.6	49.8	635.0
324	47.6	45.2	45.2	46.0	586.3
327	38.5	38.9	41.0	39.5	503.0
327	51.3	53.9	55.7	53.7	684.2
332	53.0	51.8	47.0	50.6	645.4
342	49.1	45.6	47.8	47.5	605.5
352	41.5	38.8	49.8	43.4	552.8
356	47.7	50.1	50.1	49.3	628.4
362	51.8	51.4	49.3	50.8	647.9
379	51.9	59.2	62.6	57.9	738.1
380	42.9	56.6	49.9	49.8	634.5

group of birds would be more appropriate than using theoretical values based on unknown populations.

This short feeding trial has demonstrated that measuring food intake in any study involving the manipulation of food availability to subjects requires care in order to account for the variation in body mass of individual birds within the focal group. I studied 26 females and determined a female-specific mean daily food intake to allow estimation of the daily food requirements for each of the laying females (Table 2.2). This suggests that calculating a mean food intake for the overall female group would not be advisable, as some females would be food-deprived when this was not the intention. This is especially important in studies involving daily requirements based upon the energetic contents of diets.

In experimental work on Japanese quail, it is commonly assumed that an average adult female Japanese quail weighs 120 g, consumes on average 20 g of food per day and requires, on average, 264 kJ kg^{-1} of bird per day (Shim & Vohra 1984), but frequently the mass of the individual birds is not considered in the execution of such studies. Moreover, the nutrition of Japanese quail is most commonly studied within the context of applied poultry science where the focal group can consist of hundreds of birds (e.g. Soares *et al.* 2003) and where logistics prevent initial body masses of birds from being considered. The result is that the variability that I have found in this study is often lost and with it the effectiveness of a study's approach. Furthermore, birds are often communally housed in large numbers in these studies, resulting in further problems in unbiased estimation of their daily energy requirements as a result of competition for food among birds. Such variances must be monitored and accommodated if feeding experiments are to be planned and executed robustly. I strongly recommend that it is necessary to consider both the energetic content of diets and the body mass of birds before initiating a food intake study. Often, the latter is either absent in reported studies or an

insufficient number of birds has been weighed to allow integration of inter-individual variation into calculations of their dietary requirements.

2.3. Condition-dependent strategies of eggshell pigmentation: an experimental study of Japanese quail

2.3.1. Introduction

Avian eggshells are diverse in their patterns of pigmentation and many adaptive hypotheses have been proposed culminating in a revived interest in the subject during the last twenty years (reviewed in Underwood & Sealy 2002, Kilner 2006). Across a wide range of species, the variation in eggshell colouration and patterning has been explained, among others, in the context of crypsis (Wallace 1889, Tinbergen *et al.* 1962), mimicry and defence against brood parasitism (Dawkins & Krebs 1979, Brooke & Davies 1988, Rothstein 1990), and protection of the developing embryo against solar radiation (Lahti 2008). More recently, it has been proposed that eggshell colouration could be strongly related to female physiological condition and, in particular, antioxidant capacity (Moreno & Osorno 2003, Soler *et al.* 2005, Siefferman *et al.* 2006, Hanley *et al.* 2008, but see Riehl 2011). This new assertion is founded on the investment of two main pigments: biliverdin, a blue–green antioxidant pigment, and protoporphyrin, a brown pro-oxidant pigment (Gorchein *et al.* 2009). Both may reflect the antioxidant capacity of the female and both are involved in the vertebrate haem metabolic pathway (Bloomer 1988). Their concentrations are highly correlated in the avian eggshell (Wang *et al.* 2009). Thus, it is proposed that only females with an increasingly efficient antioxidant system are able to allocate more biliverdin into their eggshells in the face of accommodating their oxidative stress (Moreno & Osorno 2003). Moreover, because of its pro-oxidant properties, protoporphyrin causes a physiological oxidative stress in the liver (Shan *et al.* 2000) and females in lower body condition and under elevated stress may passively deposit more protoporphyrin into their eggshells to facilitate reduced oxidation (Moreno & Osorno 2003, Martínez-de la Puente *et al.* 2007).

The 'sexually selected eggshell colouration' (SSEC) hypothesis (Moreno & Osorno 2003) has provoked many experimental and correlative studies that have demonstrated positive correlations between eggshell colouration and female and/or offspring body condition and immuno-competence (e.g. maternal antibodies, yolk testosterone, yolk lutein) (Moreno *et al.* 2005, Hargitai *et al.* 2008). However, findings from an increasing number of studies are now in conflict with the predictions of this hypothesis. Many studies did not find a significant correlation between eggshell colouration and female and/or egg characteristics (Cassey *et al.* 2008b, Honza *et al.* 2011, Riehl 2011). Most of these have focused on species that lay blue–green eggs but substantially less attention has been paid to brown-spotted eggshells (Riehl 2011, Dearborn *et al.* 2012). High eggshell concentrations of protoporphyrin have been positively related to thinner eggshells as a result of calcium deficiency (García-Navas *et al.* 2011) and pesticide contamination (Jagannath *et al.* 2008). Thus, the brown colouration of maculated eggshells could reflect both egg quality and female body condition. This hypothesis has been examined in domestic chickens, where older females laid lighter and less-coloured (i.e. redder) eggs because of an increase in egg size, but there was no comparable change in eggshell pigment concentrations (Odabaşi *et al.* 2007). In the house sparrow, pigment deposition decreased with age and through the laying sequence (López-de-Hierro & De Neve 2010). Furthermore, a cross-fostering experiment in house wrens (*Troglodytes aedon*) found that less-pigmented eggshells indicated heavier eggs and higher female body condition (residuals from a regression of body mass on tarsus length) (Walters & Getty 2010). It is noteworthy that there is currently little agreement in the literature about the relationship between eggshell colour and female body condition. It is common for researchers to speculate on female investment in terms of eggshell pigments (e.g. Poole 1965, Walters & Getty 2010, García-Navas *et al.* 2011) without measuring pigment concentrations.

Therefore, the aim of this study was to examine the relationship between female body

condition (through residuals from a regression of body mass on tarsus length), eggshell physical reflectance (brightness, UV chroma and chroma) or perceived discrimination by an avian visual system (chromatic and achromatic contrasts), and maternal investment in egg quality. This study is the first to use an experimental approach that mimics a naturally challenging environment, by restricting food availability to captive laying birds to investigate the relationship between female body condition, and eggshell colouration and pigmentation. If eggshell spot and background colouration indicate female body condition, I predicted that, compared with control females, food-restricted females in lower body condition would exhibit quantifiable changes in eggshell colouration. I predicted that they would deposit more protoporphyrin and less biliverdin in their eggshells, resulting in a decreased achromatic (brightness) and an increased chromatic (UV chroma and chroma) colouration. I also predicted females in lower body condition would invest less in egg quality as measured through egg mass, egg volume and yolk/albumen proportion.

2.3.2. Materials and methods

2.3.2.1. Study species and experimental procedure

Experiments were conducted on a captive population of outbred Japanese quail at the University of Glasgow (Cochno Research Centre and Farm, Scotland). Twenty-four adult females and nine adult males were randomly selected from an outbred wild-type population and were housed in single sex groups in indoor 15 m^2 aviaries for 2 weeks to allow habituation to housing conditions before the start of the experiment. Birds were fed *ad libitum* with a standard commercial diet (Layers Pellets, BOCM Ltd, Ipswich, UK) during habituation. Each female was individually identified with a uniquely numbered white leg ring and moved to an individual cage (51 cm high × 46 cm wide × 61 cm long) for 1 week of further habituation prior to the start of food manipulation. All birds were in visual and

acoustic contact with each other at all times. Each male was individually identified with a uniquely numbered coloured leg ring and males were group-housed in a single enclosure in the same room as the females under *ad libitum* feeding conditions. Ambient temperature was maintained at 18.0–18.9°C and the light regime was 14L:10D (hours).

Each female was weighed (to the nearest 1 g) on an electronic balance before the feeding trial and, again, after her last egg had been collected. Right tarsus length was measured (to the nearest 0.01 mm) with a digital calliper. All birds were returned to single-sex group-housing after the last egg collection.

2.3.2.2. Food intake measurement and manipulations

To determine daily food requirements for the treatment groups, a pilot study was previously conducted (Section 2.2; Duval *et al.* 2012). Dietary manipulation commenced 1 week after the last day of the pilot experiment. This delay allowed me to confirm that female behaviour (e.g. feather pecking, routine feeding or drinking) was not adversely affected by individual housing. Females were randomly allocated to one of three treatment groups: control (C: fed *ad libitum*, i.e. 100% daily requirements, N = 8); medium quantity [MQ: 90% daily requirements, N = 8 (one bird had to be removed from the experimental design for health reasons)]; and low quantity (LQ: 75% daily requirements, N = 8). To control for possible 'cage' or 'ceiling' effects, individuals from each experimental group were housed in such a way that across all groups equivalent numbers of birds were in cages on the floor and close to the ceiling. The respective quantity of food calculated from the pilot study for each subject was then provided every morning at the same time for the entire feeding trial.

2.3.2.3. Egg collection

I only analysed fertile eggs. A male was randomly paired with one female of each treatment group (i.e. three females in total). Sexual activity in males is highest within the first 5 minutes of presentation to a female, averaging approximately three copulations before satiation (Schein *et al.* 1972). Therefore, a male was placed in a focal female's cage for 5 minutes per day before being removed and allowed a 1 hour resting period before presentation to a subsequent female. In this way, each male was exposed to three females each day, one from each treatment group. The order of presentation to females of different experimental groups was randomly assigned each day. Egg collection began after 10 days of mating, the period required to obtain fertile eggs (Adkins-Regan 1995). Each cage was visited every morning and eggs were collected and placed in a dark box in a cold room (4°C).

Four eggs per female were collected. Specifically, they were egg numbers 1 and 2, and 13 and 14 within a clutch, with the modal clutch size of free-living birds being 14 eggs (Shousha *et al.* 2007). The eggs were analysed to assess the effect of dietary treatment on their mass, volume, eggshell colouration and pigment concentrations (Hoyt 1979). The first and last eggs (i.e. 1 and 14) were carefully opened along the longitudinal axis using dissecting scissors on the day of laying. Yolk and albumen were separated and weighed (to the nearest 0.01 g) on a digital balance to determine relative egg components by mass as a proxy for egg macronutrient content (Baumgartner *et al.* 2008). Eggshells were washed with distilled water and kept in a dark box to dry at room temperature and to avoid direct exposure to light that could cause pigment degradation (Cassey *et al.* 2011a).

2.3.2.4. Measurement of eggshell reflectance by spectrophotometry

The colouration of Japanese quail eggs varies considerably across a population with a background colour varying from white to blue–green to light yellow–brown, upon which darker speckles or spots of variable size, shape and colour occur (Sezer & Tekelioglu 2009) (Fig. 2.2a). Eggshell reflectance was measured between 300 and 700 nm in the laboratory using an Ocean Optics USB4000 Miniature Fibre Optic spectrophotometer with a DH-2000-FHS deuterium–halogen light source (Ocean Optics, Eerbek, The Netherlands). A 90 deg probe with a black plastic extension was used to ensure stability for measurement and to maintain a consistent angle and distance between the eggshell and the measuring fibre optics (Cassey *et al.* 2010b). Two spots were randomly chosen from each half of an egg (one from the apex and one from the blunt end) and three replicates of reflectance were measured from each spot (Fig. 2.2a). For eggshell background, three reflectance spectra were randomly measured in each area (apex and blunt end) (Fig. 2.2a). Spectra were expressed relative to a white Ocean Optics WS-1 and a black standard as shown in Figure 2.2b for representative spectra from spots and background.

2.3.2.4.1. Shape model

From these spectral measurements, brightness, UV chroma, blue–green chroma and red chroma were extracted as spectral shape descriptors using Avicol software (Gomez 2006, Doutrelant *et al.* 2008). Brightness was estimated as the total reflectance (*R*) between the wavelengths 300 and 700 nm. UV chroma was calculated as $R_{320–400\ nm}/R_{300–700\ nm}$, which is the proportion of the reflectance in the UV zone (320–400 nm) (Pérez-Rodríguez *et al.* 2011). Then, I calculated blue–green chroma (BGC) (Siefferman *et al.* 2006) as $R_{400–575\ nm}/R_{300–700\ nm}$, and red chroma as $R_{595–655\ nm}/R_{300–700\ nm.}$

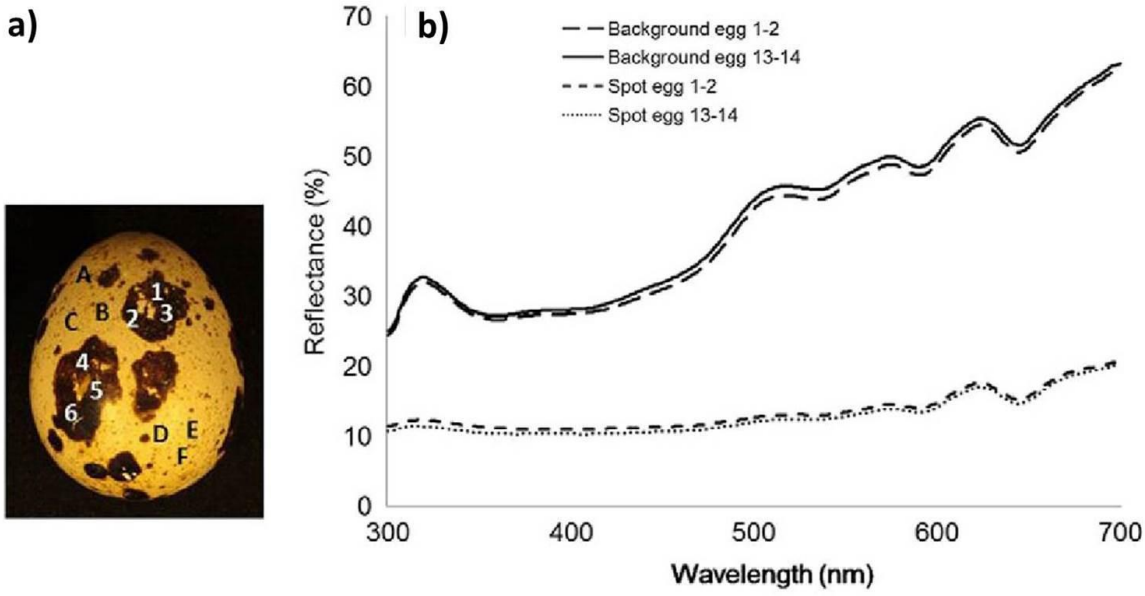

Figure 2.2. (a) Zones on a typical Japanese quail eggshell where reflectance spectra were measured by spectrophotometry. From each half of the egg, three measurements were taken from two spots (1–3 and 4–6) while six measurements (A–F) were taken from the background. (b) Mean representative reflectance spectra from the spots and background of Japanese quail eggs (egg numbers 1 and 2, and 13 and 14 in the laying sequence).

2.3.2.4.2. Vision model

To account for the avian visual system, I used the protocol of Loyau *et al.* (2007b) to compute two types of chromatic (*S*; colour) and achromatic (*Q*; brightness and forms) contrasts (Vorobyev & Osorio 1998) using Avicol software (Gomez 2006) as described by Osorio *et al.* (1999). I used the photoreceptor spectral sensitivities and relative densities data available for the domestic chicken as it is the closest species to Japanese quail in terms of photoreceptor characteristics (Hart & Hunt 2007). Chickens have tetrachromatic colour vision based on single cones containing visual pigments with specific absorption maxima of 570 nm ($\lambda_{max,red}$), 508 nm ($\lambda_{max,green}$), 455 nm ($\lambda_{max,blue}$) and 419 nm ($\lambda_{max,violet}$) (Bowmaker & Knowles 1977). They also possess double cones that mediate luminance, pattern and texture detection (Bowmaker & Knowles 1977, Vorobyev & Osorio 1998).

2.3.2.5. Determination and quantification of eggshell pigments

Eggshell pigment content was quantified in eggs 1 and 14, the same eggshells as used for the spectrophotometric measurements. Pigments were identified and their concentrations calculated using high-performance liquid chromatography (HPLC) (Mikšík *et al.* 1996). Briefly, each eggshell was weighed, and washed with distilled water and then solubilised (and esterified) in the dark for 2 days at room temperature in 15 ml of methanol containing 8.5% concentrated sulphuric acid. The resulting solution was filtered (to remove shell membranes), 7.5 ml of chloroform and 5 ml of distilled water were added and then the solution was shaken. The lower chloroform phase was washed with 5 ml of 10% sodium chloride solution, followed by distilled water until the washing water had neutral pH (typically after two washes). The extract was evaporated to dryness and reconstituted in 1 ml of chloroform. Standards for the quantification of protoporphyrin IX and biliverdin (Sigma, St Louis, MO, USA) were treated using the same procedure. Porphyrins were analysed by reversed-phase HPLC using Agilent 1100 LC system (Agilent, Palo Alto, CA, USA) consisting of a degasser, binary pump, autosampler, thermostatically controlled column compartment and multi-wavelength and fluorescence detectors. Chromatographic separation was carried out on a Gemini 5u C18 110A column (250 × 2 mm i.d.; Phenomenex, Torrance, CA, USA). The sample (20 μl) was injected into the column and eluted with a gradient consisting of (a) methanol–water–pyridine 35:65:0.25 v/v and (b) methanol–acetonitrile–pyridine 90:10:0.25 v/v (flow rate 0.3 ml min^{-1} at a temperature of 55°C). The gradient started at a–b 80:20 reaching 10:90 ratios after 15 minutes. For the next 10 minutes, the elution was isocratic (the composition of the mobile phase is unchanged during the entire Elution process) followed by another 10 minute isocratic elution at 100% b. Protoporphyrin was detected by fluorescence at 405 nm excitation/620 nm emission, whereas biliverdin was detected by absorbance as it has no fluorescence response. The two detectors were connected

in tandem. LC-MS was used (i.e. liquid chromatography was directly coupled to mass spectrometry).

2.3.2.6. Data analysis

2.3.2.6.1. Shape model

For all four colour variables (i.e. brightness, UV chroma, blue–green chroma and red chroma), mean spot and background reflectance values were calculated for each egg per eggshell area (apex and blunt areas) (Fig. 2.2a). Univariate generalised linear models (GLMs; SPSS Statistics 19.0.0) were conducted to test for the effect of eggshell area (apex or blunt end) on spot and background reflectance, with colour variables as dependent variables, and egg area (apex or blunt end) as a fixed factor. Female identity was included as a random factor. There was no effect of egg area on any dependent variable in the analysis (spot: $0.09 < Ps < 0.38$; background: $0.27 < Ps < 0.79$). Therefore, all subsequent analysis was carried out on data averaged (i.e. on eggshell means) across the whole egg.

Pearson's correlations were performed on the four colour variables, between eggs 1 and 2, and then between eggs 13 and 14, for each female to test whether eggshell reflectance was similar between eggs from the same female. As eggshell reflectance was significantly correlated between eggs 1 and 2, and between eggs 13 and 14 ($0.42 < Rs < 0.97$, all $Ps < 0.001$), mean reflectance values for each female at the beginning (mean of the two first eggs) and at the end (mean of the two last eggs) of the manipulation were calculated for the four colour variables.

2.3.2.6.2. Vision model

All spectra were interpolated to obtain a reflectance value every 1 nm from 300 to 700 nm for the first two and the last two eggs per female. I first investigated egg discriminability by

calculating the chromatic and achromatic contrasts within and between females. I computed for all pairwise comparisons contrasts or just-noticeable differences (JNDs) within a female's eggs (i.e. JND within). Then, I calculated contrasts between females by computing all pairwise comparisons between a female's eggs and all those laid by other females (i.e. JND between).

To assess whether food restriction had a perceptible effect on eggshell spot and background chromatic and achromatic variations, I then calculated contrasts between beginning/end eggs for each female. I also calculated a mean spot/background contrast for each female (Holveck *et al.* 2010) at the beginning and at the end of the manipulation to assess the effect of the treatment on this perceived contrast.

Within each change (*S* or *Q*), contrasts were compared between first and last eggs with 1 as the discrimination threshold, below which chromatic or achromatic differences are not detectable and above which they become more detectable for larger JND values (Dearborn *et al.* 2012). I assumed that light (neural noise) did not limit visual performance (Holveck *et al.* 2010). I also tested whether the average differences in egg colour within and between females were detectable by a domestic chicken's vision model by comparing the within- and between-female contrast values to the threshold 1 using one-sample t-tests (all JNDs were normally distributed). Paired t-tests were performed to test whether the within and between contrasts were significantly different for each type of contrast computed.

2.3.2.6.3. Effect of dietary manipulation on eggshell colour

As I observed a significant difference in background red chroma between the three experimental groups before the start of the dietary manipulation (Kruskal-Wallis H-test: $H_2 = 5.63$, $P = 0.04$), I examined the effect of dietary treatment on the change in body condition and on eggshell reflectance over the trial. Body condition of each female was calculated as

the residual from a linear regression of body mass on tarsus length. I then calculated the difference in body condition and in eggshell reflectance parameters between the beginning and the end of the dietary manipulation. Univariate GLMs were performed to test whether dietary treatment influenced the change in egg traits (i.e. mass, volume, yolk proportion), eggshell reflectance, pigment quantities, and chromatic and achromatic contrasts, as dependent variables, with the pre-dietary manipulation colour variables as covariates. One-sample t-tests were performed to test whether differences in colour variables differed from 0, and whether the contrasts differed from the discrimination threshold 1. All residuals were normally distributed.

Finally, Pearson correlations examined whether female body condition at the start of the feeding trial was correlated with egg traits (i.e. mean mass, volume and yolk proportion), eggshell colouration and pigment content.

2.3.3. Results

2.3.3.1. Effect of food restriction on females and their egg characteristics

Female body condition was not significantly different between groups at the start of food manipulation (Kruskal–Wallis test: $H_2 = 0.48$, $P = 0.78$). Food restriction, however, significantly affected female body condition with LQ females being in lower body condition than C or MQ females, whose mass and body condition increased throughout the manipulation (body condition: $F_{2,22} = 8.05$, $P < 0.01$; Fig. 2.3).

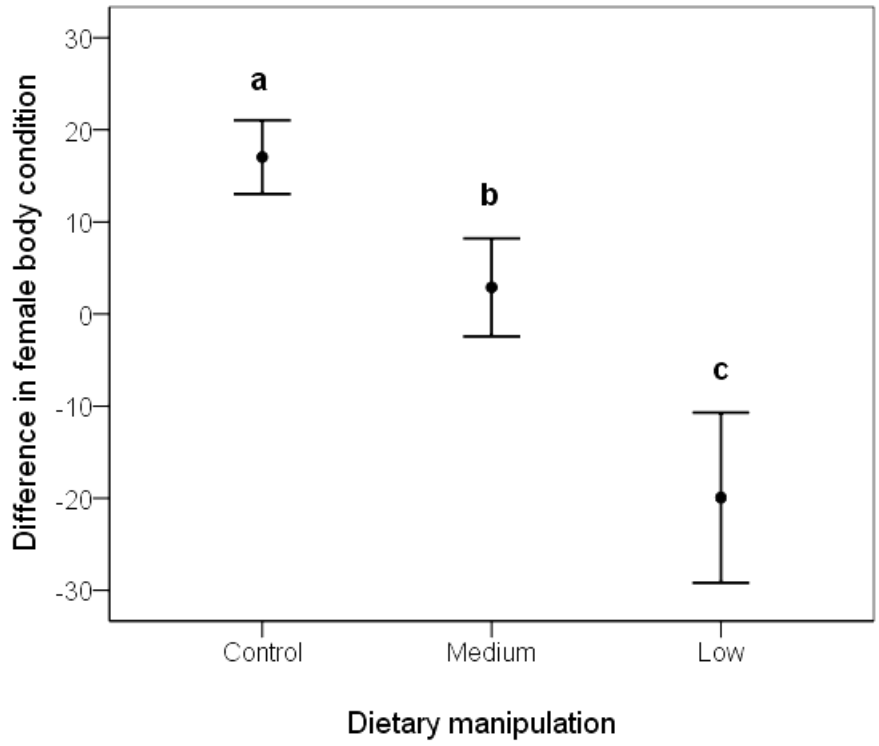

Figure 2.3. Effect of dietary manipulation on female body condition (mean ± 1 SE) of Japanese quail that were on *ad libitum* (Control), medium quantity (Medium) or low quantity (Low) food diets. The difference in body condition (residuals from regression of body mass on tarsus length) was calculated as the difference between that before the dietary manipulation and that after the last egg was laid. Different lowercase letters reflect statistically significant differences.

There was no significant effect of food manipulation on any egg characteristics (egg mass: $F_{2,22} = 0.69$, P = 0.51; egg volume: $F_{2,22} = 0.35$, P = 0.71; yolk proportion: $F_{2,22} = 1.10$, P = 0.35). However, I found that heavier females in higher body condition at the start of the food manipulation laid heavier and bigger eggs (egg mass: r = 0.44, P = 0.03; egg volume: r = 0.49, P = 0.02).

2.3.3.2. Effect of food restriction on eggshell reflectance

2.3.3.2.1. Shape model

I found no significant effect of food manipulation on eggshell colour variables (Table 2.3).

Table 2.3. Effect of dietary manipulation on eggshell colour variation of Japanese quail.

Parameter	Factor	F	P
Spot reflectance			
Brightness	Treatment	0.24	0.79
	Initial value	0.25	0.13
UV chroma	Treatment	1.03	0.37
	Initial value	5.75	**0.03**
Blue–green chroma	Treatment	0.60	0.56
	Initial value	11.00	< **0.01**
Red chroma	Treatment	0.96	0.40
	Initial value	4.32	0.05
Background reflectance			
Brightness	Treatment	0.17	0.85
	Initial value	0.22	0.64
UV chroma	Treatment	0.21	0.81
	Initial value	17.16	< **0.01**
Blue–green chroma	Treatment	0.53	0.60
	Initial value	29.28	< **0.01**
Red chroma	Treatment	0.223	0.80
	Initial value	25.37	< **0.01**

The 24 female Japanese quails were exposed to *ad libitum*, medium quantity or low quantity food manipulations (for details of dietary manipulation see section 2.3.2). Eggshell colour variation was judged as the difference in eggshell reflectance between eggs collected before the start of food restriction and after 15 days of treatment. Mean reflectance of egg numbers 1 and 2, and of egg numbers 13 and 14 was used in the statistical models. Univariate generalised linear models (GLMs) were performed to test whether dietary treatment (d.f. = 2) influenced eggshell reflectance, with the pre-dietary manipulation colour variables as covariates (initial value, d.f. = 1). Bold text indicates statistical significance at the alpha threshold of 0.05.

None differed significantly from 0 (one sample t-tests: −1.32 < all ts < 1.64, all Ps > 0.05, N = 24), suggesting that there was no natural variation in eggshell colouration during the dietary manipulation. Eggshell colour was not correlated with egg mass (spot: −0.28 < all rs < 0.21, all Ps > 0.05; background: −0.24 < all rs < 0.16, all Ps > 0.05, N = 24), nor with egg volume (spot: −0.28 < all rs < 0.20, all Ps > 0.05; background: −0.30 < all rs < 0.20, all Ps > 0.05, N = 24). However, females in higher body condition at the start of the experiment laid eggs that displayed bluer backgrounds (r = 0.45, P = 0.03, N = 24).

2.3.3.2.2. Vision model

The results from the avian vision model suggested that some of the variation measured with reflectance spectrophotometry would be detectable by the avian visual system. For each female, the mean visual contrast for eggshell background was greater when comparing eggs between females than within females (Fig. 2.4; paired t-test: S background: $t_{22} = –2.19$, P = 0.04; Q background: $t_{22} = –2.99$, P < 0.01; Fig. 2.4). However, I did not find any significant Visual (chromatic and achromatic) contrasts were not correlated with any of the eggshell reflectance variables computed with the descriptive shape model (all *P*s > 0.05). I did not find any significant effect of dietary manipulation on eggshell spot and background contrast, and on spot/background difference of reflectance (Table 2.4).

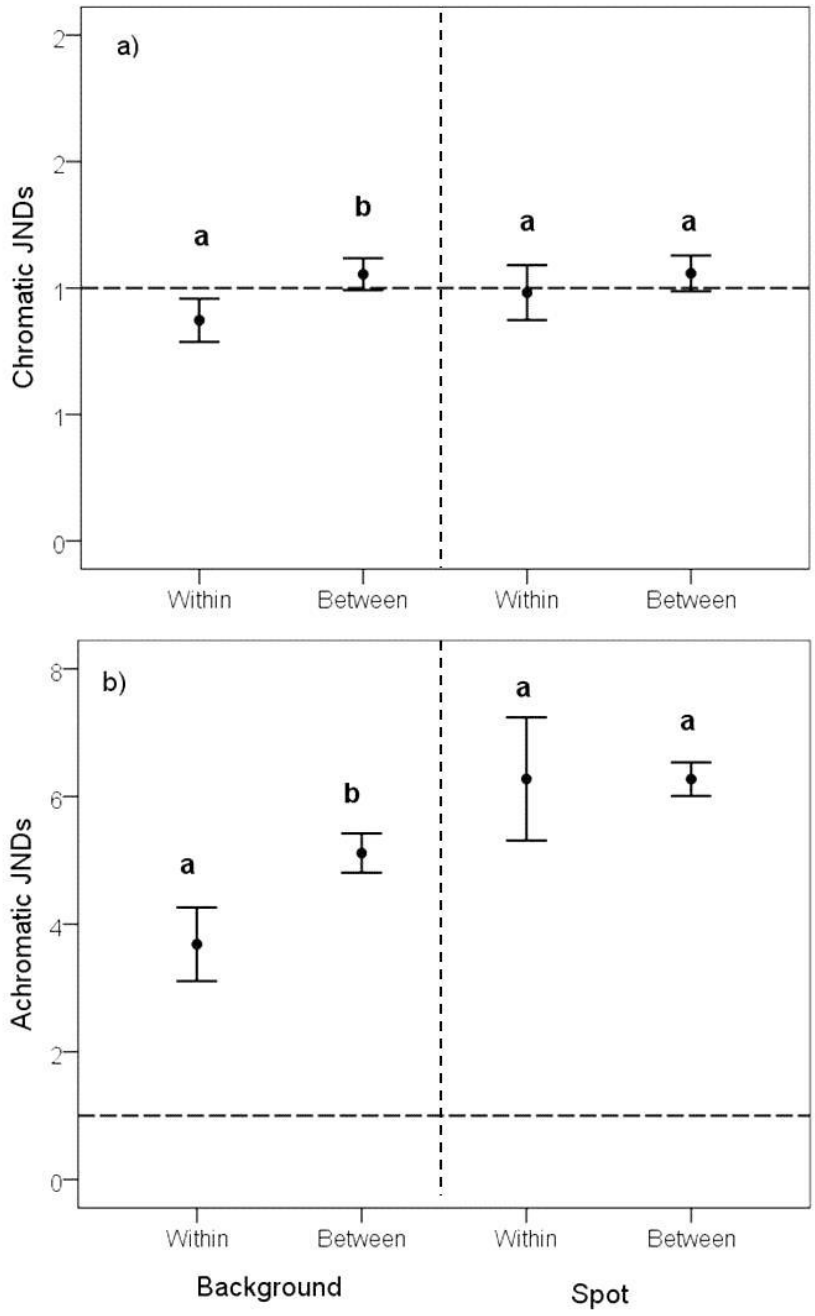

Figure 2.4. Mean detectability (± 1 SE) of chromatic (A) and achromatic (B) contrasts in pairwise comparisons of self-laid eggs (Within) and in pairwise comparisons between eggs laid by each female versus eggs laid by all the other females (Between). Discriminability was calculated for each of the 24 Japanese quail females, with values > 1 just-noticeable difference (JND; threshold shown by horizontal dashed line) representing contrasts that are likely to be detected by the bird's visual system. Visual contrasts between spots and background have not been statistically tested in the model so the different lowercase letters, which reflect statistically significant differences, have to be noted independently for spots and background.

Table 2.4. Effect of dietary manipulation (d.f.=2) on the perceived eggshell colour variations of Japanese quail through the use of an avian vision model.

Parameter	F	P
Achromatic variations		
Spot/background contrast	0.03	0.97
Spot	2.71	0.09
Background	3.16	0.06
Chromatic variations		
Spot/background contrast	2.83	0.08
Spot	0.29	0.75
Background	0.20	0.83

The 24 female Japanese quails were exposed to *ad libitum*, medium quantity or low quantity food manipulations (for details see section 2.3.2). The achromatic (brightness) or chromatic (colour) variations were calculated as the just noticeable differences (JNDs) between eggs from the beginning (mean spectrum between egg numbers 1 and 2) and those from the end of the food manipulation (mean spectrum between egg numbers 13 and 14).

2.3.3.3. Eggshell pigments

Pigment analyses revealed that eggshells of Japanese quail contained high concentrations of protoporphyrin IX (mean ± SD: 113.75 ± 57.18 μg g^{-1} of eggshell) and biliverdin (91.03 ± 48.40 μg g^{-1} of eggshell), with the two concentrations positively correlated at the start of the food manipulation (r = 0.40, P = 0.05, N = 24). I found that dietary manipulation significantly affected both protoporphyrin ($F_{2,22}$ = 3.48, P = 0.05) and biliverdin ($F_{2,22}$ = 3.67, P = 0.04) deposition in eggshells. Compared with control females, food-restricted females invested more protoporphyrin and less biliverdin in their eggshell contents (Fig. 2.5).

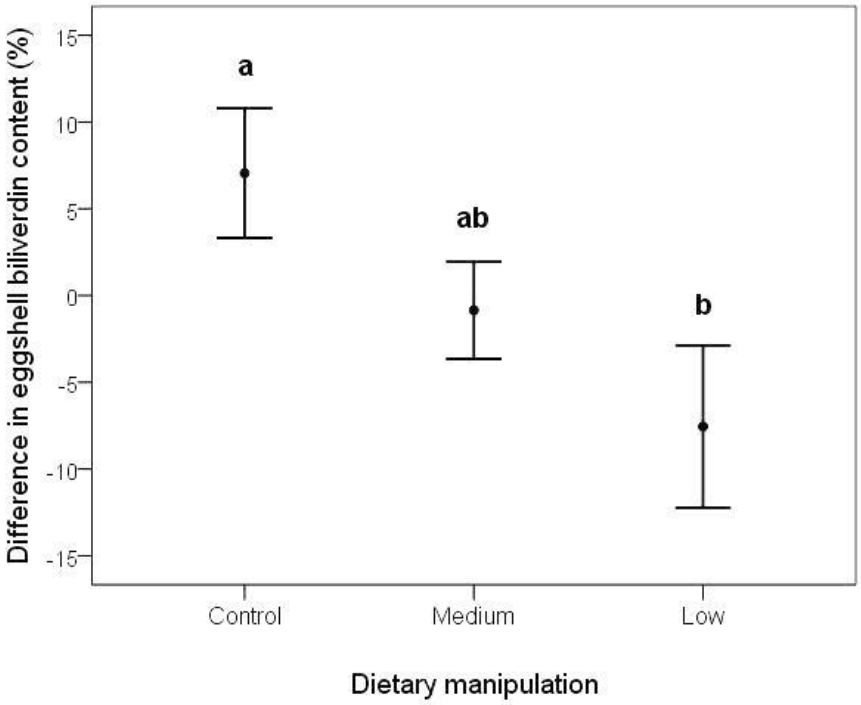

Figure 2.5. The difference (mean ± 1 SE) in biliverdin content (as a percentage of total pigment deposited) of eggshells collected between the start and the end of the laying sequence (see section 2.3.2 for details) of Japanese quails fed *ad libitum* (Control), medium quantity (Medium) or low quantity (Low) food diets. Different lowercase letters reflect statistically significant differences.

No significant correlation was found between eggshell pigment contents and any measure of female body condition or egg quality (biliverdin: $-0.07 < \text{all } rs < 0.36$, all $Ps > 0.05$; protoporphyrin: $-0.09 < \text{all } rs < 0.28$, all $Ps > 0.05$, $N = 24$). However, eggshells containing more biliverdin exhibited bluer spots with higher blue chroma values ($r = 0.50$, $P = 0.01$, $N = 24$) and those containing more protoporphyrin displayed backgrounds of lower brightness ($r = -0.50$, $P = 0.01$, $N = 24$).

2.3.4. Discussion

The results reveal that an experimental manipulation of female body condition through food restriction induced a change in eggshell pigment investment but not in the apparent colour of the eggshell. Previous studies on the function of eggshell colouration have experimentally manipulated female body condition through an increase in food availability or antioxidant content. For instance, Moreno and colleagues found that female European pied flycatchers supplemented with mealworms (*Tenebrio molitor*) laid bigger and bluer eggs (Moreno *et al.* 2006). They suggested that this experimentally demonstrated that blue–green eggshell colour and biliverdin concentration both indicated the nutritional condition of the breeding female in birds. More recently, Dearborn and colleagues manipulated the antioxidant content of food provided to Araucana chickens and found that the differences in eggshell colouration between birds were due to female identity rather than to the food manipulation, and that variation in eggshell colour was unlikely to be perceived by the chicken (Dearborn *et al.* 2012). Nevertheless, in both studies, there was neither nutrient restriction nor measurement of protein and energy intake, female body condition, oxidative stress or eggshell pigment content. The present study represents the first to induce a decline in female body condition experimentally in order to investigate the effect on eggshell colouration in a species laying heavily maculated eggs (Fig. 2.2a). I found that food restriction decreased female body condition but not measures of egg quality. Egg size or mass may be more sensitive measures of egg production than clutch size as food limitation is likely to operate initially on egg volume as opposed to egg numbers (Martin 1987, Reynolds *et al.* 2003). Moreover, the deposition of hormones, immunological compounds (e.g. carotenoids and antibodies) and nutrients by females into their eggs influences offspring growth and development (Groothuis & Schwabl 2008, Ho & Burggren 2010). Thus, egg mass is a widely used measure of egg quality, with heavier eggs being more fertile and containing more nutrients and antibodies

that are essential for chick survival (Galbraith 1988, Farooq *et al.* 2003, Grindstaff *et al.* 2005). High 'quality' females may invest more in reproduction and deposit more resources into their eggs if they are to increase offspring fitness (Pilz *et al.* 2003). Accordingly, I predicted that food-restricted females would lose body mass and body condition, resulting in smaller and lighter eggs. Yet, I found that egg mass, volume and yolk proportion were not affected by food restriction of the layer, even if heavier females generally laid heavier and bigger eggs. Similarly, Giuliano and colleagues found that female northern bobwhites (*Colinus virginianus*) and scaled quails (*Callipepla squamata*) that were food restricted (i.e. quantity, protein and energy content) lost body mass through the feeding trial but did not modify their investment in egg mass or size (Giuliano *et al.* 1996). One explanation for these findings is that females would optimise the quality of their eggs and of their chicks by reducing their own body mass and activity when food was restricted, while maintaining the size and mass of their eggs similar to those of control birds, to compensate for their decreased body condition and loss of nutrients invested in their eggs (Meijer & Langer 1995). According to the poultry science literature (e.g. Moula *et al.* 2009), an egg is composed of approximately 60% albumen, 30% yolk and 10% eggshell; this composition can vary with environmental factors such as breed, age, female health status, egg mass and female diet. Variation in the yolk compared with the albumen fraction is an index of egg quality that is used by the poultry industry to commercial ends. Thus, I predicted that food-restricted females would lay eggs with a reduced fraction of yolk/albumen compared with control females. While I successfully reduced female body condition through food restriction, I cannot attribute this to nutrient limitation as I only used an approximation of egg quality (i.e. egg and yolk mass). More detailed analyses of yolk constituents (e.g. proteins, carotenoids, hormones) and albumen (e.g. lysozyme) content would verify whether the nutritional stress induced in the females influenced resource allocation into their eggs.

I compared between- and within-clutch variation in spot and background reflectance (i.e. brightness, UV chroma, blue–green chroma and red chroma) using repeatability estimates (Lessells & Boag 1987), and found that between-female variance was markedly higher than within-female variance, suggesting that eggshell reflectance could be highly heritable and would constitute a female-specific phenotypic trait (Sezer & Tekelioglu 2009). Any variation in eggshell colouration due to female body condition could constitute a signal of female quality towards conspecifics (Moreno & Osorno 2003). I chose to analyse the reflectance data using a neural noise model (Holveck *et al.* 2010), assuming that light was not limiting visual performance. I did not compare eggshell reflectance to a background (e.g. nest) but, instead, I investigated within-individual variations (Cassey *et al.* 2009) that were subject to environmental modification such as food restriction. Under controlled laboratory conditions, birds are thought to use chromatic aspects of colour to detect large targets and achromatic aspects (that are based solely on differences in the intensity of reflected light) to detect small objects and pattern (Osorio *et al.* 1999, Spaethe *et al.* 2001). In the experimental group of birds, eggshell background contrasts were greater when comparing eggs between females than within females, whereas I did not find any significant difference between spot colour contrasts within females compared with between females. This suggests that eggshell background colour contrasts would be more detectable by an avian model than spot colour differences (see also Holveck *et al.* 2010). Moreover, all the chromatic discriminability values were small (< 1 JND) compared with achromatic contrasts (Dearborn *et al.* 2012), suggesting that differences in luminance and texture would be more easily detectable than differences in colour (Kelber *et al.* 2003, Avilés 2008). Thus, these results suggest that even in species nesting on the ground in open environments with optimised visual acuity, birds would be able to detect eggshell brightness and form variations better than colours. Female body condition at the start of the experiment predicted the intensity of eggshell background

blue–green colouration, with females in higher body condition laying bluer eggshells (e.g. higher blue chroma values). However, female body condition was not correlated with any other parameter of eggshell colouration, suggesting that there may be a strong relationship between body condition and biliverdin investment, explaining why there is no direct relationship between female body condition and spot red chroma. This confirms previous findings (Cassey *et al.* 2012a) showing that eggshell pigment concentrations were not always correlated with eggshell colour parameters in two thrushes (*Turdus* spp.). Moreover, spot and background colour and darkness might indicate an aspect of female health such as antioxidant capacity (Hanley *et al.* 2008) or physiological stress (Martínez-de la Puente *et al.* 2007), which are not signalled by female body condition alone. However, contrary to my predictions, food restriction had no significant effect on either the physical properties of eggshell spectra or the achromatic and chromatic contrasts that could be perceived by birds themselves or by conspecifics.

The analysis of eggshell pigment concentration revealed that the eggshells of Japanese quails are pigmented with protoporphyrin IX and smaller amounts of biliverdin. The two pigments are part of the same biochemical pathway and the positive correlation that I found between their quantities in whole eggshells suggests that the processes of deposition of these pigments are not independent, and that the quantities of biliverdin and protoporphyrin should change proportionately (Wang *et al.* 2009). Indeed, Moreno and Osorno (2003) suggested that the relationship between deposition into the eggshell of such pigments and female body condition was adaptive: the SSEC hypothesis proposed that eggshell colouration signals female quality to the male. Pigment deposition into the eggshell would be modulated by female antioxidant capacity and males would subsequently adjust their care in response to the intensity of eggshell colour. Thus, according to my predictions, food restriction would modulate eggshell pigment investment by the female. Indeed, food-restricted females increased their investment

in protoporphyrin and decreased the amount of biliverdin deposited into the eggshell. This result suggests that the decrease in female body condition could be associated with a decrease in antioxidant capacity or an increase in oxidative stress and, thus, that females with low antioxidant capacity in the present study passively deposited more protoporphyrin into their eggshells to remove this pro-oxidant. The fact that females in lower body condition also decreased the deposition of biliverdin into their eggshells supports this hypothesis. It confirms that only females in higher body condition can face the trade-off between pigmentation of their eggshells with biliverdin and control of oxidative stress (Moreno & Osorno 2003). Measurements of oxidative stress and antioxidant capacity in my subjects were not the focus of this investigation; therefore, I cannot confirm this hypothesis but the conclusions indicate fruitful directions for future research.

The methodology for quantifying pigments did not allow determination of pigment content in different egg regions (Fig. 2.2a), but both pigments may be responsible for spot and background colouration in mixed quantities. The spot reflectance spectra of eggshells of Japanese quail exhibit a peak at ~ 630 nm (Fig. 2.2b), which is consistent with the presence of protoporphyrin IX (Sanz & García-Navas 2009). However, background spectra show the same peak at 630 nm, and also two more peaks: one around 500 nm, similar to the reflectance spectra of blue–green eggshells (Siefferman *et al.* 2006), and one in the UV zone at 320 nm. This suggests that protoporphyrin would be mainly responsible for spot colour but both pigments may be responsible for background colour. Indeed, Poole (1964) described the eggshell background of Japanese quail as pale green and suggested that a strong genetic control for shell colour would act in that species. Moreover, eggshells would be superficially pigmented with red–brown or green–brown dots. Pigment masses would be deposited at first as dots and then spread into blotches on the shell surface by contractions of the shell gland and rotation of the egg (Tanaka *et al.* 1977). Yet, I found that eggshells containing higher

concentrations of protoporphyrin displayed darker backgrounds. This could suggest that protoporphyrin, being a red–brown pigment and darker than biliverdin, might be responsible for the lightness of eggshell background rather than influencing the colour itself as suggested by the lack of relationship between protoporphyrin content and background red chroma. I also found that eggshells containing more biliverdin displayed bluer spots. These results confirm the complexity of eggshell pigment distribution in different areas of the eggshell (Sparks 2011). Eggshell pigment synthesis and deposition are still hotly debated mechanisms in heavily spotted eggs, but the proposition that protoporphyrin IX and biliverdin are implicated in haem synthesis is now gaining credence (e.g. Milgrom 1997, De Coster *et al.* 2012). Both pigments circulate in the bloodstream, and are metabolised in the shell gland (Poole 1965, Wang *et al.* 2009, Honza *et al.* 2012). Pigments are deposited a few hours before oviposition (Poole 1965) and the two pigments could be differentially allocated on the eggshell according to female body condition. Future work should certainly examine ways to differentiate between pigments destined for the two eggshell 'components' of maculation and base colour as initial steps in investigation of their synthesis in relation to their deposition.

To the best of my knowledge, this is the first study in which female body condition has been modified to investigate spotted-eggshell colouration by quantifying not only eggshell reflectance of spots and background independently, but also pigments concentrations in the eggshell. Many previous studies have examined avian species with post-natal paternal care and have focused on the SSEC hypothesis (reviewed by Riehl 2011). However, males of species that do not invest care post-hatching could also be subject to selection. Where predation pressure is high and egg colouration is linked not only to female body condition but also to crypsis, the assumptions of the SSEC hypothesis might also apply. To date, no study has investigated the relationship between eggshell pigmentation and female body condition in such a species. In addition, few studies have experimentally manipulated female body

condition to examine its direct effects on eggshell pigmentation (García-Navas *et al.* 2011, Morales *et al.* 2011). I suggest that eggshell colouration could be used in making eggs cryptic in Japanese quails, and that females would be able to maintain the appearance of their eggshell constant despite fluctuating environmental conditions (e.g. food availability). Further analysis on spot coverage might help to understand how eggshell maculation can be influenced by female body condition and what would be the implications for egg crypsis in such a ground-nesting species.

2.4. Maternal influence on eggshell maculation: implications for cryptic camouflaged eggs

2.4.1. Introduction

To maximise their reproductive success, female birds are able to modulate their investment not only through the size of their clutch but also through the quality of their eggs (Bernardo, 1996). Many different egg components determine chick quality and these include hormones (testosterone, CORT) (Petrie *et al.* 2001, Mazuc *et al.* 2003, Loyau *et al.* 2007a), and antibacterial (lysozyme) (Saino *et al.* 2002) and antioxidant factors (carotenoids, vitamins) (McGraw & Ardia 2003, Costantini 2010) that are deposited by mothers into their eggs (e.g. into the yolk and albumen). These components are known to influence embryonic development, hatching success, chick growth, survival and immunity (Birkhead & Nettleship 1982, Arnold *et al.* 1991, Hill 1993, Amat *et al.* 2001). The mother modulates the quantities of these components in response to seasonal and breeding parameters, including male attractiveness (Loyau *et al.* 2007a), but, initially, in response to their own body condition (Hanssen *et al.* 2003).

Much less is known about the role of maternal investment into eggshell specific components, including into pigmentation. The eggshell is vital in: protecting the embryo from mechanical damage; controlling water loss (Board & Halls 1973, Handrich 1989); regulating gas exchange between the developing embryo and the environment (Tullet 1984); preventing contamination by bacteria (Board 1980) and other pathogens; and providing a source of nutrients, primarily calcium, to the developing embryo (Reynolds & Perrins 2010). Subsequently, females adjust their resource allocation to the eggshell according to their

specific physiological condition at breeding (Gosler *et al.* 2005, Higham & Gosler 2006) and hence many eggshell-specific traits are considered condition dependent.

Females also transfer colourful pigments into their eggshells, and the function of pigment deposition is currently highly debated. Avian eggshell pigmentation has been studied repeatedly in the context of camouflage, mimicry, egg recognition, female signalling, maternal inheritance, and eggshell strength (reviewed in Kilner 2006, Cassey *et al.* 2011b). It may protect the embryo from environmental threats or promote its development by photo-acceleration (reviewed in Maurer *et al.* 2011a). In addition, eggshell pigment concentrations may be related to female physiological condition due to the potential antioxidant/pro-oxidant properties of the two main pigments involved: biliverdin (McDonagh 2001) and protoporphyrin (Shan *et al.* 2000). In brown-spotted eggs, protoporphyrin, which is the main pigment responsible for eggshell maculation, may have structural properties and could compensate for a lack of calcium in the eggshell as it is structurally similar to phthalocyanine, a lubricants commonly used in solid-state engineering (Solomon 1997, Gosler *et al.* 2005). Calcium is a crucial nutrient for birds with it constituting approximately 98% of eggshell dry mass (Reynolds *et al.* 2004). Dietary calcium must be available during egg formation (Graveland & van Gijzen 1994, Bureš & Weidinger 2003), but eggshell structural defects develop when it is a limited resource (i.e. thin and spongy shells, abnormal pigmentation, absent cuticle and shell breakage; Graveland *et al.* 1994, Eeva & Lehikoinen 1995, Graveland 1996). Thus, eggshell maculation patterns might depend on female body condition during reproduction. For instance, female blue tits exhibiting lower body condition and higher stress levels (as signalled for example by high Heat Shock Protein concentrations in the blood) laid more maculated eggshells (i.e. with more brown spots). However, in great tits, heavier females laid paler and less maculated eggs (Stoddard *et al.* 2012). In the same species, another study did not find any significant relationship between female body condition and

age with eggshell pigmentation pattern (Gosler *et al.* 2000). Thus, the relationship between eggshell maculation and female body condition remains poorly studied and invites experimental manipulation to further our knowledge.

Recently, I experimentally decreased female body condition by restricting the diet of Japanese quails (Section 2.3; Duval *et al.* 2013). Both colouration and pigment content were measured in whole eggshells. Food-restricted females were in lower body condition and deposited more protoporphyrin but less biliverdin into their eggshells compared with control females. However, eggshell reflectance was not affected by the 'switch' in eggshell pigment deposition. I proposed that eggshell colouration would be strongly preserved in Japanese quails in order to maximise eggshell crypsis. I also suggested that further investigation of eggshell maculation could explain why studies to date have failed to detect a direct relationship between eggshell colouration and pigment content.

In this study, I quantified eggshell maculation of the eggs collected in the previous study (Section 2.3) as the percentage of spot coverage from digital photographs from eggs of females that had experienced access to different food availabilities. If eggshell brown spots (maculation) are mainly due to the presence of protoporphyrin, I predict that food-restricted females that deposit more protoporphyrin and less biliverdin into their eggshell should lay eggshells with a higher percentage of maculation. To the best of my knowledge, this is the first study to investigate experimentally eggshell maculation as a condition-dependent trait.

2.4.2. Materials and methods

2.4.2.1. General methods

I used the eggshells obtained from the previous experiment (Section 2.3) to form the basis of my maculation analysis (Duval *et al.* 2013). The experimental design is described in greater detail in Duval *et al.* (2013) (Section 2.3.2).

2.4.2.2. Digital photography

Using calibrated digital photography, I characterised eggshell spot coverage by quantifying pixels corresponding to the spots and background areas for each photograph. Constant lighting and long exposures, rather than flash photography, were used to protect the eggshell pigments from light degradation. A calibrated CANON EOS 450D camera with a 105 mm SIGMA AF lens was used and was activated remotely using a CANON RC1 infrared control. Eggs were placed beneath the camera on black velvet usually used as a standard background in photography. Before each photographic session a picture of a colour chart and a grey standard (Colour Confidence, Spectrum Point, Birmingham, UK) were obtained for calibration. Four eggs per female were photographed. For each photograph the camera was adjusted on its stand so that the egg filled the entire frame. The picture of the egg was taken including a label with the date and female identity and a size standard. Each 90-degree rotation of the egg was photographed providing four such views per egg. For the subsequent three photographs the focus was maintained but the egg was given a 90-degree turn to the right. All digital egg images were saved in standardised RAW format that is beneficial for colour analyses (Cassey *et al.* 2012b). The characterization of the camera's spectral sensitivities and the calibration process were as described in Lovell *et al.* (2005). The linear RAW images were converted to XYZ (CIE XYZ colour-space coordinates (CIE, 1986)), and subsequent conversion from XYZ to CIELAB space was implemented using Matlab image

processing toolbox (2008, The MathWorks, Natick, MA, USA). Variations in the illumination of the photographed scene were controlled for by normalizing the luminance values (the L channel) to 0 for the darkest area of the black velvet background and to 60 for the white graph-paper.

The area of the photograph occupied by the egg was identified (Fig. 2.6a), and for pixels in this area a histogram of the spread of luminance values was plotted, giving a bi-modal distribution of luminance values corresponding to spots and background (Fig. 2.6b).

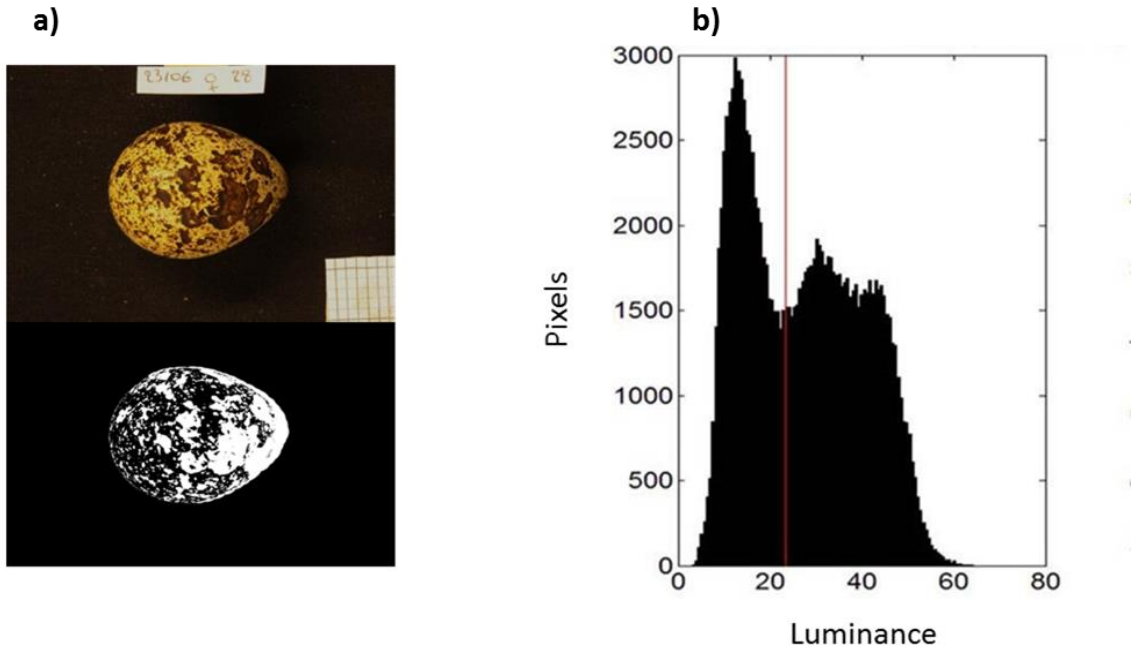

Figure 2.6. (a) Example of a photograph of a Japanese quail egg on a black velvet surface (top) and the corresponding egg mask (bottom). (b) Example of a histogram of luminance values where the red line shows the cut-off between dark areas of maculation (to the left) where the luminance is low, and light areas of background (to the right) where the luminance is high.

The cut-off between the maculation and background areas for each photograph was then visually selected, all eggs being analysed blind to the treatment. Finally, the spot coverage percentage was calculated as the number of pixels in the brown spots region divided by the

total number of pixels constituting the egg in the photograph, multiplied by 100 (average: 73.7%; range: 48.7% - 90.6%).

2.4.2.3. Statistical analysis

I calculated intra-class correlation coefficient (*r*) repeatability estimates (Lessells & Boag 1987), and compared between- and within-clutch variation in spot coverage at the beginning ($r = 0.78$, $P < 0.001$, $N = 48$) and at the end of the diet manipulation ($r = 0.69$, $P = 0.004$, $N = 48$), and then used the mean spot coverage per female in the analyses. I compared spot coverage between groups before the treatment using an independent samples Kruskal-Wallis test. I used a Pearson's correlation to investigate the relationship between eggshell spot coverage and its protoporphyrin concentration at the beginning of the diet manipulation. I tested the effect of food manipulation on the change in spot coverage over the experiment by calculating the difference between the pre- and post-food restriction eggs. I used a General Linear Model (GLM) with the difference in spot coverage as the dependent variable, the treatment-group as fixed factor and the difference in the protoporphyrin proportion as covariate to account for variation between groups. *Post hoc* analyses for main effects were performed using a Bonferroni method. Model residuals were found to be normally distributed. All statistical analyses were performed in SPSS Statistics 19.0.0.

2.4.3. Results

Eggshell spot coverage did not differ between groups before the treatment (Kruskal-Wallis: $H = 0.58$, $P = 0.74$, $N = 24$). Spot coverage was negatively correlated with eggshell protoporphyrin concentration (Pearson correlation: $r = - 0.43$, $P = 0.04$, $N = 24$) before the beginning of the diet manipulation (Fig. 2.7). The dietary treatment significantly influenced eggshell spot coverage: control females decreased spot coverage (pre-treatment mean ± 1 SD:

74.77 ± 7.11; post-treatment mean ± 1 SD: 70.23 ± 7.17), compared to food-restricted females which maintained a spot coverage similar to their pre-treatment levels (MQ pre-treatment mean ± 1 SD: 77.10 ± 10.28; MQ post-treatment mean ± 1 SD: 76.16 ± 8.33; LQ pre-treatment mean ± 1 SD: 72.47 ± 14.80; LQ post-treatment mean ± 1 SD: 74.82 ± 11.32 (Fig. 2.8) (GLM: Group: $F_{2,22} = 6.24$, P = 0.01; *post hoc*: C *versus* LQ: P = 0.01; C *versus* MQ: P = 0.22; MQ *versus* LQ: P = 0.17).

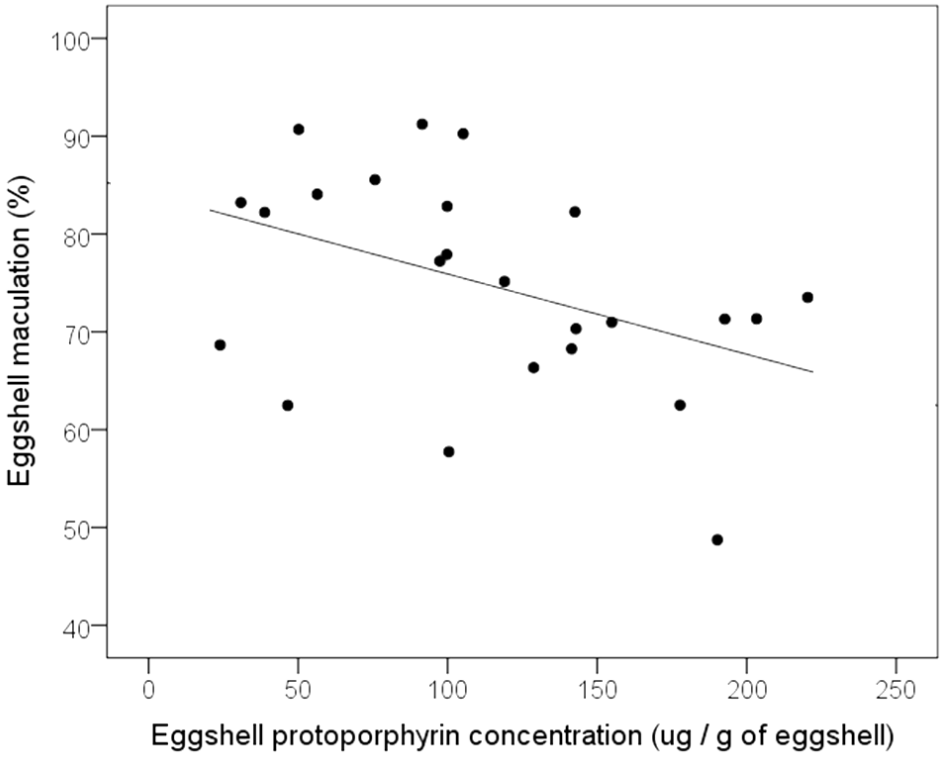

Figure 2.7. The bivariate relationship between eggshell maculation (spot coverage) and eggshell protoporphyrin concentration in all Japanese quail eggs (N = 24) before dietary treatment.

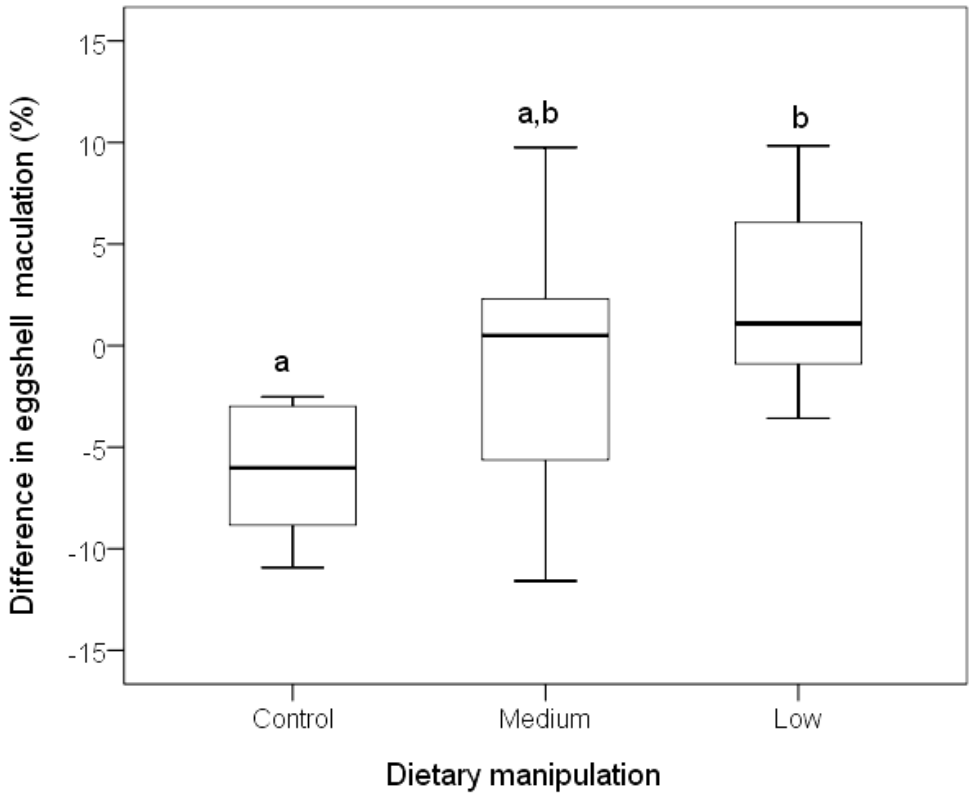

Figure 2.8. Variation in eggshell maculation (spot coverage) calculated as the difference in spot coverage between eggs (N = 24) collected prior to and after dietary treatments (Control; Medium quantity diet; and Low quantity diet – see section 2.3.2 for further details). Different lowercase letters reflect statistically significant differences.

2.4.4. Discussion

My data demonstrate for the first time that eggshell maculation is influenced by maternal body condition in the Japanese quail, but in a direction that at first glance is counter-intuitive. In a previous published study (Duval *et al.* 2013), I proposed that eggshell reflectance in the Japanese quail may have evolved to maintain eggshell crypsis in changing environments and my results here are in line with this hypothesis. However, in this previous analysis, I did not take into account the extent of eggshell maculation. Reflectance spectrophotometry focuses on a single small point (no larger than 2 mm) on the eggshell that allows the precise quantification of wavelengths at specific locations. Yet, the exposed surface of the egg will

be the first thing that conspecifics and predators visually detect. Thus, eggshell maculation is an important consideration (Stoddard & Stevens 2010). For instance, in the house sparrow egg rejection increased significantly when spot patterns rather than eggshell colour were experimentally modified (López-de-Hierro & Moreno-Rueda 2010).

I used digital photography to investigate eggshell maculation as its spot coverage under restricted food availability. Before any manipulation, eggshell spot coverage was negatively correlated with eggshell protoporphyrin concentration, which would suggest that protoporphyrin is not used to increase the amount of visible brown spots on the eggshell. This slightly counter-intuitive result might be due to a complex interaction between protoporphyrin and the eggshell matrix. Indeed, protoporphyrin in Japanese quail is deposited throughout the shell integument from the shell membrane through the cuticle (Tamura & Fujii 1967), and is not only present on the surface of the eggshell. In a previous study (Section 2.3; Duval *et al.* 2013), I showed that eggshells containing more protoporphyrin displayed darker backgrounds. However, spot brightness was not correlated to eggshell protoporphyrin content.

Over the clutch, control females decreased their proportion of maculation compared to food-restricted females, which maintained similar maculation levels. This suggests that there is a natural variation in eggshell maculation in control females that may be an optimal strategy to maintain eggshell reflectance following variations in eggshell pigments concentrations. Indeed, I have previously shown that females in higher body condition (i.e. controls) decreased their deposition of protoporphyrin over the clutch (Section 2.3; Duval *et al.* 2013), suggesting that they might have had better antioxidant capacities at the end of the experiment, probably due to a decreased activity and a food provided ad libitum. Thus they might have been able to sustain higher concentrations of protoporphyrin, explaining why they decreased its deposition in the eggshell. Decreasing eggshell maculation when eggshell protoporphyrin

is diminished may help females to maintain constant eggshell reflectance (e.g. intensity of colour patterns) despite variation in the concentration of eggshell protoporphyrin deposited. In addition, females may vary eggshell maculation without compromising the camouflage of their eggs as they only decreased eggshell maculation degree by 4.54 % (mean ± 1 SD: -4.54 % ± 6.66 %), which is unlikely to be perceived by potential predators. However, further analysis using perceptual visual models may be necessary to confirm this hypothesis. In contrast, eggshell maculation did not change in the food-restricted group. I predicted that food-restricted females may have been able to redistribute protoporphyrin across the different regions of the eggshell.

My results suggest a complex interaction between maintaining invisible (cryptic) eggshell maculation and the concentration of the pigment protoporphyrin. Indeed, the negative correlation between eggshell maculation and its protoporphyrin concentration is counter-intuitive and may imply that both parameters vary in opposite directions. This suggests that additional study of mechanisms of eggshell pigment deposition would allow us to understand how female body condition influences pigment allocation in different parts of the eggshell (Butcher & Miles 2011). However, such complex within-shell allocation of pigments cannot be measured by simple digital photography of the eggshell surface, or whole eggshell analysis of pigment concentrations. It will require sophisticated techniques such as the use of a layer-by-layer dissolution method to study the deposition velocity of pigments in different layers of the eggshell (Wang *et al.* 2007).

This is the first experimental demonstration in a species laying spotted eggs of eggshell maculation depending on female body condition. My results have three major implications at methodological and evolutionary levels. First, the relationship between pigment concentration and eggshell colour is complex and spot colour is likely due to an interaction between these two pigments. Combined with previous findings (Section 2.3; Duval *et al.*

2013), I show that eggshell reflectance and maculation cannot be used as proxies of eggshell pigment content in Japanese quail. Further comparative studies measuring both colouration and pigment concentrations from specific fragments of eggshell of differently patterned eggshells from various species would help us to understand better the spatial distribution of pigments across eggshells of many species. Further considerations of the physiology associated with eggshell pigments, and knowing precisely how it and they contribute to variation in eggshell colour, will clarify how (or whether) eggshell colour can act as an honest signal of female body condition.

Secondly, it has recently been proposed that eggshell pigments may have multiple implications for embryonic development. Indeed, Maurer *et al.* (2011a) proposed several hypotheses to explain the diversity of eggshell patterns from the "embryo's view" such as thermoregulation, protection against UV-B radiation, photo-acceleration of embryo development, functional asymmetry and lateralization of the chick, establishment of the circadian clock, DNA repair by photo-activation, and antimicrobial defences. Thus, as eggshell pigments might directly affect chick growth and development, eggshell maculation might be one of the egg parameters influenced by maternal effects (Mousseau & Fox 1998).

Finally, the 'nest-crypsis' hypothesis proposes that selection for egg crypsis has not strongly evolved in species laying conspicuous nests, predators which search preys visually, detect nests first and then the eggs (Skutch 1976). However, optimizing egg camouflage (e.g. via egg matching with background; Lee *et al.* 2010) might be fundamental to the survival and breeding success of ground-nesting species that do not make nests to conceal their eggs (Götmark 1992, Götmark 1993, Kilner 2006). Accordingly, it has recently been shown that female Japanese quails match egg maculation colour with the background they lay on to maximise egg camouflage, independently of egg maculation degree (Lovell *et al.* 2013). This might reinforce the idea that egg reflectance plays a major role in egg camouflage in this

species and adjusting eggshell maculation to maintain its reflectance constant might be the optimal camouflage strategy adopted by better females.

Overall, I propose that eggshell maculation is dependent on body condition with any change in female body condition during laying potentially impairing a female's capacity for camouflaging her eggs effectively. This female capability could be an extended phenotypic trait with only females in higher body condition able to maintain eggshell reflectance and maculate their eggs in order to maximise egg crypsis. This makes the optimization of the proportion of maculation essential.

2.5. Chapter Two - Summary and perspectives

In this chapter, I experimentally investigated the relationship between female body condition and pigmentation in brown-spotted eggshells laid by Japanese quails. The pilot study highlighted the importance of measuring the food intake of individual birds (Boswell *et al.* 2002) instead of setting the daily individual food intake for a group of subjects based upon a population mean. The quails in this experiment were significantly heavier than NRC guidelines (NRC 1994) and body mass was highly variable between individuals resulting in high variation in daily food intake across the small population of birds. In a study where birds are individually housed, this would lead to an underestimation of the food requirements of heavier birds, and an overestimation of the food requirements of lighter birds. In addition, measuring individual food intake is essential in all experimental designs, as food competition occurs between birds that are group-housed and this confound may lead to bias in food requirement calculations.

The results of the food restriction manipulation showed that restricted females with a reduced body condition deposited more protoporphyrin and less biliverdin into their eggshells,

contrary to control females which were in a higher body condition, showed the opposite response and were able to deposit more of the anti-oxidant pigment, biliverdin, in their eggshells. Interestingly, eggshell reflectance remained constant in both groups. However eggshell maculation decreased in control females over the course of a clutch and remained unchanged in food-restricted birds. My findings suggest that there is a complex interaction between both pigments that results in the spotted pattern observed on quail eggshells, and that maintaining eggshell colour despite a switch in pigment allocation could be an adaptive behaviour that has evolved to facilitate egg camouflage and decrease predation risk in species that lay spotted eggs and nest on the ground. In addition, diminishing eggshell maculation when the concentration of protoporphyrin (mainly responsible for the maculation of spotted eggs; Kennedy & Vevers 1976) decreases, may help females in higher body condition to maintain eggshell reflectance and maximise egg camouflage.

I encourage additional experimental studies restricting different maternal resources such as food, calcium or antioxidants (e.g. carotenoids, Vitamin E) to help to clarify which of these specific nutrients is the most limiting and drives eggshell pigment deposition strategies. In addition, sudden environmental change such as food restriction can be perceived as a stressor by individuals (Lynn *et al.* 2010). Thus, further experimental manipulation of female stress status may help to understand whether eggshell pigmentation can indicate maternal stress exposure during reproduction and how this could potentially influence female capacity to maximise egg camouflage in stressful contexts.

In conclusion, I showed in Chapter Two that eggshell pigments deposition is condition-dependent in Japanese quail, and that female can modulate eggshell maculation and maintain constant eggshell reflectance, to potentially maximise egg crypsis. In Chapter Three, I will investigate how female exposure to physiological stress during reproduction, as well as the stress that they might have experienced early in life, could influence eggshell pigmentation.

Chapter Three

EFFECTS OF FEMALE STRESS EXPOSURE ON EGGSHELL PIGMENTATION

3.1. Abstract

Stress has short and long-term effects on individual physiology such as their antioxidant capacity, and might influence eggshell pigmentation process in adulthood. Evidence in favour of this hypothesis is scarce. I first investigated whether repeated exposure to stress hormones influenced female Japanese quails stress level, antioxidant defences and eggshell pigmentation, using corticosterone (CORT) supplementations. CORT-fed females should suffer from an increased oxidative stress and decreased body condition; deposit more protoporphyrin and less biliverdin in the eggshell, leading to an increased maculation but a constant reflectance. Three eggs before and after CORT supplementation were analysed, and I found that CORT-fed birds laid brighter eggs; however female physiology or eggshell maculation were unaffected. This suggests that spot reflectance may be a key factor affected by females CORT exposure. I then investigated the effects of developmental stress on eggshell pigmentation in adulthood. Given the above results, eggs laid under adverse current conditions should be brighter but eggshell maculation should remain constant. Females stressed during development should be programmed and less affected by stress during breeding than females experiencing stress for the first time at adulthood. Eggs collected from 30 females that had been exposed to developmental stress or not were analysed, and I found that pre and post-natal stress differentially influenced eggshell pigmentation. Pre-natal stress helped females to maintain eggshell maculation and protoporphyrin concentrations during stressful breeding, which may suggest enhanced oxidative stress tolerance. Post-natal stress facilitated the deposition of biliverdin in eggshells under adverse breeding conditions but changed eggshell reflectance, potentially conferring antibacterial protection to the offspring, at the risk of impairing egg camouflage. Many factors trade-off to produce eggshell patterning and these trade-offs change depending upon prevailing environmental conditions.

3.2. Eggshell appearance does not signal maternal corticosterone exposure in Japanese quail: an experimental study with brown-spotted eggs

3.2.1. Introduction

Throughout their life, birds have to cope with a range of stressful stimuli such as elevated predation risk, food shortage, and habitat disturbance that can affect their fitness via costs to health, reproduction and survival. Birds have evolved behavioural and physiological responses (i.e. allostasis) in order to reduce the negative effects of such stressors on their survival (Wingfield *et al.* 1998, McEwen & Wingfield 2003, Landys *et al.* 2006). Any stressful stimulus induces the activation of the Hypothalamic-Pituitary-Adrenal (HPA) axis, ultimately resulting in the release of glucocorticoid hormones (Wingfield 1994, Romero 2004) such as CORT in birds. CORT induces an increase in glucose release to maximise the energy available for the optimisation of life-saving behavioural strategies (Munck *et al.* 1984, Wingfield *et al.* 1998). Acute exposure to stress results in a transient increase in glucocorticoid secretion; however, elevated CORT can be sustained for a long period of time when individuals are faced with chronic stress and unable to return its concentration to a basal level (Angelier & Chastel 2009). Chronic stress exposure can have negative effects on the nervous system and cause deficiencies of the immune system and physiological functions such as antioxidant capacities (McEwen & Stellar 1993, Sapolsky 2000, de Kloet *et al.* 2005, Costantini *et al.* 2011). For example, in adult common kestrels, oral administration of CORT to mimic a physiological stressor induced an oxidative stress with birds showing a 32% increase in circulating reactive oxygen metabolites (Costantini *et al.* 2008). Moreover, recent evidence in broiler chickens showed that chronic administration of CORT induced an increased level of lipid peroxidation suggesting the formation of Reactive Oxygen Species

(ROS) and a decreased antioxidant capacity (Lin *et al.* 2004). Several antioxidant enzymes exist and Superoxide dismutase (SOD) and Glutathione peroxidase are commonly measured in oxidative stress studies (Weydert & Cullen 2009, Montgomery *et al.* 2012, Marasco *et al.* 2013). Both enzymes act as free radicals scavengers: SOD catalyzes the dismutation of superoxide (O2-) into oxygen, and plays a major role in controlling the cellular level of free radicals (Bowler *et al.* 1992). Likely, Glutathione peroxidase is an enzyme that minimises the cellular levels of hydrogen peroxide using Glutathione (GSH) as a reductant, leading to the formation of its oxidized dimer, GSSG that can be cytotoxic if not reduced by another enzyme, the Glutathione reductase (Hayes & McLellan 1999). Thus, measures of blood SOD activity and GSH and GSSG concentrations can be good preliminary indicators of individuals' antioxidant response efficiency that could be affected by exposure to CORT.

During reproduction, birds are particularly sensitive to stress as breeding individuals face a trade-off between resources allocated to their current reproductive investment and to their own survival (Stearns 1992). The endocrine stress response regulates reproductive effort, and for instance both baseline and stress-induced CORT levels are highest during reproduction compared with non-reproductive events (Romero 2002). During egg formation, stress can affect both the mother and her offspring as female birds can modulate their investment in different egg components such as hormones (e.g., testosterone, CORT) (Mazuc *et al.* 2003, Loyau *et al.* 2007a), and antibacterial (lysozyme) or antioxidant factors (carotenoids, vitamins) (Saino *et al.* 2002, Hargitai *et al.* 2009, Costantini 2010), according to their own physiological condition during laying ("maternal effects", Mousseau & Fox 1998). Less is known about maternal investment in eggshell components and especially in eggshell pigments.

Many avian species lay spotted eggs which have been studied repeatedly in the context of mimicry, brood parasitism, signalling towards male, maternal inheritance, and eggshell

strength (reviewed in Kilner 2006). Eggshell spotting is predominantly pigmented by the tetrapyrrole protoporphyrin (Gorchein *et al.* 2009), which is a molecule derived from haemoglobin anabolism and is thought to be synthesised in the uterus and then deposited into the eggshell just prior to oviposition (Sparks 2011). Porphyrins are known to possess pro-oxidant properties and they can induce an oxidative stress response, eventually resulting in liver damage (Afonso *et al.* 1999). Moreover, it has been shown *in vitro* that protoporphyrin can directly stimulate the synthesis of haem oxygenases (HOs) such as HO-1 or HSPs (Shan *et al.* 2000), which are synthesized after cellular stress and function as molecular chaperones to prevent proteins from misfolding (Åkerfelt *et al.* 2010). A second pigment also found in spotted eggs, biliverdin, is thought to possess the opposite (i.e. antioxidant) properties and, therefore, may help individuals to cope with oxidative stress (McDonagh 2001). Thus, protoporphyrin and biliverdin deposition into eggshells might vary according to the status of the female's immune system and, in particular, to her antioxidant capacity. Indeed, Moreno and Osorno (2003) proposed the SSEC hypothesis which postulates that females with high antioxidant capacities produce eggs with more biliverdin which gives them a 'bluer' appearance. Females with lower antioxidant capacity may suffer from physiological stress and passively deposit higher amounts of protoporhyrin into their eggshells (Moreno & Osorno 2003). Yet, to date the deposition of both pigments remains poorly considered in quantitative studies of eggshell colouration (but see Duval *et al.* 2013).

Organisms are continually exposed to stressors in their environment that challenge homeostasis. Previous studies of poultry have suggested that in layers of brown eggshells, stress can result in eggshell whitening following premature termination of shell pigment deposition and delayed oviposition (Mills *et al.* 1991, Nys *et al.* 1991). Different forms of stress (e.g., higher cage densities, increased handling, and louder noises) can induce a loss of pigmentation on the eggshell (Butcher & Miles 2011). Eggshell colouration in blue tits has

been correlated with female stress; females laying more spotted eggs were in lower body condition, had higher cellular concentrations of the stress protein HSP70 and tended to have lower total plasma immunoglobulin levels (Martínez-de la Puente *et al.* 2007). If eggshell pigment deposition is related to the body condition of the breeding female, a chronic stress response may suppress immune functions such as their antioxidant capacity, inducing an oxidative stress, and this may be reflected in eggshell colouration. However, the majority of correlative research has not, to date, quantified eggshell pigment concentration, assuming that eggshell colouration is a proxy for its pigment content. This assumption remains a contentious issue (Cassey *et al.* 2012a).

Manipulating experimentally stress hormones levels during breeding may help us to understand the relationship between environmental stress and eggshell pigmentation in birds more fully. In this study, I administered CORT by feeding adult female Japanese quails with CORT-injected mealworms (Marasco *et al.* 2012) over a 15-day period to investigate the effects of simulated chronic stress on female physiology and eggshell appearance. I measured female basal CORT concentration as well as two antioxidant agents, namely blood superoxide dismutase (SOD) and glutathione, and also eggshell reflectance, maculation, and pigment content. I predicted that CORT supplementation would mimic a chronic stress and increase oxidative stress, reduce body condition and lead to an increase in protoporphyrin deposition that females would endeavour to eliminate due to its pro-oxidant properties. In addition, I predicted a decrease in biliverdin investment into the eggshell as females would benefit from its antioxidant properties for their own antioxidant response. Considering the stability of eggshell reflectance (Duval *et al.* 2013), I expected an increase in eggshell maculation in stressed females following the increase in protoporphyrin deposition, but I did not predict *a priori* any modification in eggshell reflectance following the CORT supplementation (Duval *et al.* 2013).

3.2.2. Materials and methods

3.2.2.1. Study species and experimental procedure

The experiment was conducted at the University of St Andrews from November to December 2011 and all of the procedures were agreed by the Local Ethics Committee at the University of St Andrews. The experiment was conducted under the Animals (Scientific Procedures) Act 1986 (under PIL 30/8939 held by CD and PPL 60/4068 held by KAS).

Thirty wild-type female and nine male Japanese quails were purchased at 9 weeks of age from two different private suppliers ('Chinesepaintedquails' in Wigan and 'Wetheriggs zoo' in Penrith, UK). The birds were kept at 20-22°C under a light regime of 14L:10D. All birds were identified with a white numbered leg ring and were housed in single-sex groups in indoor aviaries (3 m^2 floor area) for 2 weeks to allow quarantine and habituation to housing conditions before the experiment commenced. During habituation, birds were fed *ad libitum* with a standard commercial diet (Layer pellets, ARGO Feeds).

Females were weighed (to the nearest gram) on an electronic balance before the start of any experimental manipulations, and on the last day of the supplementation, and the length of their right tarsus was measured (to the nearest 0.01 mm) with a digital calliper.

3.2.2.2. CORT dosage calculations

Due to high variability in females body mass (range: 197-360g, SD = 50.3g), I used two categories of females: small-bodied (< 300 g) or large-bodied (> 300 g) when I calculated a dose of CORT to administer to each experimental group. I based my calculations on the CORT physiological doses and plasma concentrations for Japanese quails and zebra finches (*Taeniopygia guttata*) (Marasco, unpublished data; Spencer & Verhulst 2007, Spencer *et al.* 2009) that I scaled by the mean body mass in each category of females (category 1 mean =

243 g, SD = 44 g, N = 14; category 2 mean = 339 g, SD = 23 g, N = 16), in order to mimic an increase in plasma CORT that was within a natural range.

The daily dose to administer to the stressed birds in category 1 was 0.088 mg of CORT (Sigma Aldrich, Poole, UK), dissolved in peanut oil (concentration of 1.76 mg/mL) via two 25 µl doses (at least 6 hours apart). In category 2, the daily dose to administer was 0.122 mg of CORT, dissolved in peanut oil (concentration of 2.44 mg/mL) via two 25 µ l doses (at least 6 hours apart).

3.2.2.3. Experimental design and CORT administration

Three weeks before the experimental manipulation commenced, females which were all laying were moved to individual cages (61 cm × 44.5 cm × 50.8 cm), fed *ad libitum* (Standard Layer Pellet, BOCM, UK) with a supplement of freshly dead mealworms every morning for 1 week (i.e. from day -7 to day -1), and were randomly assigned to one of two groups (Control: N = 11 and CORT-supplemented: N = 11). Individuals were in visual and acoustic contact with the other females at all times. Males were group housed in the same room as the females under *ad libitum* feeding conditions and were then randomly paired with one female of each treatment group (i.e. two females in total for each male) to provide fertile eggs. Sexual activity in males is highest within the first 5 minutes after presentation to a female, averaging approximately three copulations before reaching satiation (Schein *et al.* 1972)**.** Hence, a male was placed in a focal female's cage for 5 minutes per day before being removed and allowed a 1-hour resting period before presentation to a subsequent female. Pair encounters finished after the last day of CORT supplementation on day 14.

CORT treatment began on day 0 and ceased on day 14. Each female was supplemented with two mealworms each day, one in the morning between 9am and 12am GMT, and one in the afternoon between 1pm and 5pm GMT. Mealworms were injected on the day they were used,

between two dorsal segments. A 27-gauge needle (12 mm × 0.45 mm) was used to avoid any leakage of oil from the site of puncture. Mealworms fed to the control group were injected with two 25 µl doses of peanut oil only. Note that from the 30 initial females, 8 had to be removed from the experiment just before the start of supplementation (N = 8) because they were not laying or not laying every day. The final sample size was 22 females. Females were observed until they had ingested the mealworm which took only few seconds for each individual.

Blood samples were collected from the 22 females by puncture of the brachial vein and withdraw of up to 300 µl of blood in heparinized microcapillary tubes. All blood samples were collected within 3 minutes of bird capture (Romero & Reed 2005) and they were kept on ice and centrifuged as soon as possible at 3,500 rpm for 5 minutes at 4°C. Plasma was removed after centrifugation with a Hamilton syringe and both plasma and red blood cells (RBCs) were frozen at -80°C. Females were blood sampled once on day 0 to measure the plasma CORT baseline and their antioxidant capacity just before the start of the supplementation, and once at day 20, 3 days after the last day of CORT supplementation. In order to validate the CORT supplementation and confirm that the CORT treatment mimicked a repeated acute stressful event within a natural range of the species, one female from each group was randomly chosen each day between day 1 and day 13 and bled 10 minutes after a mealworm was consumed.

3.2.2.4. Radioimmunoassay

Plasma CORT was extracted in dichloromethane from each aliquot of 4 to 20 µl of plasma (mean ± SD = 18.82 ± 2.28 µl of plasma) (N = 22). Plasma CORT concentrations were measured by radioimmunoassay using anti-CORT antiserum code Esoterix Endocrinology USA B3-163 (1:100 dilution in assay buffer: 0.01M PBS pH = 7.4, 0.25% BSA; Esoterix,

Austin, TX) and [1, 2, 6, 7-^{3}H]-CORT label (Perkin Elmer, NET 399) as described in Spencer *et al.* (2009). The mean extraction efficiency was 48 ± 0.07%, the detection limit for this assay was 0.08 ng ml^{-1} and the assay was run with 50% binding at 1.85 ng ml^{-1}. All samples were run in duplicate in the same assay and the intra-assay coefficient of variation was 13%.

3.2.2.5. Antioxidant analysis

Antioxidants were measured using the spare RBCs available for each female after the radioimmunoassay was performed. SOD activity was measured in RBCs of 21 females using the Arbor Assays SOD Colorimetric Activity Kit (Arbor Assays, Inc., Ann Arbor, MI) following the vendor's instructions. Two randomly chosen samples were diluted by 100, 200, 400, and 800 in order to determine the best dilution which was 1:100. The mean intra-assay coefficient of variation was 7.4%, and the inter-assay coefficient of variation was 6.7%. Briefly, RBCs were lysed by adding ice cold deionized water to them and centrifuging at 3,500 rpm for 30 minutes at 4ºC to remove debris. RBCs were then diluted 1:100 in assay buffer prior to assaying. All standards and samples were assayed in duplicate. The reaction was initiated by adding 25 µl of xanthine oxidase to each well, and then the plate was incubated at room temperature for 20 minutes. The absorbance of each standard and sample was read at 450 nm using a microplate reader (ANTHOS 2010, AnthosLabtec Instrument). SOD activity was calculated from the equation of a four-parameter logistic curve obtained from the standard values. One unit of SOD is defined as the amount of enzyme causing half the maximum inhibition of the reduction of 1.5 mM nitro blue tetrazolium in the presence of riboflavin at 25ºC and at pH 7.8. All samples from a single individual were quantified in the same assay and treatment groups were equally represented within each assay (two plates).

Glutathione (GSH) concentration was also measured in lysed RBCs (see previous Methods) of 17 females using the Arbor Assays Glutathione Colorimetric Detection Kit (Arbor Assays,

Inc, Ann Arbor, MI) following the vendor's instructions. RBCs were deproteinized and diluted 1:40 in SSA (aqueous 5-sulfo-salicylic acid dehydrate) solution prior to assaying. Two random samples were preliminary diluted by 40, 80 and 160 times before being tested in order to determine the best dilution for the assay; which was 1:40. The mean intra-assay coefficient of variation was 3.01%, and the inter-assay coefficient of variation was 9.7%. Samples were either treated with 2-Vinylpyridine (2VP) to block free GSH by alkylation or left untreated, in order to measure oxidized Glutathione (GSSG). All standards and samples were assayed in duplicate and 25 µl of Colorimetric Detection reagent was added to each well. The reaction was initiated by adding 25 µl of the reaction mixture to each well, and then the plate was incubated at room temperature for 20 minutes. The absorbance of each standard and sample was read at 405 nm using a microplate reader (ANTHOS 2010, AnthosLabtec Instrument). GSH concentrations (µM) were calculated from the equation of a four-parameter logistic curve obtained from the standard values. GSSG concentrations (µM) of the samples were determined from the data obtained from the 2VP- treated samples read off a 2VP-treated standard curve. Free GSH concentrations (µM) were obtained by subtracting the GSSG measures obtained from the 2VP-treated standards and samples from non-treated standards and samples (i.e., the total GSH). All samples from a single individual were quantified in the same assay and treatment groups were equally represented within each assay (four plates).

3.2.2.6. Egg collection

The mass of all eggs was measured (to the nearest 0.01 g) using a Mettler AE163 electronic balance. Eggs that were collected on day -2 to day 0 were used to measure pre-treatment egg characteristics (i.e. mass, eggshell colouration and pigment concentration) and then on day 12 to day 14 (i.e. final supplementation day) to allow enough time for maternal CORT to be

transferred to the eggs (Hayward & Wingfield 2004) and to assess the effect of CORT supplementation on egg characteristics.

3.2.2.7. Analysis of eggshell maculation by digital photography

Photographs of all eggs on their laying day were taken in a windowless room using a light-box and two bulb lights positioned at equal distances from each side of the light box, as the only constant light source. Constant lighting and long exposures, rather than flash photography, were used to protect the eggshell pigments. A Nikon D90 camera with a 105mm lens was used and was activated using a remote control. For the photographs, each egg was placed on a stand against a black card as a photographic standard background and next to a colour chart (Macbeth Mini Color Checker) and white graph-paper inside the light-box. Six eggs per female were photographed, and the picture of each egg was taken including a label identifying the date and the female. Each 90° rotation of each egg was photographed providing four images in total. The camera was focused on one side (i.e., quarter) of the egg and for the three subsequent images the focus was maintained.

All digital images of eggs were saved in standardized RAW format that is beneficial for colour analyses. The characterization of the camera's spectral sensitivities and the calibration process is described in Lovell *et al.* (2005). The linear RAW images were converted to XYZ (CIE XYZ colour-space coordinates (CIE, 1986)), and subsequent conversion from XYZ to CIELAB space was implemented using Matlab image processing toolbox (2008a, The MathWorks, Natick, MA, USA). Variations in the illumination of the photographed scene were controlled-for by normalizing the luminance values (the L channel) to 0 for the darkest area of the black card and to 60 for the white graph-paper. The area of the photograph occupied by the egg was identified, and for pixels in this area a histogram of the spread of luminance values was plotted, giving a bi-modal distribution of luminance values

corresponding to maculated (darker values) and background (lighter) regions. I manually selected the cut-off between the foreground and background areas for each photograph. Finally, the degree of maculation (spot coverage) present in an egg photograph was computed as the percentage of the foreground and background regions. The darker regions were assumed to be the foreground 'spots' and the spot percentage was calculated as the number of pixels in the foreground region divided by the sum of total pixels in the background and in the foreground regions (mean: 67.1%; range: 37.3-89.3%).

All eggs were then carefully opened along the longitudinal axis using dissecting scissors. The eggshells were collected, washed with distilled water and stored in a dark box to dry at room temperature and to avoid direct exposure to light that causes pigment degradation (Cassey *et al.* 2011a).

3.2.2.8. Measurement of eggshell colouration by spectrophotometry

3.2.2.8.1. Shape model

On the three days following the last day of CORT supplementation (days 15, 16 and 17), eggshell reflectance was measured between 300 and 700 nm in the laboratory using an Ocean Optics USB4000 Miniature Fibre Optic spectrophotometer with a DH-2000-FHS deuterium-halogen light source (Ocean Optics, Eerbek, The Netherlands). A 90-degree probe with a black plastic extension was used to ensure stability for measurement and to maintain a consistent angle and distance between the eggshell and the measuring fibre optics. Two spots were randomly chosen from each half of an egg, one in each area of the half (top and bottom), thereby totalling four spots per egg. One reflectance measurement was performed at each of these four spots. For eggshell background, two measures were taken on each eggshell half, one at the top and one at the bottom (i.e., four background reflectance measures per

egg). Spectra were expressed relative to a white Ocean Optics WS-1 and a black standard that were measured before each session of spectrophotometric measurement.

As described in Duval *et al.* (2013) (Chapter Two, Section 2.3.2.4), brightness, UV chroma, blue-green chroma and red chroma were extracted from these spectral measurements as spectral shape descriptors using the software Avicol (Gomez 2006, Doutrelant *et al.* 2008). I compared between and within-clutch variation in spot and background reflectance of the three eggs per female (total N = 62) at the beginning and at the end (total N = 65) of the experiment by calculating intra-class correlation coefficient (r) repeatability estimates (Lessells & Boag 1987) and found that spot and background colour variables were highly repeatable within a female at the start ($0.71 < \text{all } rs < 0.90$, all $Ps < 0.05$) and at the end of the experiment ($0.64 < \text{all } rs < 0.82$, all $Ps < 0.05$) for each colour variable.

3.2.2.8.2. Vision model

To account for the avian visual system, I used the photoreceptor spectral sensitivities and relative densities data available for the domestic chicken to compute both chromatic (ΔS; colour) and achromatic (ΔQ; brightness and forms) contrasts (Duval *et al.* 2013) using the software Avicol (Gomez 2006) (see Chapter Two section 2.3.2.4 for further details).

3.2.2.9. Eggshell pigment determination and quantification

The pigment content of the whole eggshell was analysed for each female. These were identified and quantified using HPLC (Mikšík *et al.* 1996) following (Cassey *et al.* 2012a, Duval *et al.* 2013) (see Chapter Two section 2.3.2.5 for a detailed protocol).

3.2.2.10. Data analysis

As previously reported by Duval *et al.* (2013) and in Chapter Two, eggshell reflectance did not vary between eggshell areas (i.e. top vs. bottom). Therefore, the mean spot and background reflectance values per egg were calculated for all four colour variables (i.e. brightness, UV chroma, blue-green chroma and red chroma). All subsequent analysis was conducted on means data across the whole egg.

Body condition of each female was calculated as the residual from a linear regression of body mass on tarsus length. Repeated-measures ANOVAs (SPSS Statistics 19.0.0) were performed to test whether CORT supplementation influenced female characteristics (i.e. body condition, plasma CORT baseline, SOD activity and GSH concentration). Time of bleeding (initial and after the CORT supplementation period) was the within-subject factor, and the treatment group was the between-subjects factor. I tested the effect of CORT supplementation on the 10 minutes peak of CORT concentration in both groups, using an independent samples Mann-Whitney test.

After checking for normality of residuals, I used Generalized Linear Mixed Models (GLMMs) fitted with a linear distribution to test for the effect of CORT supplementation on egg mass, eggshell maculation and eggshell reflectance, which were added as dependent variables. Time (before and after the CORT supplementation period), (CORT and Control) and the interaction term (time × group) were included as fixed factors, and egg ID nested in female ID (i.e. female ID (egg ID)) was included as a random factor to account for multiple eggs from the same female.

Average egg detectability and discriminability were examined. I tested whether the average differences in the mean egg colour within and between females were perceived by a chicken visual model by comparing the within and between-female contrasts using one-sample *t*-tests

(all JNDs were normally distributed). Paired *t*-tests were performed to test whether the within and between-female contrasts were significantly different for each type of contrast computed. A Kruskal-Wallis analysis was performed to test whether the chromatic and achromatic contrasts between the eggs laid before and after CORT supplementation were different between the experimental treatment groups. Using the same avian visual model, I computed spot/background contrast before and after CORT supplementation, and repeated-measures ANOVA was performed to test whether the treatment, time and the time × treatment term had statistically significant effects on this specific contrast.

As biliverdin concentrations were significantly different between control and CORT-supplemented females at the start of the experiment ($H = 8.68$, $P = 0.01$, $N = 61$), I used univariate general linear mixed model (GLMMs) to test for the difference in biliverdin and protoporphyrin concentrations, between the three eggs collected before the start of CORT supplementation, and between the three eggs collected after the CORT supplementation, with pigment concentration as dependent variables, egg number (i.e.,1, 2 or 3) as the fixed factor and female as a random factor. As egg number had no significant effect on pigment concentration before and after the CORT supplementation ($0.17 <$ all F-values < 1.85, all P-values > 0.05), I calculated the mean concentration for each pigment per female before and after the treatment. I performed a Pearson correlation between the mean concentration of biliverdin and the mean concentration of protoporphyrin for all females to test for the degree of interrelation between the two pigments.

I tested for the effect of CORT supplementation on the change (Δ) in eggshell pigment concentration and protoporphyrin proportion (protoporphyrin/total pigment) over the supplementation period by calculating the difference in biliverdin and protoporphyrin concentrations and protoporphyrin proportion between the pre- and post-supplementation eggs. I used a univariate GLMM with the difference in biliverdin and protoporphyrin

proportions as dependent variables, group as a fixed factor and pigment concentration and proportions at the start of the experiment as covariates. All statistical analyses were performed in SPSS Statistics 19.0.0.

3.2.3. Results

3.2.3.1. Effect of CORT supplementation on females

Female body condition was normally distributed and not different between groups before CORT supplementation after checking for variances equality (t-test: $t = -1.04$, $P = 0.31$, $N = 22$), and did not change significantly with the treatment (repeated-measures ANOVA: time: $F_{1,21} < 0.001$, $P = 1.00$; group: $F_{1,21} = 0.72$, $P = 0.40$; time × group: $F_{1,21} = 0.42$, $P = 0.52$, $N = 22$).

Female basal plasma CORT, RBC's SOD activity, total GSH concentration or GSSG concentration were normally distributed after checking for variances equality, and were not different between groups before CORT supplementation (t-test: $t = -1.20 <$ all t-values < -0.25, $0.25 <$ all P-values < 0.80, N (SOD) = 21, N (GSH, GSSG) = 17). In addition, CORT supplementation did not have any significant effect on these female physiological parameters (see Table 3.1).

Table 3.1. Effect of CORT supplementation (see text for details) on female physiological parameters of Japanese quail (GLMM). All females were exposed to ad libitum food with peanut oil supplementation or CORT supplementation. Time corresponds to the blood sampling performed before and after the CORT supplementation, and group corresponds to CORT-fed or control birds.

Trait	Factor	dfs	F	P
Basal CORT	Time	1,21	0.03	0.96
	Group	1,21	0.51	0.48
	Time × group	1,21	0.16	0.69
SOD activity	Time	1,20	1.91	0.18
	Group	1,20	0.94	0.34
	Time × group	1,20	0.49	0.49
Total GSH	Time	1,16	4.44	0.07
	Group	1,16	1.76	0.20
	Time × group	1,16	0.33	0.57
GSSG	Time	1,16	2.36	0.15
	Group	1,16	0.63	0.44
	Time × group	1,16	0.62	0.44

There was a significant effect of CORT supplementation on the plasma CORT concentration after 10 minutes of mealworm ingestion, with a peak significantly higher in CORT-fed females compared with controls (U_{22} = 88, P = 0.02, N = 22). In addition, the 10 minutes peak of CORT concentrations in CORT-fed females blood (range: 1.75–52.31; mean ± SD = 16.56 ± 14.65 ng/ml) was within a physiological range and comparable with stress-induced concentrations in similar-aged birds (range: 1.43–62.16; mean ± SD = 19.52 ± 16.50 ng/ml; KAS unpublished data). The basal CORT concentrations of controls (range: 2.18–12.14; mean ± SD = 4.82 ± 3.63 ng/ml) were also comparable to similar-aged birds (range: 0.87–26.47; mean ± SD = 8.16 ± 7.91 ng/ml) (KAS unpublished data).

3.2.3.2. Effect of CORT supplementation on eggs

I found no significant effect of CORT supplementation on egg mass (GLMM: time: $F_{1,123}$ = 0.05, P= 0.82; group: $F_{1,123}$= 0.43, P = 0.51; time × group: $F_{1,123}$ = 0.04, P = 0.84). There was no effect of CORT supplementation on eggshell maculation (GLMM: time: $F_{1,95}$ = 2.15, P = 0.15; group: $F_{1,95}$ = 1.40, P = 0.24; time × group: $F_{1,95}$ = 1.43, P = 0.23). Eggshell colour variables were also unaffected by CORT supplementation, except for spot brightness, which significantly increased in CORT-supplemented females (Table 3.2, Fig. 3.1).

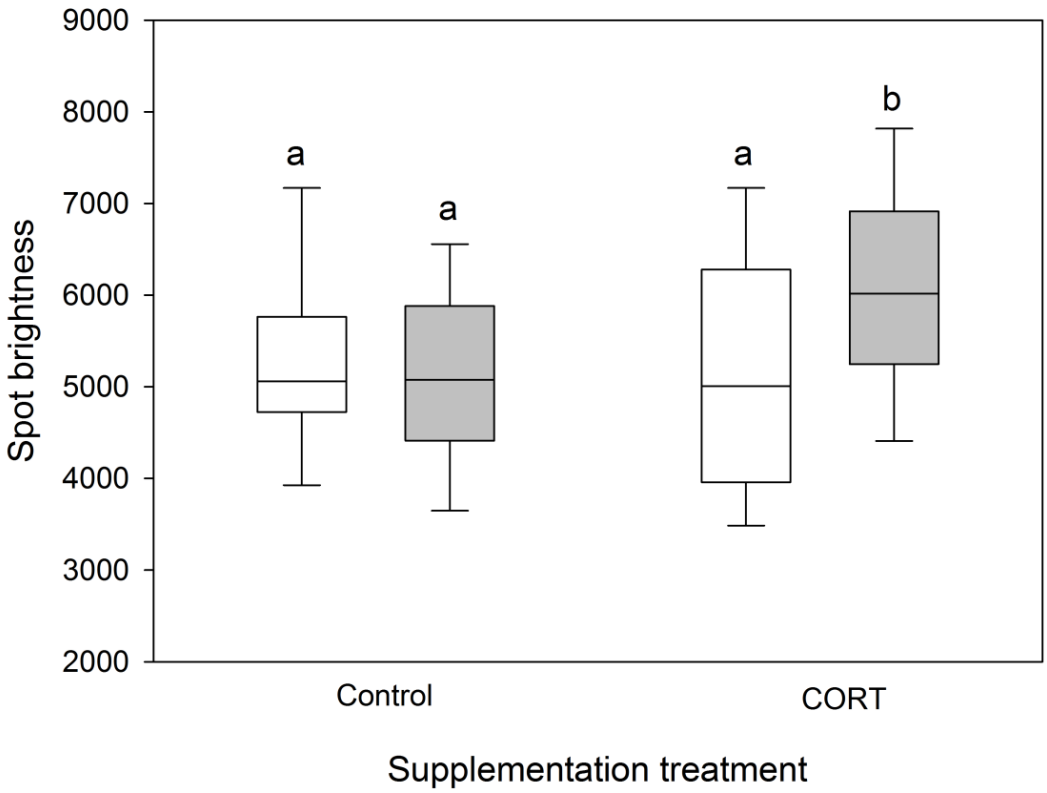

Figure 3.1. Effect of CORT supplementation on eggshell spot brightness (mean ± 1 SE; N = 123). Female Japanese quails were either fed with peanut oil alone (controls) or with CORT within peanut oil (see text for details). Open bars and grey bars represent pre-treatment and post-treatment effects, respectively. The boundary of the box closest to zero indicates the 25th percentile, the line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers indicate standard errors. Different lowercase letters reflect statistically significant differences.

Table 3.2. Effect of CORT supplementation (see text for details) on eggshell colour parameters (descriptive model) of Japanese quail (GLMM, df = 1, N = 123). The 22 female Japanese quails were exposed to ad libitum food with peanut oil supplementation or CORT supplementation. Time corresponds to the measurements performed before and after the CORT supplementation, and group corresponds to CORT-fed or control birds. Bold text indicates statistical significance.

Parameter	Factor	F	P
Spot reflectance			
Brightness	Time	3.61	0.06
	Group	3.66	0.058
	Time × group	**7.33**	**< 0.01**
UV Chroma	Time	3.25	0.07
	Group	0.26	0.61
	Time × group	0.23	0.63
Blue-Green Chroma	Time	**8.06**	**0.01**
	Group	0.10	0.75
	Time × group	0.07	0.79
Red Chroma	Time	**4.99**	**0.03**
	Group	0.21	0.64
	Time × group	0.34	0.56
Background reflectance			
Brightness	Time	0.24	0.63
	Group	0.16	0.69
	Time × group	0.48	0.49
UV Chroma	Time	**7.2**	**< 0.01**
	Group	1.83	0.20
	Time × group	0.08	0.78
Blue-Green Chroma	Time	0.007	0.93
	Group	0.16	0.69
	Time × group	0.13	0.72
Red Chroma	Time	**8.24**	**< 0.01**
	Group	1.99	0.16
	Time × group	0.07	0.79

Moreover, there was a significant effect of time on some characteristics of eggshell reflectance (Table 3.2) with a decrease in spot and background red chroma, an increase in their UV chroma, and an increase in spot blue-green chroma (Table 3.2).

My vision model suggested that some of the colour variation, measured with reflectance spectrophotometry, would be more detectable than others by an avian visual system. For each female, the average contrast for eggshell colour was greater when comparing eggs between-females than within-females for background (paired *t*-test: within vs. between ΔS: t_{21}= -11.2, $P < 0.001$; within vs. between ΔQ: t_{21}= -9.8, $P < 0.001$) and for spots (paired t-test: within vs. between ΔS: t_{22} = -3.8, P = 0.001; within vs. between ΔQ: t_{22}= -2.1, P = 0.05) contrasts. Background contrasts were greater than 1 JND compared to spot contrasts that were lower than 1 JND, suggesting that background colour differences would be more detectable by an avian visual model than spot colour differences. However, I found no significant effect of CORT supplementation on the perceived eggshell spot and background chromatic and achromatic contrasts (Kruskal-Wallis: spot ΔS: H = 0.28, P = 0.60; spot ΔQ: H = 0.74, P = 0.39; background ΔS: H = 0.06, P = 0.81; background ΔQ: H = 0.06, P = 0.81). There was no significant effect of female treatment on eggshell spot/background chromatic (repeated-measures ANOVA: time: F = 0.99, P = 0.33; group: F = 0.13, P = 0.72; time × group: F = 1.08, P = 0.31) and achromatic (time: F = 0.91, P = 0.35; group: F = 0.16, P = 0.70; time × group: F = 0.75, P = 0.39) contrasts.

Pigment analyses revealed that eggshells contained both protoporphyrin IX (113.89 µg g^{-1} eggshell, SD = 41) and biliverdin (104.46 µg g^{-1} eggshell, SD = 48.16), both quantities being positively correlated (Pearson correlation: r = 0.68, P< 0.001, N = 44). This result was supported by the use of a bootstrap simulation in R 2.14.0 (R Development Core Team 2011), that demonstrated after 1000 bootstraps of size N = 22 (total number of females) chosen with replacement, that 80% of the simulated coefficients were greater than r = 0.7.

Controlling for the initial pigment concentrations, I did not find any significant effect of CORT supplementation on the mean change in pigment concentrations and proportions (univariate GLMs: Δbiliverdin: $F_{1,21} = 2.31$, P = 0.14, observed power = 30%; Δprotoporphyrin: $F_{1,21} = 3.15$, P = 0.10, observed power = 40%; Δprotoporphyrin proportion: $F_{1,21} = 0.06$, P = 0.81, observed power = 56%).

3.2.4. Discussion

I experimentally exposed female Japanese quails to physiological doses of CORT (Spencer *et al.* 2009) and found that elevated stress hormones did not have any effect on their basal CORT concentration, antioxidant capacity or eggshell pigment content. However, contrary to my predictions, and despite the consistency of eggshell reflectance in the species (Duval *et al.* 2013, Chapter Two), I found that stressed birds laid eggs with significantly brighter spots but with maculation that remained constant compared with control birds. This is the first study that experimentally investigated the relationship between eggshell pigmentation and female stress exposure in an ecological context.

Contrary to my predictions, I did not find any effect of CORT supplementation on the concentration of eggshell pigments deposited. Interestingly, Duval *et al.* (2013) demonstrated that female Japanese quail in lower body condition deposited more protoporphyrin, but less biliverdin, into their eggshells under food restriction. In the present study, I found no significant effect of CORT supplementation on either female body condition or eggshell characteristics. I did, however, observe a peak in plasma CORT in CORT-fed birds after 10 minutes of oral dosing (mean ± SD: 16.56 ± 14.65 ng/ml). This suggests that the CORT treatment mimicked a repeated acute stressful event in the experimental group within a natural range of the species. However, the increase in plasma CORT concentration did not induce any change in eggshell pigment concentration. One explanation could be that the dose

I administered was not sufficiently high to induce a change in female physiology and body condition. Indeed, other studies have found contradictory results on the effect of CORT administration on food intake or variation in body mass of individuals (Silverin 1986, Malheiros *et al.* 2003, Lin *et al.* 2004). Similar to our findings, CORT did not influence body mass during the period of supplementation, in common kestrels (Costantini 2008).

CORT is one factor that can influence red-ox balance in birds (Costantini *et al.* 2008). In chickens chronic CORT administration is associated with increased plasma lipid peroxidation, plasma antioxidant activity and uric acid, but not with SOD activity (Lin *et al.* 2004). Despite the lack of experimental evidence for the roles of biliverdin and protoporphyrin in avian red-ox balance, it has been proposed that both pigments are related to female oxidative stress due to the pro-oxidant properties of protoporphyrin and the antioxidant properties of biliverdin (Moreno & Osorno, 2003). Some correlative and experimental studies of blue-green eggs have found ambiguous results regarding the relationship between eggshell colouration and female antioxidant capacities. For example, there was no effect of antioxidant (carotenoid) supplementation on eggshell colouration of Araucana chickens (Dearborn *et al.* 2012). Cassey *et al.* (2008a) found no evidence for a signalling function for blue-green eggshell colouration in the context of maternal investment (yolk carotenoids) in thrushes. However, female gray catbirds which laid eggs with higher blue-green chroma also showed higher total antioxidant capacity (Hanley *et al.* 2008). In European pied flycatchers, females which laid more colourful eggs showed lower plasma total antioxidant levels (Trolox equivalent antioxidant capacity) after an experimentally increased reproductive effort through nest removal. This would suggest that eggshell pigmentation is a costly process for the antioxidant system, and that females face a trade-off in investment between the two traits (Morales *et al.* 2008). Thus, the relationship between

eggshell pigment deposition and female oxidative stress remains unclear, particularly in brown-spotted eggs.

I predicted that CORT supplementation would increase female oxidative stress resulting in a decrease in eggshell biliverdin investment and an increase in protoporphyrin deposition. However, I did not find any significant effect of CORT on antioxidant capacities which might explain the lack of change in eggshell pigment concentration, given the properties of biliverdin and protoporphyrin. This may suggest that CORT supplementation did not disturb the oxidative stress balance in my study birds. However, I did not directly measure parameters reflecting the production of free radicals and the degree of oxidative damage and plasma antioxidant capacity. Thus, I must be conservative in my conclusions about female oxidative stress (Costantini & Verhulst 2009).

In my study, eggshell colour analysis partly contradicted the findings of Duval *et al.* (2013) and Chapter Two that showed a high constancy of eggshell reflectance despite variations in eggshell pigment content in Japanese quail. The observed decrease in eggshell spot and background red chroma between the start and the end of the laying sequence, combined with the increase in spot blue-green chroma in both groups, suggests a potential reallocation of pigments throughout the experiment. Indeed, protoporphyrin could have been re-distributed across the eggshell and in particular away from spots, resulting in a more even distribution of brown colouration manifested as a greater relative 'blueness' due to reflectance of the pigment biliverdin. Surprisingly, even though there were no significant changes in total eggshell pigment concentration, I found that CORT-supplemented birds laid eggshells with brighter spots than the controls. As brightness is defined as the total light reflected by eggshell spots or background in my study (Montgomery 2006), the observed increase in eggshell spot brightness could be attributed to a change in the shape of the spectra that I did not measure. Indeed, I chose to measure blue-green chroma (400–575 nm) and red chroma

(595–655 nm) as they correspond to the maximum reflectance generated by the pigments biliverdin (Ding & Xu 2002) and protoporphyrin (Scalise & Durantini 2004). However, reflectance changes in other portions of the spectrum such as the green-yellow region (570–610 nm) could have also occurred but remained undetected by my shape model analysis. Alternately, this may support the hypothesis of potential reallocation of protoporphyrin across the eggshell as the change of brightness between the start and the end of the laying sequence was stronger in stressed females, and could be associated with a change in eggshell structure itself due to CORT supplementation rather than to only pigment deposition, but this remains speculative. Measuring the local distribution of pigment concentration across the eggshell remains untested, to date, and would allow important insights into the process of pigment deposition under variations in environmental conditions.

I did not find any significant effect of CORT supplementation on female body condition or maternal investment (i.e. in egg mass), but I cannot rule out that my treatments modified the assimilation and metabolism of certain nutrients such as calcium, an element that is fundamental to the integrity of the eggshell strength as suggested by the structural function hypothesis (Gosler *et al.* 2005) to explain eggshell pigmentation. Thus, it is possible that CORT treatment may have affected some aspects of the eggshell matrix structure that are not directly related to eggshell pigments (Mills *et al.* 1991, Nys *et al.* 1991, Butcher & Miles 2011) but that might change eggshell gloss (Maurer *et al.* 2011b) and thus explaining why I found a change in eggshell brightness but not in eggshell red or blue-green chroma due to the treatment. Nevertheless, neither chromatic nor achromatic visual contrasts were influenced by CORT supplementation, suggesting that the change in eggshell reflectance due to the treatment would be undetected by an avian visual model.

In conclusion combined with previous findings revealing how eggshell pigment content is a condition-dependent trait in Japanese quail (e.g. Duval *et al.* 2013; Chapter Two), the present

study supports the idea that eggshell reflectance in spotted eggs varies over the laying sequence, and in particular that eggshell spot reflectance is a key factor affected by females exposure to stress during reproduction, even if the changes were not detected by a photoreceptor noise-limited colour opponent model of avian visual perception in my study. It is conceivable that stress may potentially impair egg crypsis in a species which maximises choices of laying substrate in order to maximise camouflage such as the Japanese quail (Lovell *et al.* 2013), but this remains speculative.

3.3. Stress during early life affects eggshell pigmentation strategy under stress during reproduction

3.3.1. Introduction

Eggshell colouration is thought to have many adaptive roles (reviewed in Reynolds *et al.* 2009) and the presence of biliverdin and protoporphyrin, the two main eggshell pigments, might be related to the body condition of the breeding female (Duval *et al.* 2013; Section 2.3, Chapter Two). Their investment might potentially benefit the embryo via protection against harmful effects of solar radiation or enhancement of its development by photo-acceleration (reviewed in Maurer *et al.* 2011a). Few experimental studies have manipulated female body condition to investigate the effect on eggshell pigmentation (Moreno *et al.* 2006, Morales *et al.* 2011, Dearborn *et al.* 2012, Duval *et al.* 2013, Hargitai *et al.* 2013) but have found mixed results and need further investigation. Duval *et al.* (2013) found that food-restricted female Japanese quails that were in lower body condition, elevated the deposition of protoporphyrin compared to biliverdin into their eggshells (Section 2.3, Chapter Two) and that control females (on *ad libitum* food) were able to adjust the degree of maculation on their eggshells (section 2.4) and to maintain reflectance characteristics within a natural range of variation (section 2.3). Thus, eggshell appearance may be strongly influenced by changes in female body condition during egg formation.

One major factor that can alter phenotype over the short and long-term is the stress experienced at different stages of individual's life. As already discussed above (section 3.2.1), many environmental factors can act as stressors, resulting in an increase in individual CORT. Chronic stress has been shown to affect individual physiology such as antioxidant defences (Costantini *et al.* 2011). When stress arises at a particularly sensitive period such as

reproduction, mothers can alter their maternal care but also transfer GCs via the egg yolk (Hayward & Wingfield 2004, Love *et al.* 2008, Almasi *et al.* 2012) through maternal effects (Mousseau & Fox 1998, Räsänen & Kruuk 2007). Any change in mothers' environmental conditions during egg-laying can affect the developing embryo, in particular its hatching mass, size and growth (Hayward & Wingfield 2004, Love & Williams 2008b, reviewed in Sheriff & Love 2013). For instance, an experimental injection of CORT into eggs directly can induce embryonic mortality, impaired embryonic development, altered embryonic vocalizations, reduced hatching mass and chick growth rates, and reduced begging display (Eriksen *et al.* 2003, Heiblum *et al.* 2001, Mashaly 1991, Rubolini *et al.* 2005). Yet, negative effects of stress on hatching mass and chicks growth rate can be compensated by a period of catch-up growth, which may potentially have its own costs for the growing individual (reviewed in Metcalfe & Monaghan 2001). Long lasting effects have also been associated with pre-natal stress, such as modifications of the HPA-axis stress response (Hayward & Wingfield 2004), altered reproductive organs in males leading to negative consequences on their fitness (Satterlee *et al.* 2007), or behavioural changes such as anxiety, fearfulness and an inability to compete for food (Janczak *et al.* 2007, Davis *et al.* 2008, Marasco *et al.* 2012, Zimmer *et al.* 2013).

Post-natal stress, such as changes in parental behaviour, nutritional state, sibling interactions, and parasite loads, are factors that increase the exposure of a developing organism to GCs, and can affect offspring phenotype in the short-term (Schoech *et al.* 2011). For instance, many studies that employ food restriction, brood size manipulation or direct administration of exogenous CORT have shown associated changes in chick growth rate, begging behaviour, and immune response (Saino *et al.* 2003, Kitaysky *et al.* 2006, Loiseau *et al.* 2008, Sears & Hatch 2008, Honarmand *et al.* 2010). In the long-term, early life exposure to GCs can impact on song characteristics (Spencer *et al.* 2003), neophobia (Spencer & Verhulst 2007),

cognitive abilities (Kitaysky *et al.* 2006, Spencer & Verhulst 2007, Schoech *et al.* 2009) and induce permanent changes in the HPA axis (Spencer *et al.* 2009, Marasco *et al.* 2012).

Thus, both pre- and post-natal stress may induce negative enduring effects on an individual's phenotype and physiology that could alter its fitness (Metcalfe & Monaghan 2001, Tschirren *et al.* 2009). However, an alternative hypothesis has suggested that early life stress may be beneficial and adaptive for the offspring (Nesse & Young 2000). Indeed, maternal stress hormones may shape offspring's phenotype to programme it to better cope in a hostile post-natal environment that matches its mother's one (Bateson *et al.* 2004, Gluckman & Hanson 2004). A mismatch between the maternal and post-natal environmental conditions may explain the long-term negative effects of pre-natal stress on offspring phenotype (Monaghan 2008). In addition, both pre- and post-natal stress may act in an additive or interactive ways to shape adult phenotypes to modulate the long-term effects of early-life stress on individual physiology such as their redox balance (Marasco *et al.* 2013) or behaviour such as risk-taking (Zimmer *et al.* 2013).

As eggshell appearance is an extended phenotypic trait intrinsically linked to a female's body condition, it might be influenced by both her developmental history and the environmental conditions under which she breeds (Love *et al.* 2008, Cohen *et al.* 2012). Whilst many studies have shown pleiotropic effects of maternally derived stress (MDS) on egg compounds and offspring phenotype (Henriksen *et al.* 2011, Sheriff & Love 2013), much less is known about the effects of stress experienced in early life on eggshell characteristics, and in particular eggshell pigmentation. Interestingly, Martínez-de la Puente *et al.* (2007) found that in blue tits, females laying eggs with more spots showed a lower body condition, and had higher cellular concentrations of HSP70, and marginally lower total immunoglobulin blood levels than those laying less spotted eggs. My previous work (section 3.2, Duval *et al. in press*) showed that female exposure to stress during reproduction induced a change in eggshell

appearance. Nevertheless, not only might the stress experienced during reproduction influence female physiology, but also the stress that they experienced early in life. Yet, no study has investigated whether eggshell patterning could reflect any stress experienced by females during their development.

In the present study, I tested the effect of early life – both pre and post-natal development on eggshell characteristics (i.e. maculation, reflectance and pigments concentrations) and how these interact with adult breeding conditions to further influence these traits. I studied Japanese quail eggs laid by females that had been exposed to pre- and post-natal stress or to no such stressor, and that were exposed to stress during reproduction using random food-removal (see detailed methods in Zimmer *et al.* 2013). If current stress affects eggshell spot reflectance only (see section 3.2, Duval *et al. in press*), I predicted that females breeding under stress would lay brighter eggshells but maintain their eggshell pigment concentrations and maculation. In addition, if the adaptive view of developmental stress is correct then females that experienced both pre- and post-natal stress should be better prepared for stressful environments during breeding, thus they should be less affected by stress during reproduction and should maintain eggshell pigment concentrations, colour and maculation.

3.3.2. Materials and methods

3.3.2.1. Pre- and post-natal stress

Unrelated Japanese quail eggs (N = 76) were randomly assigned to one of two groups: CORT injection (CORT: N = 38) or Control (Ctrl: N = 38). Fertile eggs in the CORT group were then injected with 10 µl of a sterile solution of CORT that elevated endogenous CORT concentration within the yolk by a factor of 1.8 above controls within a natural range in the breeding population (Ctrl: 8.7 ± 5. (SD) ng ml^{-1}; CORT: 17.1 ± 8.3 (SD) ng ml^{-1}; Boogert *et al.* 2012). Controls were injected with 25 µl of sterile peanut oil alone. After 14 days, all eggs

were transferred to a hatcher maintained at 37°C until hatching (after 18 days). The 59 chicks that hatched (preCORT: N = 31; preCtrl: N = 28) were allocated to a post-natal food treatment at 4 days of age: either food removal for 25% of daylight hours (i.e. 3.5 hours) on a random daily schedule for 15 days (postFood-: N = 28), which would increase stress hormones in birds (Cuthill *et al.* 2000, Buchanan *et al.* 2003), or *ad libitum* food at all times (postCtrl: N = 31) (see Table 3.3 for a full description of the experimental groups). The experiment was repeated and conducted in two batches (batch 1 = 31 chicks; batch 2 = 28 chicks). Birds were fed with a standard commercial diet (Layer pellets, ARGO Feeds).

Table 3.3. Matrix of the combinations between pre- and post-natal treatments and sample sizes of the four experimental groups of Japanese quails (male and female) created.

Pre-natal condition	Post-natal condition	Groups
Control = preCtrl	Control = postCtrl	preCtrl/postCtrl (N = 15)
		preCtrl/postFood- (N = 13)
CORT-injected = preCORT	Food removal = postFood-	preCORT/postCtrl (N = 16)
		preCORT/postFood- (N = 15)

3.3.2.2. Adult stress during reproduction

Food removal from adult females began between 12 and 16 weeks of age. Females (N = 30) from the four previous experimental groups (group 1: preCtrl/postCtrl, N = 6; group 2: preCtrl/postFood-, N = 6; group 3: preCORT/postCtrl, N = 12 and group 4: preCORT/postFood-, N = 6) were allocated to one of two further groups (housed in two identical rooms): unpredictable food availability (AdFood-) or ad libitum food (AdCtrl) (with Ad referring to Adult). Females in the unpredictable group experienced removal of all food items for 25% of daylight hours (3.5 hours) on a random schedule between the hours of 8AM

and 8PM (GMT). Controls were provided with *ad libitum* food throughout this breeding period. At the same time, one male was allocated to four females and put into a cage with a female for 20 minutes a day in order to obtain fertile eggs. Each female laid two clutches (with a break of 2 months) under the two different conditions (AdCtrl and AdFood-), which allowed repeated-measures within individuals, and the order of each treatment was randomized to control for any effect of the first clutch treatment on the response for the second clutch.

3.3.2.3. Egg collection

Three eggs per female were randomly collected after 10 days of mating which is the minimum time needed to obtain fertile eggs (Adkins-Regan 1995), and this was repeated for the second clutch. Eggs were collected, washed with distilled water and stored in a dark box to dry at room temperature and to avoid direct exposure to light that could cause pigment degradation (Cassey *et al.* 2011a).

3.3.2.4. Analysis of eggshell appearance and pigments concentrations

Photographs were taken using the protocol described in the previous section of this chapter (section 3.2.2.6, Duval *et al. in press*). The degree of maculation (spot coverage) present in an egg photograph was estimated as percentage of the foreground and background regions (average 32.8 %; range 5.5-54.1 %). Eggshell reflectance was then measured following the protocol described previously in section 3.2.2.8.1 (see Chapter Two for further details). Finally, HPLC chromatography (Mikšík *et al.* 1996) was used to identify and quantify the whole content of eggshell pigments and was analysed in all the eggshells collected for each female, following the protocol described previously in section 2.3.2.4 (see Chapter Two for further details).

3.3.2.5. Statistical analyses

I tested the effect of stress during reproduction on female body mass change between the start and the end of each clutch by using a GLMM. Female body mass change (end – start of the clutch) was added as a dependent variable and adult treatment (AdCtrl or AdFood-) was added as a repeated factor. Female was added as a random factor.

I tested the effect of pre-natal, post-natal stress, and adult stress on eggshell maculation (e.g. spot coverage), pigment concentration and reflectance using GLMMs. Eggshell maculation, pigment concentrations and reflectance were specified as dependent variables. Pre-natal treatment (preCtrl or preCORT) and post-natal treatment (postCtrl or postFood-) were added as fixed factors. Adult treatment (AdCtrl or AdFood-) was added as a repeated factor, and the interaction terms were added as fixed factors in the model. Female and egg number were included as random factors.

Finally, a Pearson's correlation was used to test the relationship between biliverdin and protoporphyrin concentrations.

GLMMs were fitted using the Mixed PROC in SAS (SAS Institute Corporation), after checking for normality of residuals. The REML (Residual Maximum Likelihood) was used as the estimation method. Tukey-Kramer multiple comparison adjustment was applied to obtain corrected P values. An alpha threshold of 0.05 was used and data are presented as means ± SEMs.

3.3.3. Results

3.3.3.1. Effect of breeding stress on female body mass

During reproduction, females under *ad libitum* food gained body mass (mean body mass change ± 1 SE: 12.67 ± 3.83 g), but slightly lost it when food was removed (mean body mass change ± 1 SE: -0.55 ± 4.83 g) (GLMM: $F_{1, 36.8} = 5.65$, P = 0.02).

3.3.3.2. Effect of pre-natal stress on adult reproduction

There was a significant effect of the interaction between pre-natal stress and adult food removal on eggshell maculation. Although multiple comparisons did not show significant differences between treatment groups, pre-natal control females tended to lay more maculated eggshells under adult food removal condition during breeding than under control condition. In contrast, pre-natally stressed females maintained eggshell maculation across the two clutches (pre-natal: $F_{1,26} = 0.89$, P = 0.35; post-natal: $F_{1,26} = 0.07$, P = 0.80; adult: $F_{1,117} = 0.10$, P = 0.75; pre-natal × adult: $F_{1,117} = 5.10$, P = 0.03, Fig. 3.2).

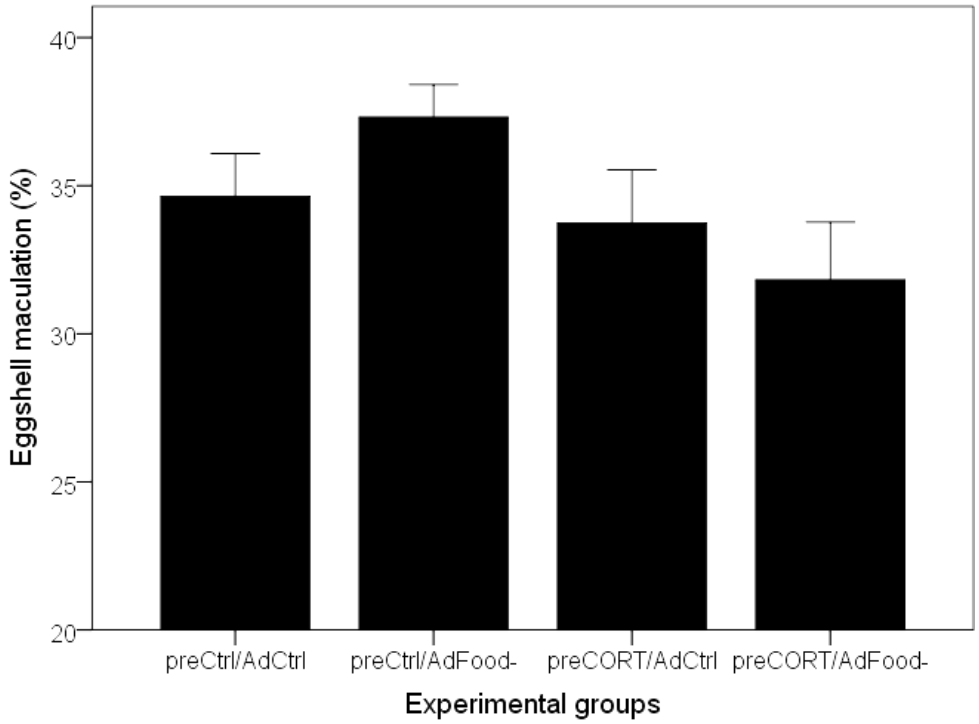

Figure 3.2. Effect of pre-natal stress and breeding stress on mean (+ 1 SE) eggshell maculation of Japanese quail eggs from four different experimental groups: preCtrl/AdCtrl (pre-natal control, reproduction control), preCtrl/AdFood- (pre-natal control, reproduction stress), preCORT/AdCtrl (pre-natal stress, reproduction control), and preCORT/AdFood- (pre-natal stress, reproduction stress) (see text for further details).

In addition, there was a significant interaction between pre-natal and adult stress on eggshell protoporphyrin concentration. Pre-natal control females deposited more protoporphyrin under food removal conditions during breeding than under control conditions, compared to pre-natally stressed birds that did not change pigment concentration across the two treatments (Tukey-Kramer test: $t = -3.90$, $P = 0.001$) (Fig. 3.3, Table 3.4). Eggshell biliverdin and protoporphyrin concentrations were positively correlated under both control ($R^2 = 0.77$, $P < 0.0001$, $N = 55$) and food-removal ($R^2 = 0.68$, $P < 0.0001$, $N = 57$) treatments.

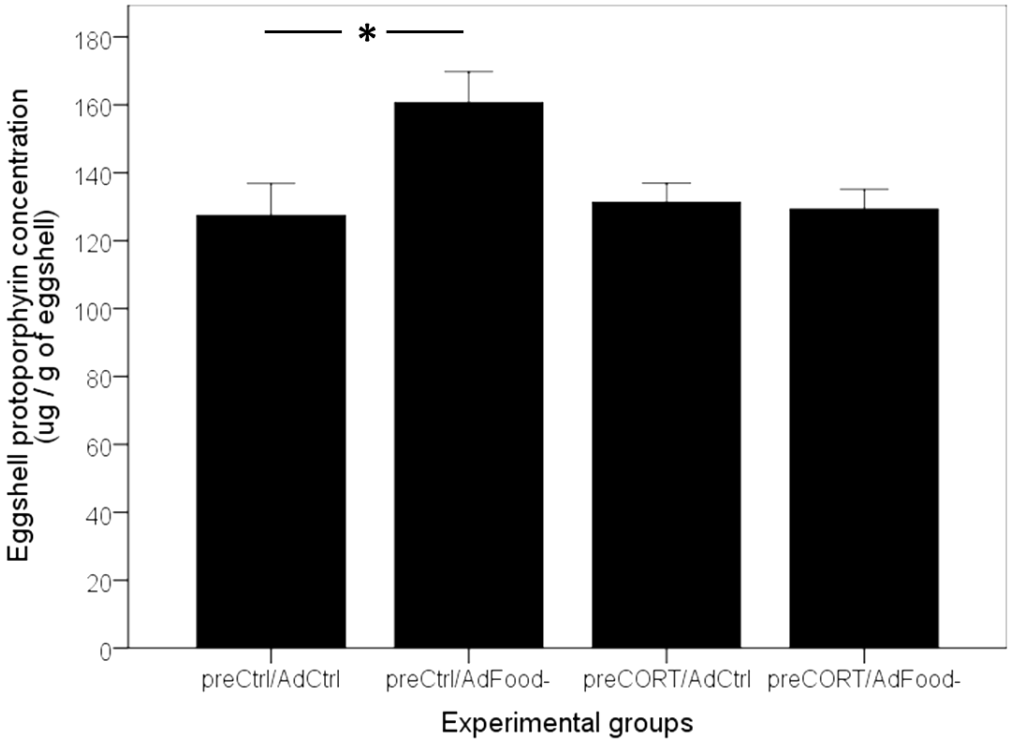

Figure 3.3. Effect of pre-natal stress and breeding stress on mean (+ 1 SE) eggshell protoporphyrin concentration of Japanese quail eggs from four different experimental groups: preCtrl/AdCtrl (pre-natal control, reproduction control), preCtrl/AdFood- (pre-natal control, reproduction stress), preCORT/AdCtrl (pre-natal stress, reproduction control), and preCORT/AdFood- (pre-natal stress, reproduction stress) (see text for further details). Asterisk denotes statistically significant differences between groups.

Table 3.4. Effects of pre-natal and post-natal stress and breeding stress on eggshell colouration and pigment concentrations of female Japanese quails (see text for further details). GLMMs were performed to test whether stress influenced eggshell reflectance and pigments concentrations. Bold text indicates statistical significance.

Eggshell trait	Parameter	Factor	dfs	F	P
Spot reflectance	Brightness	Pre-natal stress	1, 26.2	1.98	0.17
		Post-natal stress	1, 26.2	0.12	0.73
		Adult stress	1, 127	1.16	0.28
		Post-natal × adult stress	1, 127	3.33	0.07
	Red chroma	Pre-natal stress	1, 25.3	0.11	0.75
		Post-natal stress	1, 25.3	0.00	0.96
		Adult stress	1, 129	0.52	0.47
		Post-natal × adult stress	**1, 129**	**5.95**	**0.02**
	Blue-green chroma	Pre-natal stress	1, 26	0.07	0.79
		Post-natal stress	1, 26	0.02	0.89
		Adult stress	1, 126	0.03	0.86
		Post-natal × adult stress	1, 126	1.54	0.22
Background reflectance	Brightness	Pre-natal stress	1, 26.1	3.64	0.07
		Post-natal stress	1, 26.1	0.08	0.78
		Adult stress	1, 128	0.11	0.74
		Post-natal × adult stress	1, 128	2.21	0.17
	Red chroma	Pre-natal stress	1, 27.7	3.28	0.08
		Post-natal stress	1, 27.7	0.90	0.35
		Adult stress	1, 132	0.03	0.86
		Post-natal × adult stress	**1, 132**	**5.16**	**0.02**
	Blue-green chroma	Pre-natal stress	1, 26.3	1.07	0.31
		Post-natal stress	1, 26.3	0.37	0.54
		Adult stress	1, 128	2.17	0.14
		Post-natal × adult stress	1, 128	0.19	0.66
Eggshell pigment	Protoporphyrin	Pre-natal stress	1, 26.4	1.60	0.22
		Post-natal stress	1, 26.4	0.29	0.60
		Adult stress	**1, 81.4**	**9.67**	**<0.01**
		Pre-natal × adult stress	**1, 81.4**	**4.46**	**0.04**
	Biliverdin	Pre-natal stress	1, 25.2	0.63	0.43
		Post-natal stress	1, 25.2	0.70	0.41
		Adult stress	**1, 47.7**	**19.55**	**<0.01**
		Post-natal × adult stress	**1, 47.7**	**7.38**	**<0.01**

3.3.3.3. Effect of post-natal stress on adult reproduction

There was a significant interaction between post-natal and adult stress on eggshell reflectance (Table 3.4). Although multiple comparisons did not show significant differences between treatment groups, post-natally stressed females tended to lay eggs with redder spots and backgrounds (Fig. 3.4) under food removal conditions during breeding than under control conditions (Table 3.4). However, neither brightness nor blue-green chroma were affected by stress (all Ps > 0.05) (Table 3.4). In addition, pre-natal stress did not have any influence on eggshell colouration (all Ps > 0.05) (Table 3.4).

In addition, eggshell biliverdin concentration was significantly affected by the interaction between post-natal stress and adult breeding stress (Table 3.4). Post-natally stressed females deposited more biliverdin into their eggshells under food removal conditions during breeding than under control conditions (Tukey-Kramer test: $t = -4.36$, $P = 0.0002$) (Table 3.4, Fig. 3.5). In contrast, pre- and post-natal control females did not change their biliverdin allocation strategy when food was removed during reproduction.

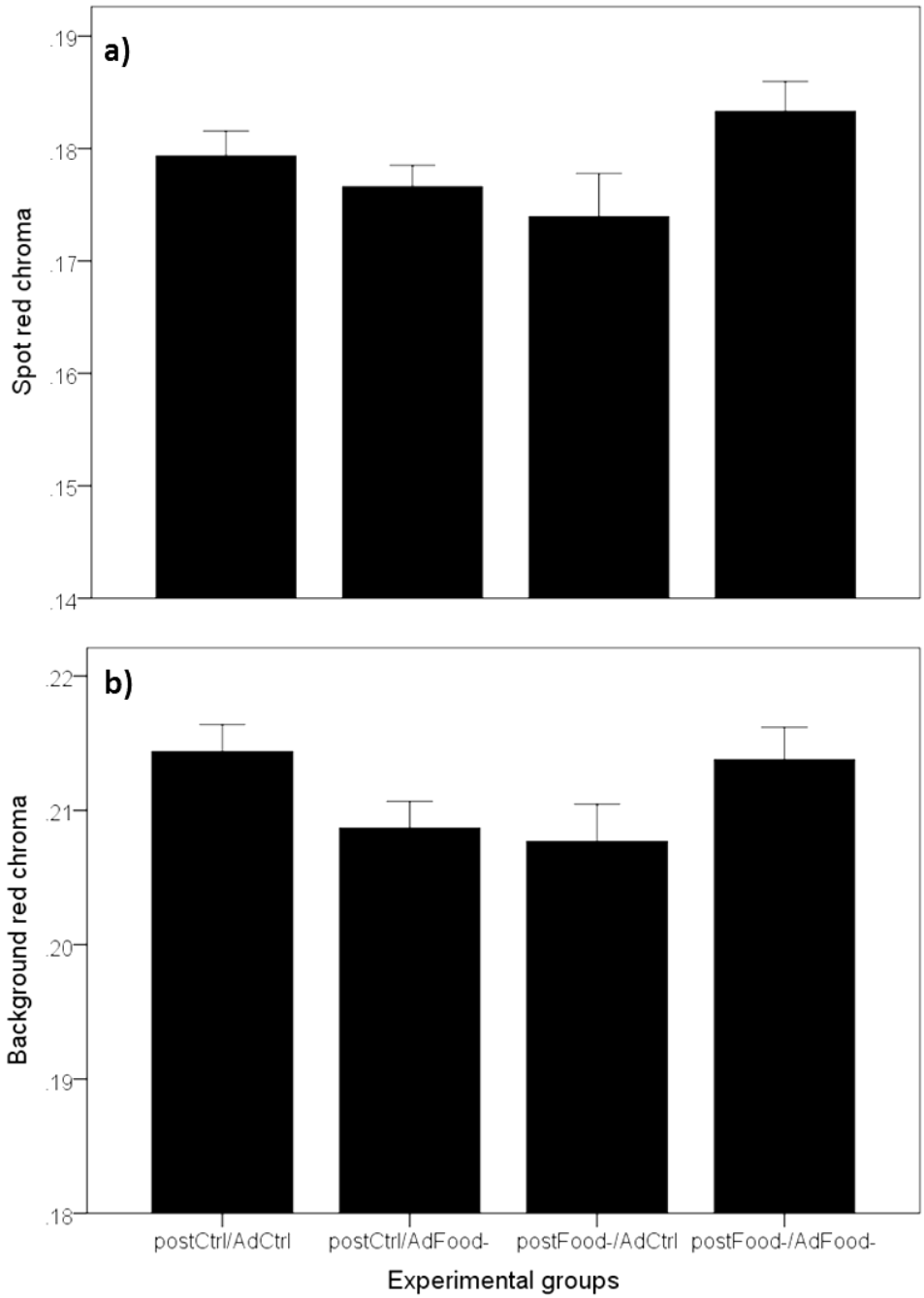

Figure 3.4. Effect of post-natal stress and breeding stress on mean (+ 1 SE) (a) spot red chroma and (b) background red chroma of Japanese quail eggs from four different experimental groups: postCtrl/AdCtrl (post-natal control, reproduction control), postCtrl/AdFood- (post-natal control, reproduction stress), postFood-/AdCtrl (post-natal stress, reproduction control), and postFood-/AdFood- (post-natal stress, reproduction stress) (see text for further details).

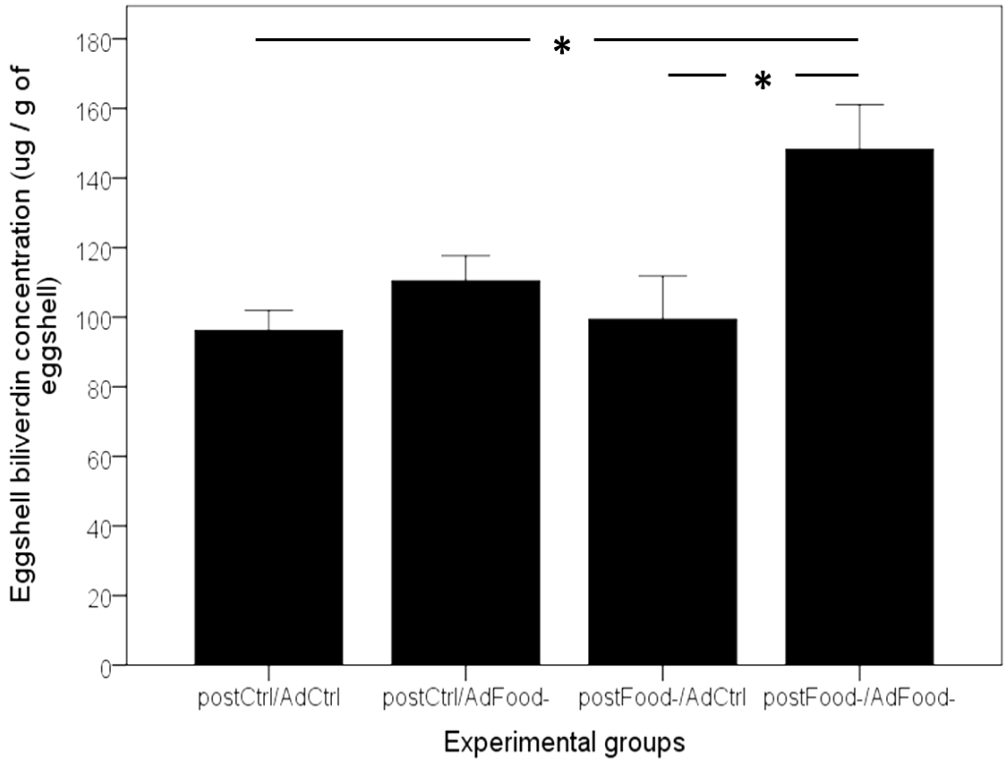

Figure 3.5. Effect of post-natal stress and breeding stress on mean (+ 1 SE) eggshell biliverdin concentration of Japanese quail eggs from four different experimental groups: postCtrl/AdCtrl (post-natal control, reproduction control), postCtrl/AdFood- (post-natal control, reproduction stress), postFood-/AdCtrl (post-natal stress, reproduction control), and postFood-/AdFood- (post-natal stress, reproduction stress). Asterisk denotes significant differences.

3.3.4. Discussion

In this study, I showed for the first time that developmental stress interacts with the response to adult reproductive conditions to alter eggshell pigmentation. The degree and direction of eggshell changes differ depending upon the timing of stress. Stress *in ovo* might facilitate egg camouflage in a stressful breeding environment, whereas post-natal stress enhanced eggshell biliverdin investment, potentially enhancing fitness but to the detriment of egg camouflage. This study gives potential evidence in favour of the 'environmental matching hypothesis'.

I found that pre-natal stress significantly influenced eggshell maculation and protoporphyrin deposition in adulthood. Females which had experienced pre-natal stress maintained eggshell maculation and concentration of protoporphyrin in their eggshells across the two clutch conditions. In contrast, females which had not experienced pre-natal stress increased eggshell maculation and protoporphyrin deposition under stressful breeding conditions compared to their control clutch. Protoporphyrin is a pro-oxidant pigment (Afonso *et al.* 1999) responsible for the brown maculation visible on spotted eggshells. The removal of food during breeding caused a slight reduction in female mass, whereas control conditions resulted in females showing a slight body mass increase (see also Buchanan *et al.* 2003). My results indicate that pre-natal control females may have suffered from an increased oxidative stress following the food removal manipulation during reproduction, and increased the deposition of the brown pigment into the eggshell to eliminate it. This suggests that experiencing pre-natal exposure to stress might have enhanced a female's physiological ability to respond to stress during reproduction, as suggested by the 'environment matching hypothesis' (Monaghan 2008). It is possible that these females might be able to cope physiologically with an increased allostatic load during reproduction and may show better immune response such as antioxidant capacity (Marasco *et al.* 2013). This may enable individuals to tolerate high concentrations of the pro-oxidant pigment while enduring stress during reproduction, during which they maintain eggshell maculation. The stress experienced by females pre-natally may shape their ability to camouflage their egg later in life, which may be a crucial component of Japanese quail clutch survival strategy (Lovell *et al.* 2013).

I found that post-natal stress significantly influenced eggshell reflectance and biliverdin deposition during adulthood. When reproducing under stressful conditions, females that had experienced post-natal stress laid redder eggshells containing more biliverdin. However, eggshell brightness was not affected by female stress exposure contrary to my predictions. In

contrast, post-natal control females kept eggshell reflectance and biliverdin concentration constant across the two clutch conditions. Biliverdin is a blue-green pigment that possesses antioxidant properties (McDonagh 2001) and might signal female antioxidant capacity (Moreno & Osorno 2003). However, the role of biliverdin colouration in brown-spotted eggs remains largely untested, as most of the focus has been on protoporphyrin which is mainly responsible for brown-spots (i.e. maculation) in these species. Yet, in Japanese quail, biliverdin may also play a role in brown-spotted eggshells (Duval *et al.* 2013) and, for instance, interact with eggshell structure. Pigment function and allocation into different compartments of the eggshell remain highly speculative and further studies should investigate pigment deposition in eggshell spots and background independently. Nevertheless, I found that post-natal stress facilitates biliverdin deposition in the eggshell under conditions of breeding stress, which may be related to female antioxidant capacity. Recently, it has been shown that post-natal diet restriction diminishes oxidative damage in yellow-legged gull chicks (*Larus michahellis*) (Noguera *et al.* 2011). It is conceivable that post-natally stressed females may have enhanced antioxidant capacity that allowed them to face oxidative stress during reproduction and deposit more biliverdin into their eggshells. However, I also found that eggshell reflectance was modified in post-natally stressed females under stressful breeding conditions, which suggests that they might not be able to maximise the camouflage of their eggs. This implies that biliverdin allocation might be costly to the female under stressful conditions and may confer advantages to the embryo, such as favouring its development as in amphibians (Falchuk *et al.* 2002) and ensuring protection against bacterial infection (Ishikawa *et al.* 2010) or solar radiations (Lahti 2008). Post-natally stressed females might optimise embryo development under stressful reproduction at the risk of impairing egg camouflage and suffering from potential costs in term of oxidative stress that could induce delayed effects on female antioxidant response over a long-term.

To conclude, the interaction between development and adult environments is crucial for shaping phenotypic traits such as eggshell appearance. Both pre- and post-natal stresses have independent effects on female eggshell pigmentation strategy but my data did not show any interactive effects. Protoporphyrin is mainly responsible for eggshell maculation in Japanese quail, and might be essential for egg camouflage (Lovell *et al.* 2013). Thus, it is possible that shaping eggshell maculation strategy as early as at the pre-natal stage might be essential as it will determine egg and chick survival later in life in future generations. In contrast, biliverdin deposition strategy in adults may be more sensitive to post-natal stress, would be costly to females and may impair other processes such as their ability to keep eggshell reflectance constant.

3.4. Chapter Three - Summary and perspectives

In this chapter, I experimentally investigated the relationship between maternal stress exposure at different life stages and eggshell pigmentation, in brown-spotted eggshells laid by Japanese quails. The study of females exposed to stress during reproduction showed that CORT-fed females laid eggs displaying brighter spots compared to control females whose eggs showed an unchanged reflectance. My findings suggest that stress may have affected female assimilation of certain nutrients such as calcium that we did not measure, influencing some aspects of the eggshell matrix structure that are not directly related to eggshell pigments, but that might change eggshell gloss, explaining the change in eggshell brightness. In contrast, unstressed females were able to keep a constant eggshell reflectance, which might be crucial in hiding eggs from predators. Recent work has shown that female quails maximise their choice of laying substrate in order to maximise camouflage (Lovell *et al.* 2013). If stress has an impact on eggshell spot reflectance, further studies should manipulate female stress

and examine the effect on laying substrate choice within the context of the use of egg camouflage as a signal of female quality.

The results of the early stress exposure in the life of the bird showed that pre- and post-natal stress influence eggshell pigmentation strategies later in life under stressful breeding conditions and the effects are independent of one another. Eggshell protoporphyrin concentration and maculation were affected by pre-natal stress, compared to eggshell reflectance and biliverdin concentration that were influenced by post-natal stress. My findings imply that pre-natal environment plays a key role in determining eggshell characteristics that are crucial in egg camouflage in Japanese quail (Lovell *et al.* 2013). In contrast, the stress experienced during an individual's post-natal development might impact on its decision to deposit compounds into eggs that may favour embryo development but at the risk of impairing egg camouflage later in life.

I encourage further studies that investigate in a more specific way the role of both protoporphyrin and biliverdin in eggshell structure and embryo development (reviewed in Maurer *et al.* 2011a). Investigating the effect of pre- and post-natal stress on the development of the female reproductive system could help us to understand the variation in eggshell pigmentation strategy in response to changes in the breeding environmental conditions.

Overall, I have shown that eggshell pigmentation of Japanese quail indicates the stress experienced by the female at different stages of her life.

Chapter Four

TESTING THE IMPORTANCE OF EGGSHELL PIGMENTATION FOR EGG CRYPSIS IN BROWN-SPOTTED EGGS

4.1 Abstract

Resembling the background (background matching) or visually breaking contours (disruptive colouration) are two keys strategies used for camouflage. It has been shown that captive Japanese quails choose the laying substrate colour that maximizes egg crypsis via disruptive colouration. Whether substrate heterogeneity enhances egg camouflage has never been experimentally explored so far. I investigated whether female Japanese quails maximise egg camouflage when choosing between heterogeneous laying substrates to ascertain whether 1) the previous study is repeatable and 2) whether birds use other clues than substrate colour when choosing their laying area. Nineteen females were offered eight differently coloured and patterned substrates on which to lay. Females should match egg maculation colour, conceal egg outline and contrast egg background colour. If females match laying-background appearance, they should prefer heterogeneous substrates. Using digital photography analyses, I confirmed that female quails maximize disruptive colouration when they lay, but independently on egg maculation degree. Female choice may not be explained by substrate heterogeneity either, however further analysis on substrate texture is required to bring new knowledge on the importance of substrate heterogeneity on female decisions to lay in the context of egg camouflage in ground-laying species.

4.2. Introduction

Many hypotheses have been proposed to explain the diversity of eggshell colour and patterns observed in avian species. These include egg recognition and mimicry, eggshell strength, thermoregulation (reviewed in detail by Underwood & Sealy 2002), signalling of female quality (reviewed in Riehl 2011), and embryo protection (reviewed in Maurer *et al.* 2011a). Camouflage was among the first hypotheses proposed to explain the adaptive significance of eggshell colour in birds (Wallace 1889). Visual crypsis of eggs is effective when egg appearance (e.g. size, colour and pattern) matches the background such that detection by a predator is compromised (Endler 1978). To make their eggs undetectable, birds have evolved different strategies depending on their nesting environment. Indeed, Hewitson (1838) noted that cavity birds most likely laid white eggs, which would enhance egg detectability by parents in dim light (Lack 1958). Similarly, white or blue eggs are laid by species that construct nests that are domed or cover the clutch to conceal the eggs (Wallace 1889). Thus, eggshell patterning may have evolved as an adaptation to the specific micro-environment of each nest to avoid predation (Wallace 1889), in particular in ground-nesting species, that do not always cover their eggs. In those species in particular, matching egg colour with the colour of the nest background may enhance egg crypsis (Tinbergen 1962, Collias & Collias 1984).

There is currently mixed support for egg crypsis, mainly due to methodological limitations, such as the use of dummy eggs (e.g. painted) or artificial nests that do not always mimic natural ones (reviewed in Underwood & Sealy 2002). Brown and spotted eggshells may be less predated in several species with varying nesting strategies. To test the difference in predation rates of song thrushes nests, Götmark (1992) used quail eggs to mimic thrush's eggs, and painted them either in blue, white, or blue with dark brown spots, and placed them in trees, either concealed or exposed to predators such as corvids. The author found that

spotted eggs were less predated than blue and white eggs, for both concealed and exposed nests (Götmark 1992). In a similar experiment, Yahner and Mahan (1996) built artificial nests containing brown chicken eggs, white chicken eggs, or Northern bobwhite eggs, and investigated the rate of nest disturbance by predators. The authors showed that nests containing brown chicken eggs were less disturbed than the ones containing white chicken or Northern bobwhite eggs (Yahner & Mahan 1996). In a similar study in the ground-nesting Red-legged partridges, it was shown that brown and brown-spotted eggs had higher survival advantage compared to white and white-spotted eggs, and this was related to the type of predator (i.e. mammalian or avian) and type of habitat (forest or fallow) (Castilla *et al.* 2007). Westmoreland (2008) recently tested the nest-crypsis hypothesis using american robins nests, containing eggs of red-winged blackbirds (*Agelaius phoneiceus*), brewer's blackbirds (*Euphagus cyanocephalus*), and yellow-headed blackbirds (*Xanthocephalus xanthocephalus*) across three successive predation trials. All eggs differed in both eggshell colour and pattern. The author found that clutches survival was equivalent, but the Red-winged blackbird eggs that were more reflective, were discovered sooner by predators, suggesting a higher risk of predation for conspicuous eggs (Westmorland 2008). However, most experimental studies using painted eggs actually found no difference in predation rates between natural and painted eggs (e.g. Tinbergen *et al.* 1962, Montevecchi 1976, Weidinger 2001). Artificial nests were predated significantly more often than American robin nests tested in their natural environment, even when using brown-spotted Japanese quail eggs to attract predators (Ortega *et al.* 1998).

Despite these limitations, there is some support for the benefit of the nest background matching strategy for egg camouflage and clutch survival. In Stone curlews which build nests on the ground with very little material, Solís and De Lope (1995) examined the efficiency of the background-matching strategy using natural nests. They showed that eggs that did not

match the nest background colour were more predated by avian predators, compared to the eggs that were matching nest background appropriately, enhancing hatching success in those nests. In semi-palmated plovers, eggs with cryptic colour were less conspicuous (Nguyen *et al.* 2003). Similarly, in natural nests of black-tailed gulls (*Larus crassirostris*) which lay greenish eggs with dark brown spots, eggs that matched nest background colour were more likely to survive through to hatching. This was particularly true for eggs laid in nests with poor concealment (Lee *et al.* 2010). Thus, both nest concealment and egg colouration may be crucial to avoid predation and ensure egg survival (Underwood & Sealy 2002). However, none of these studies considered egg maculation (i.e. presence of brown markings) as a potential factor involved in egg camouflage strategy, and instead focused on egg background colouration.

Recently, a study on laying substrate choice in Japanese quail showed that females laid on the substrate that resembled egg maculation colour and concealed the outline of the egg. In particular, females laying egg with a high degree of maculation selected the substrate that contrasted eggshell background colouration the most, leading to camouflage through disruptive colouration (Lovell *et al.* 2013). Disruptive colouration is defined as the development of markings that create the appearance of deceptive edges and obstruct the recognition or detection of an object, and is the second main strategy for enhancement of animal camouflage (Stevens & Merilaita 2009). For instance, disruptive colouration may have evolved in mammals (Stoner *et al.* 2003), fishes (Kelman *et al.* 2007), snakes (Beatson 1976), cephalopods (Chiao *et al.* 2005, Kelman *et al.* 2007), and birds (Graul 1973, Götmark & Hohlfält 1995). Lovell *et al.* (2013) study added to the knowledge that female birds have some prior awareness of their own egg colouration, as previously shown in species subject to brood parasitism (Lyon 2003, Petrie *et al.* 2009). Japanese quails are able to recognize their own eggs (Pike 2011) and Lovell and colleagues demonstrated that they use disruptive

colouration to make their egg less visually detectable (Lovell *et al.* 2013). Interestingly, they based their experimental design on four substrates that differed only in their colour; however, it is possible that substrate heterogeneity (or complexity) such as the presence of stones or other materials, may also influence female choice to nest. Indeed, in such a species that lays heavily maculated eggs, laying on visually complex substrates that look similar to the pattern of the eggshell may enhance egg camouflage via background-matching strategy (Westmoreland & Kiltie 1996, Colwell *et al.* 2011), in addition to disruptive colouration already demonstrated previously (Lovell *et al.* 2013). For instance, in species that lay in open nests, specific characteristics of the substrate such as the presence of egg-size stones may enhance egg crypsis (Colwell *et al.* 2011) but this remains poorly tested so far. Additionally, within-clutch variation in egg colour has been positively related to nest survival in the namaqua sandgrouse (*Pterocles namaqua*) (Lloyd *et al.* 2000), suggesting that pattern variability between eggs may also decrease their detectability compared to uniformly coloured eggs.

In this study, I experimentally investigated the importance of substrate colour and heterogeneity using coloured sands where I added differently coloured and sized stones, in a laying choice experiment using Japanese quail females. As both substrate matching and disruptive colouration appear important in enabling egg camouflage, depending upon the degree of eggshell patterning (Lovell *et al.* 2013), I expected that female would lay on the substrate that matched egg maculation colour, concealed egg outline and contrasted egg background colouration. In particular, I predicted that female would lay preferentially on the heterogeneous substrates that potentially resemble the egg patterning, to enhance the matching between egg/laying substrate appearance, and should choose the substrate with stones that matched the size of egg spots.

4.3. Materials and methods

4.3.1. Study species and experimental procedure

All experiments were carried out with ethical approval from the University of St Andrews and under Home Office Project License 60/4068 held by Dr Karen Spencer, and Personal License 30/8939 held by me.

I used 19 female Japanese quails that were provided by a private supplier from Scotland (Hillfoots Hutz and Henz, Dollar, UK), all identified with a white numbered leg ring. Before the start of the experiment, birds were all laying and were housed in an indoor aviary (110 cm high × 300 cm wide × 300 cm long) for 1 week to allow quarantine and habituation to new housing conditions before the experiment commenced. During habituation, birds were fed *ad libitum* with a standard commercial diet (Layer pellets, ARGO Feeds), the room temperature was maintained between 20 and 22°C and the light regime was 14L:10D. I provided the females with cardboard trays (29.2 cm long × 21.5 cm wide × 4.2 cm deep) (Tiny Box Company, West Sussex, UK) that were then used as laying arenas in my experiment, filled with white, brown and black sands in turns as the substrate (Trixie 76130 Desert Sand for Terrariums, TRIXIE Heimtierbedarf GmbH & Co, Germany) to habituate them to this new material.

All quails were then housed singly in arenas (100 cm long x 60 cm wide x 92 cm high) and were in acoustic contact with the other females at all times. To avoid any imitation of laying choice between females that may happen if females can see each other, an opaque plastic screen was placed on the side of each arena to prevent visual contact between birds. Within each arena, a female was provided with eight differently coloured and patterned (i.e. heterogeneous) sand substrates (Fig. 4.1) in cardboard trays.

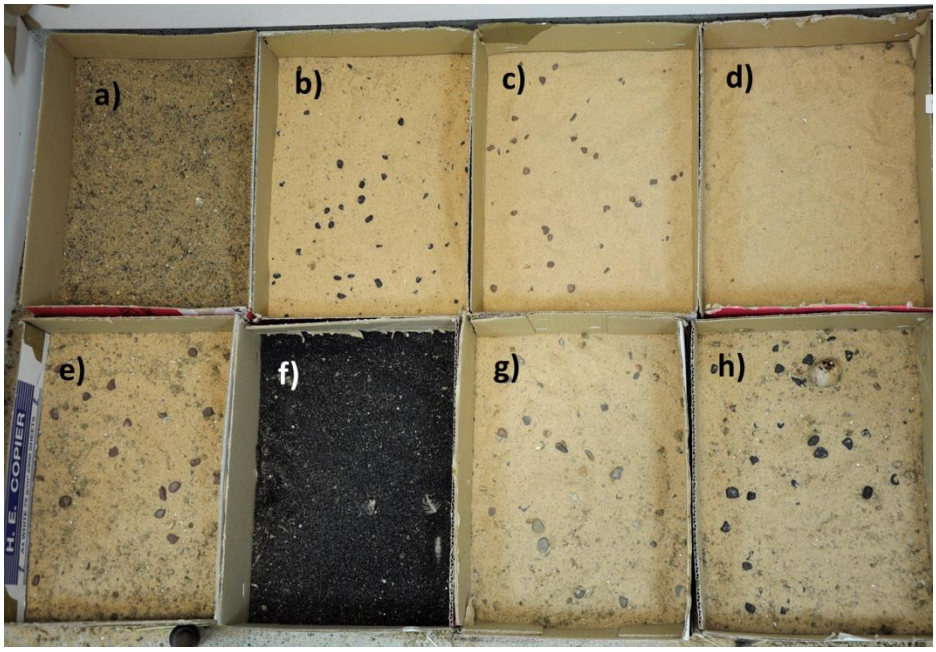

Figure 4.1. The photograph of the laying arena with eight different coloured and patterned substrates that were offered to Japanese quails in a choice experiment: (a) plain brown, (b) small black stones, (c) small brown stones, (d) plain white, (e) big brown stones, (f) plain black, (g) random stones and (h) big black stones.

Heterogeneous substrates were made of the same sand as for the plain substrates, but 20 small-sized (0.5 cm) or big-sized (1 cm) gravels were added to each substrate. To test whether females were able to discriminate between gravel particles by their colour, the stones were either painted brown (Figs. 4.1c and 4.1d) or black (Figs. 4.1b and 4.1g) (ECOS Organic Paints, Heysham, Lancs UK). In addition, one substrate was patterned with a mix of gravels of both sizes and unpainted (Fig. 4.1f). The position of each substrate was randomly changed every day to avoid any location effect. Each female experienced one laying trial that lasted for 7 days, most females laid 7 eggs except for two females which laid three and six eggs and a total of 128 eggs were laid during the experiment.

4.3.2. Digital photography

Eggs were collected every day and the substrate where each egg was laid was recorded. Photographs were taken using a calibrated Nikon D90 camera with a Nikon 105 mm lens activated using a remote control (see Chapter Two for further details). Constant lighting and long exposures, rather than flash photography, were used to protect the eggshell pigments from photo-degradation.

Two digital photographs of all 8 substrates, one including the egg on its laid position (Fig. 4.2a) and one with the egg placed upon a black card (Fig. 4.2b) were taken every day, including a colour chart in the image (Macbeth Mini ColorChecker) to allow a normalisation of estimated chromaticity values to the mean of the measured Macbeth tiles values, controlling for a potential variation in illumination across the different cages.

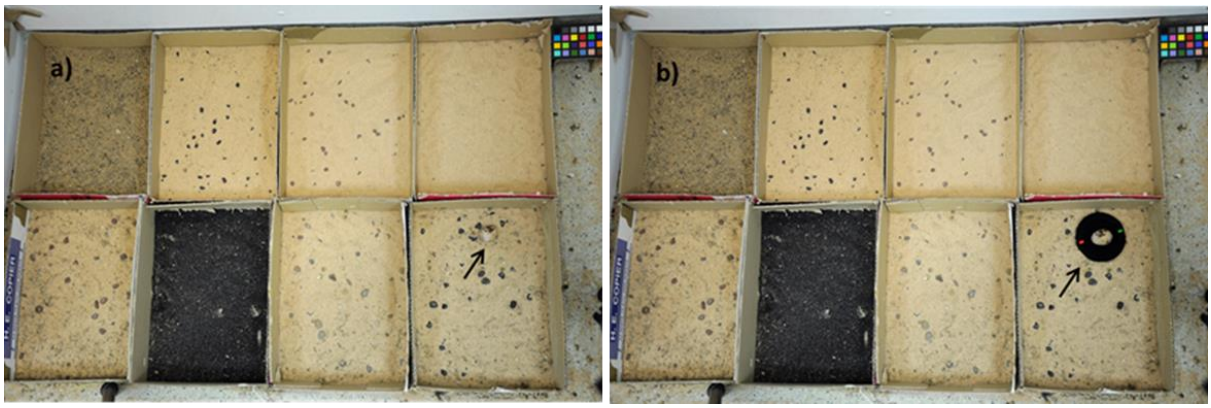

Figure 4.2. Photograph of the laying arena with one egg its laid position (a), and the same egg on a black disk (b) where the red dot represents the pointy end, and the green dot represents the blunt end of the egg. The Macbeth colour chart was positioned on the top right corner of the laying arena.

My aim was to evaluate the degree of egg crypsis on each of the eight substrates available. Thus it was necessary to artificially isolate the egg and to position it on the other seven non-chosen substrates. To do this, I created a binary image of each substrate using a region of interest (ROI) in Matlab, and repeated this selection for the egg on its black disk and for the

Macbeth colour chart (Fig. 4.3). Selected regions of interest were not shrunk or modified so that the proportion between the egg area and the sand area was identical to the real set up.

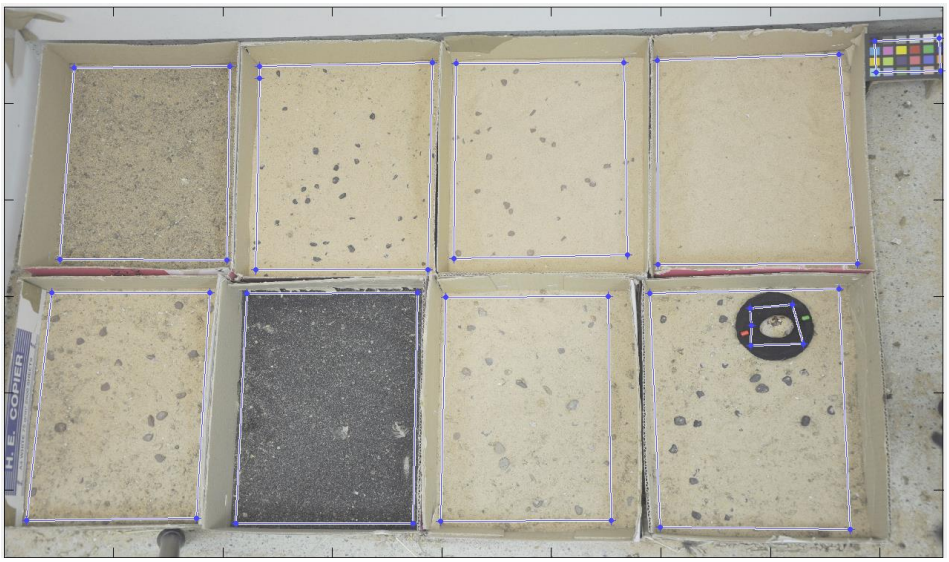

Figure 4.3. Matlab image of the Regions of Interest (blue lines) selected on each substrate, around the MacBeth colour chart and around the egg. The ROI corresponding to the MacBeth colour chart was positioned on the centres of the corner coloured tiles. The ROI corresponding to the egg was positioned upon the black disk, and for each substrate, shadows were avoided.

The RGB (Red Blue Green colour model) egg masks were checked in Gimp 2.8.2 software (http://www.gimp.org) to insure that the area that corresponded to the egg on each photograph was correctly delimited. The binary images created were used as masks and chimeric images were built by artificially placing the parts of the egg photograph into the central area of photographs of the potential laying substrates. All test images were constructed automatically within Matlab.

Chromatic analyses were conducted in CIELab space which is defined by L* (Luminance), a* (red-green) and b* (yellow-blue) colour dimensions. The CIELab space is modeled on the human visual system, and the values are perceptually uniform. Consequently, changes of

similar numerical values in the L*, a*, and b* axes will be perceived as having a similar perceptual difference (Martinkauppi 2002).

Once each chimeric image was built (Fig. 4.4), the maculated (i.e. brown-spotted) and background regions of the egg were identified.

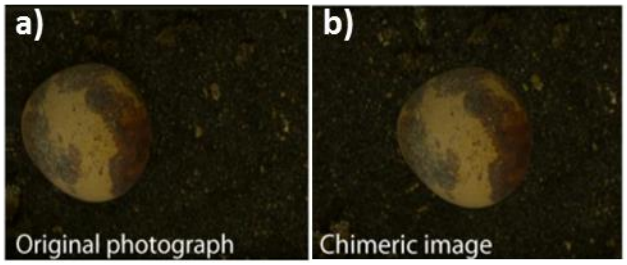

Figure 4.4. (a) Original photograph of an egg upon the chosen laying substrate. (b) Constructed chimeric egg photograph used in all subsequent analyses (modified from Lovell *et al.* 2013).

To do this, a k-means clustering algorithm in Matlab was applied to the CIELab pixel values for the egg area of each image, assuming two predominant colours within the sampled region (k = 2, giving a target of two centroids) (Lovell *et al.* 2013). Once egg maculate, egg background and substrate areas were segregated, the maculation degree (i.e. percentage of dark pixels) was calculated, and the mean CIELab values for each region were computed (i.e. the mean chromaticity for egg maculation and egg background, and for the substrate) by taking the mean Lab values for all pixels in each zone. Then, the Euclidian distance between these averaged Lab values (Delta E) was calculated to analyse the chromatic differences between the substrate and egg regions (i.e. maculate and background), for the chosen and non-chosen substrates. Higher Delta E values indicated a higher difference in colour and luminance between the egg region and the laying substrate, and thus a lower camouflage efficiency.

Egg contour (i.e. outline) was assessed using a visibility ratio variable (VisRat) and was first detected using the Canny edge-detection algorithm in Matlab (Lovell *et al.* 2013). Then, contour pixels were scored as part of the egg if they were in an area near the edge of the egg mask (four pixels into the mask and eight pixels beyond the mask; equivalent to a range of 1 mm), or as part of the substrate if they were outside the egg mask and beyond the 1 mm egg boundary area. The ratio between substrate contours and the amount of the egg's own contour was calculated and was used for the rest of the analysis as the visibility ratio VisRat (VisRat = egg edges / substrate edges) (Fig. 4.5). Higher VisRat values indicated a higher visibility of egg outline, thus lower camouflage efficiency. This variable is of a particular interest as this characterizes a camouflage strategy that involves placing the egg into a more heterogeneous substrate to diminish its detectability (Dimitrova & Merilaita 2012).

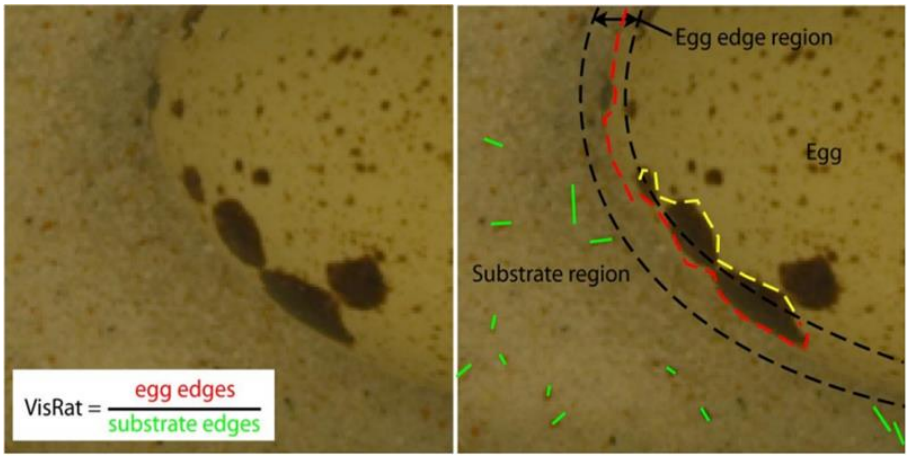

Figure 4.5. Schematic illustration of the calculation of the visibility ratio (VisRat) for an egg. Contours within the egg edge region (red segments within the two black dashed lines) were scored as being part of the detected egg contour, and the corresponding value became the numerator (i.e. egg edges). Contours within the substrate region (green segments) were summed and became the denominator in the VisRat calculation (i.e. substrate edges). In the current model the yellow contours were ignored (modified from Lovell *et al.* 2013).

Egg maculation (i.e. brown spots) size was calculated using the ellipse fitting function in Matlab. Ellipses were fitted around egg spots and the number of pixels in each ellipse was counted. As the maculation size was not normally distributed within an egg, the maculation size at the 50^{th} percentile of the distribution was used to represent the most abundant size of maculation found on each egg.

4.3.3. Statistical analyses

I first analysed the distribution of laying choices among the substrates available to the females using a Chi square test for all females. Laying choice repeatability within female was statistically significant but low ($r = 0.21$, $P = 0.001$) (Lessells & Boag 1987).

To test whether CIELab differences (Delta E) for each egg region (egg background and maculation) differed between chosen and non-chosen substrates, I performed a repeated-measures GLM with CIELab differences (Delta E) for each egg as the dependent variable, egg number as the repeated factor, egg region (background or maculation) and substrate (chosen or mean of non-chosen) as between subjects factors, and egg region × substrate as the interaction term. The mean of all non-chosen substrate was used in the analysis.

To examine what parameters drive female choice for a specific laying substrate, I examined every laying choice and ranked each substrate from 1 to 8 according to its camouflage efficiency, 1 being the most optimal (i.e. most camouflaging) and 8 being the least. I applied this ranking to the three variables examined: Delta E maculation, Delta E background, and VisRat. I then conducted a Chi-square analysis to examine the distribution of optimality ranks for the chosen substrates for all females. I also performed a Chi square analysis to test the effect of eggshell maculation degree on the distribution of optimality ranks, tabulating maculation degree against choice rank for all three variables: Delta E maculation, Delta E

background and VisRat. For this analysis, maculation degree was grouped into four percentiles (Table 4.1).

Table 4.1. Table that shows the amount of egg maculation (% of dark pixels) in each percentile group.

Quartile	Minimum	Mean	Maximum
1	16.5%	29.7%	38.3%
2	38.4%	41.8%	45.5%
3	46.0%	49.9%	54.1%
4	54.4%	62.9%	83.4%

I only used the mean CIELab (i.e. L, a and b) values for all pixels in these regions as Lovell *et al.* (2013) did not find any differences in biologically relevant variables using the same camera, when they performed the analyses using two other approaches, CIE luminance (L) data and the raw green pixel outputs from the camera (camera sensitivity peak = 537 nm, action spectra 71 nm FWHM).

To examine the effect of substrate heterogeneity on female choice, I ran the same analyses as previously, but only on eggs (N = 34) that were laid on the heterogeneous substrates with stones. I ran a Chi square analysis to examine the distribution of optimality ranks for the chosen heterogeneous substrates. In addition, I used a Kruskal-Wallis one-way analysis of variance to compare the maculation size at the 50^{th} percentile with the size of the stones on the chosen heterogeneous substrates, to test whether egg maculation size had an effect of female choice between the three categories of stones that were available on the heterogeneous substrates.

4.4. Results

4.4.1. Distribution of laying choices

Laying choices were not evenly distributed between the substrates, with 26.6 % of the eggs being laid on the heterogeneous substrates, 52.3 % on the plain substrates, and the remaining 21.1 % off arena ($X^2 = 77.03$, df = 8, $p < 0.0001$; Fig. 4.6).

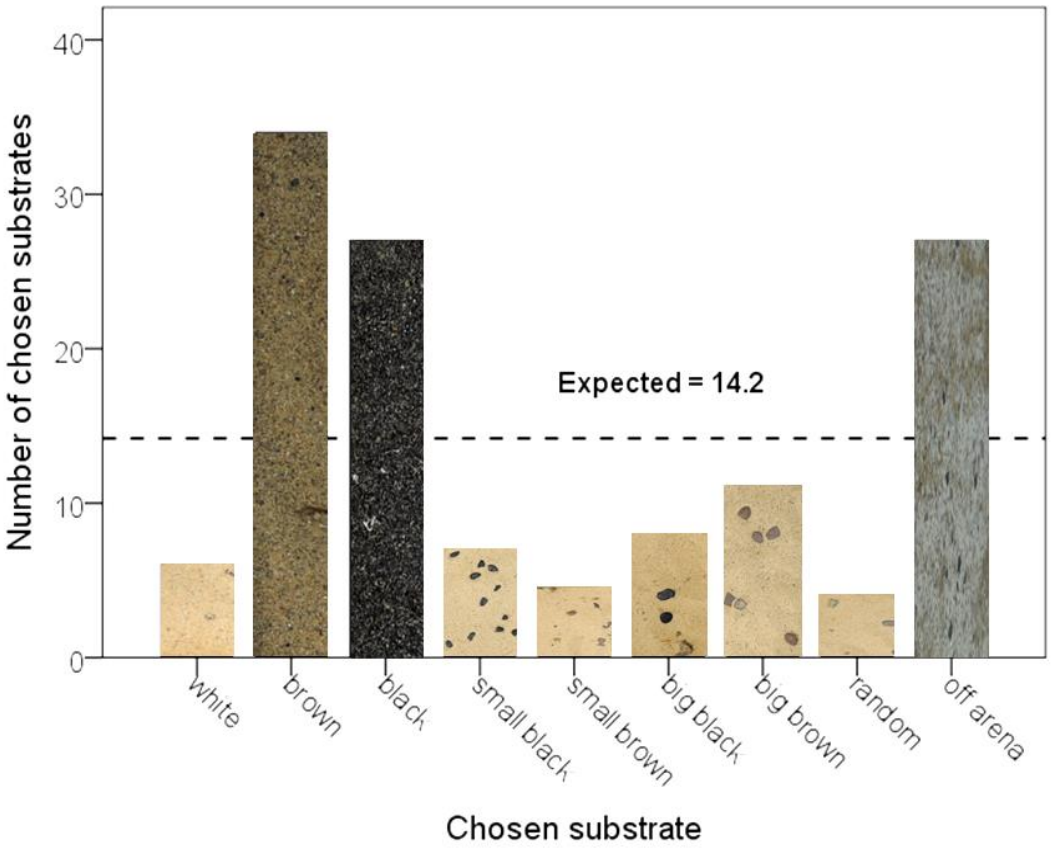

Figure 4.6. Distribution of laying choices between the substrates available to female Japanese quail, including off the arena: plain white, plain brown, plain black, small black stones, small brown stones, big black stones, big brown stones, random stones and off arena (total N = 128 eggs).

4.4.2. Chromaticity analysis

Females chose to lay on the substrate that most matched the chromaticity of their egg maculation (i.e. there was a lower Delta E maculation on chosen substrate), but that

contrasted with the colour of their background (i.e. there was a higher Delta E background on chosen-substrate) (Fig. 4.7). There was a significant interaction between substrate (chosen or non-chosen) and egg region (background or maculation) (repeated-measures GLM: substrate: $F_{1,64} = 1.08$, P = 0.30, egg region: $F_{1,64} = 0.13$, P = 0.72, substrate × egg region: $F_{1,64} = 4.88$, P = 0.03). This suggests that quails chose laying substrates in accordance with their egg colour, and this might be driven by the heavily maculated eggs as the interaction in not significant anymore when removing the eggs from the 4th quartile (heavily maculated) from the analysis (substrate: $F_{1,20} = 0.92$, P = 0.35, egg region: $F_{1,20} = 0.12$, P = 0.73, substrate × egg region: $F_{1,64} = 0.66$, P = 0.42).

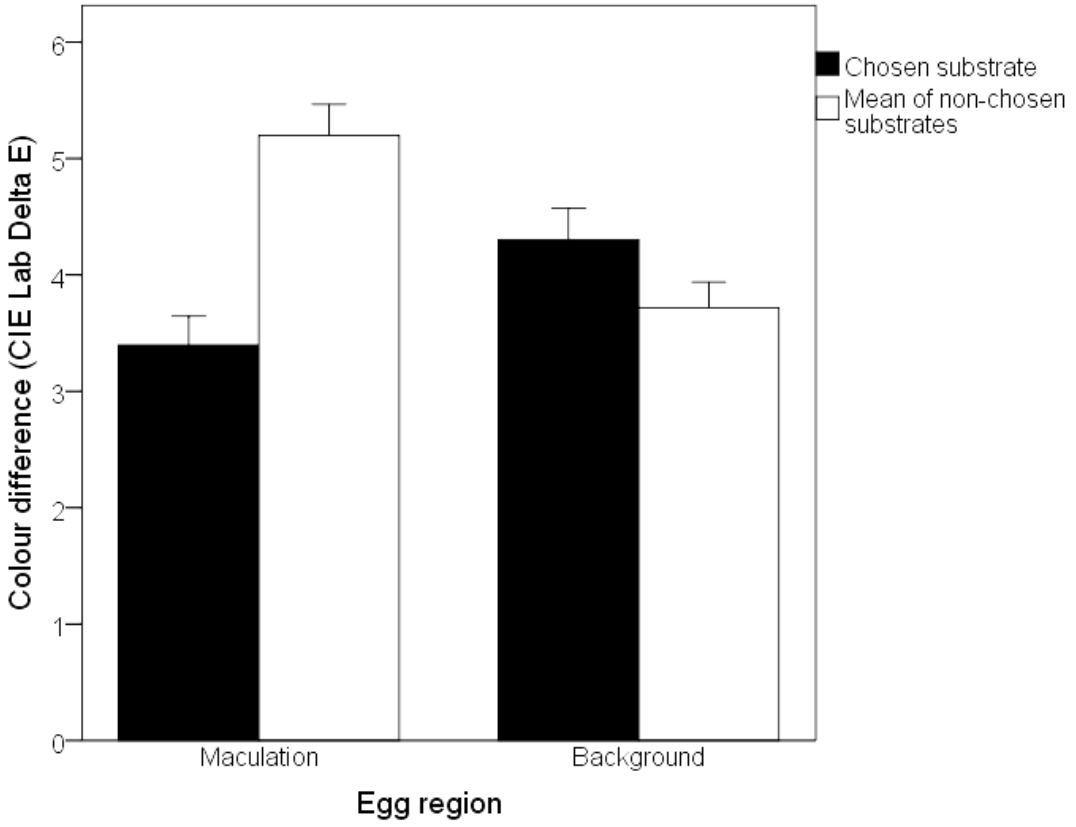

Figure 4.7. Mean Delta E values (chromatic differences) (± 1 SE) for maculation and background regions of Japanese quail eggs when comparing both chosen and non-chosen substrates are shown.

4.4.3. Distribution of the optimality of laying choices

There were significant differences between optimality choices, as the distribution of laying ranks (1 = the most camouflaging, 8 = the least camouflaging) was skewed for all three variables that were computed (VisRat, Delta E maculation and Delta E background) (Table 4.2.a). There were significantly more eggs ranked at position 1 (i.e. most optimal substrate) and fewer laid on position 8 (i.e. least optimal substrate) than expected (expected value = 16) for both VisRat (Fig. 4.8.a) and Delta E maculation (Fig. 4.8.b). However, I found an inverse relationship for Delta E background, as more eggs were ranked at position 8 and fewer at position 1 (Fig. 4.8.c). This suggests that females chose to lay on substrates that concealed the outline of their eggs and matched the colour of egg maculation, but contrasted the colour of egg background.

Table 4.2. Statistical results from (a) a Chi square analysis of ranks optimality for each of the three variable analysed: VisRat, delta E maculation and Delta E background and (b) Chi square analysis to examine the effect of egg maculation degree on female choice optimality for the three camouflage variables.

Variable	df	X^2	P
a)			
VisRat	7	93.00	**< 0.0001**
Delta E maculation	7	106.12	**< 0.0001**
Delta E background	7	37.12	< 0.0001
b)			
VisRat	21	18.22	0.66
Delta E maculation	21	22.67	0.36
Delta E background	21	27.88	0.14

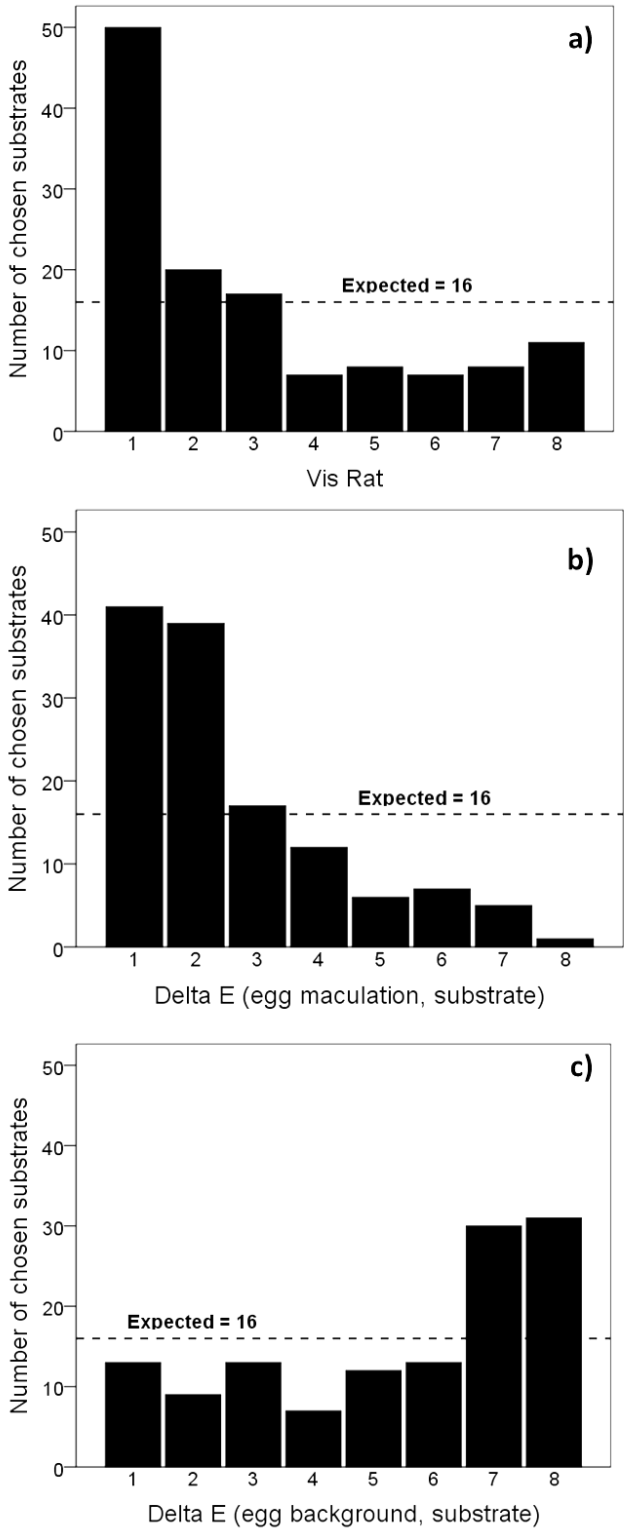

Figure 4.8. Distribution of optimality ranks of laying choices of Japanese quail for (a) VisRat, (b) Delta E maculation and (c) Delta E background (see text for details). Rank 1 corresponds to the most optimal choice (i.e. most camouflaging substrate) and rank 8 the least optimal (i.e. least camouflaging substrate). The horizontal dotted line represents the expected values if the optimality choices were evenly distributed.

I then tested the effect of egg maculation degree on female optimality of laying choice as there was a high variance of maculation degree (16.5% - 83.4%). There was no significant effect of egg maculation degree on the distribution of VisRat (Fig. 4.9.a) and Delta E maculation (Fig. 4.9.b) (Table 4.2.b). Within each maculation percentile, VisRat and Delta E distributions were skewed towards rank 1, suggesting that independently of the degree of maculation of their egg, females chose to maximise egg camouflage by laying on the substrate that concealed egg outline and matched egg maculation colour. The distribution of Delta E background tended to be skewed towards the rank 8 for maculation percentile 3 and 4 (i.e. highly maculated eggs, between 46% and 83.4%) (Fig. 4.9.c), with choices more evenly distributed for lightly maculated eggs (1^{st} and 2^{nd} percentiles of maculation [i.e. between 16.5% and 45.5%]) but the effect of maculation degree was not statistically significant (Table 4.2.b).

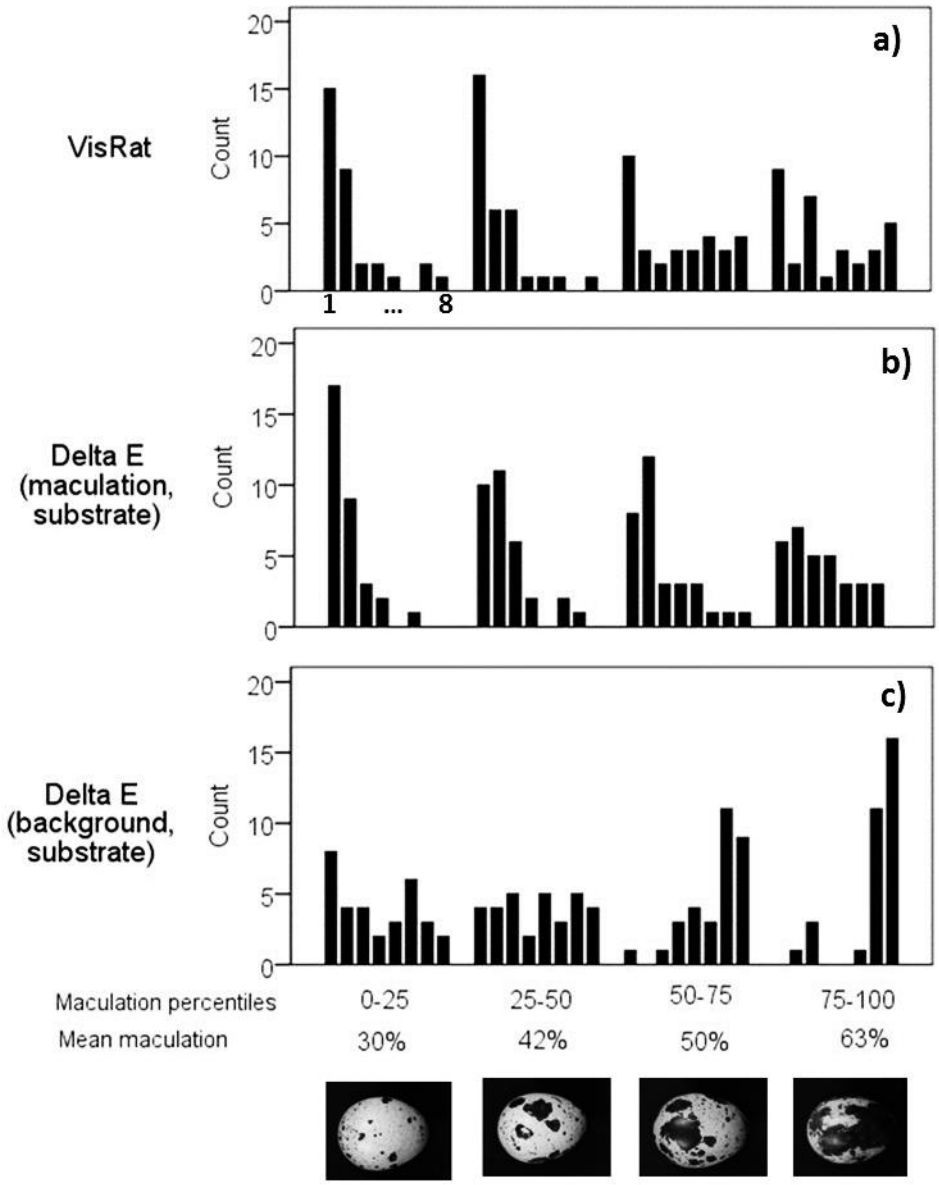

Figure 4.9. Japanese quail laying choices (rank from 1 to 8 with 1 being the most camouflaging) for (a) VisRat, (b) Delta E maculation and (c) Delta E background in relation to egg maculation degree. Each variable is split between the four percentiles of maculation degree.

4.4.4. Laying choices on heterogeneous substrates

When only examining the eggs that were laid on the substrates with stones, the distribution of Delta E maculation was marginally skewed towards the rank 3 ($X^2 = 12.94$, df = 6, p = 0.05; Fig. 4.10). However, there were no significant differences between optimality choice for

either VisRat ($X^2 = 10.06$, df = 6, p = 0.12) or for delta E background ($X^2 = 8.41$, df = 6, p = 0.21).

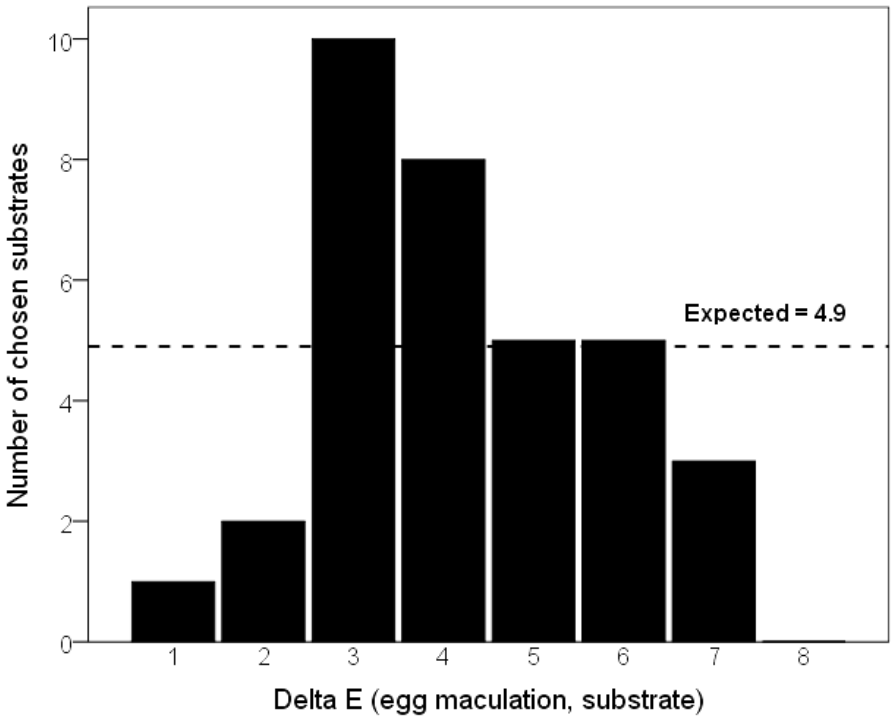

Figure 4.10. Distribution of optimality ranks of laying choices of Japanese quail on heterogeneous substrates only (N = 34), for Delta E maculation. Rank 1 corresponds to the most optimal choice (i.e. most camouflaging substrate) and rank 8 the least optimal (i.e. least camouflaging substrate). The horizontal dotted line represents the expected values if the optimality choices were evenly distributed.

I then examined the relationship between egg maculation size and the size of the stones that were put on the heterogeneous substrates (i.e. small, random sized and big). There was no significant effect of egg maculation size at the 50th percentile of the distribution, on female preference for small, big, or randomly sized stones where the egg was laid on the heterogeneous substrate (Fig. 4.11).

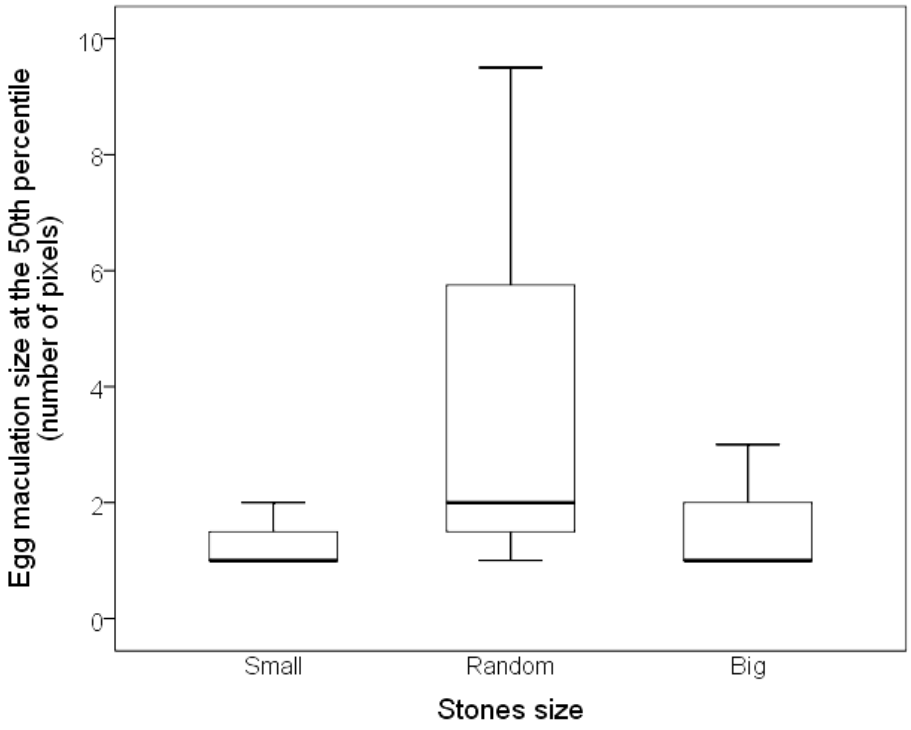

Figure 4.11. Relationship between egg maculation size at the 50th percentile of the distribution of maculation sizes, and the size of the stones on the heterogeneous substrates. The dark line in the middle of the boxes is the median of egg maculation size. The bottom of the box indicates the 25th percentile and the top of the box represents the 75th percentile. Whiskers indicate the minimum and maximum.

4.5. Discussion

In this study, I showed that Japanese quails chose laying substrates that maximise the camouflage of their eggs, enhancing maculate colour matching and background colour contrast between the egg and the laying substrate, and concealing egg outline, leading to disruptive colouration. However, egg camouflage was not maximised on heterogeneous substrates, and female appeared to have avoided these specific substrates. Substrate colouration rather than its patterning (i.e. presence of stones) may drive female decision to lay in this species. This suggests that disruptive colouration rather than substrate matching might have evolved as an egg camouflage strategy in Japanese quail.

The chromaticity analysis showed that females mostly laid on the substrates that matched the colour of eggshell maculation (i.e. brown spots) and concealed egg outline. In addition, when comparing to the non-chosen substrates, females made the best choice based on the model of optimality ranks and laid on the most camouflaging substrates. However, they contrasted the colour of eggshell background (i.e. immaculate area of the eggshell). Matching between an individual's appearance and its background is a strategy that is widely distributed across the animal kingdom, namely "background-matching", and helps to decrease the risk of being detected by predators (Wallace 1889, Poulton 1890, Beddard 1895). For instance, many experiments using real (Sumner 1934, Popham 1942) or artificial backgrounds and predators (Pietrewicz & Kamil 1977) have shown that prey that least resembled the background were attacked more often than those that matched it. In birds, it has been suggested that nest background-matching has evolved to decrease egg detectability in species which do not conceal eggs within a built nest (Lee *et al.* 2010). However, this hypothesis has only been tested in the context of a match between the eggshell immaculate region (i.e. eggshell background) and the appearance of the laying area, ignoring the presence of brown maculation on the eggshell. If background-matching is defined as a resemblance with the laying area, both eggshell background and maculation should match the appearance of the laying substrate. My results do not support the nest background-matching hypothesis, as female Japanese quail only matched the colour of egg maculate with the one of the laying substrate, and concealed egg outline, but did not match eggshell background colour; in fact they actively contrasted with it. As highlighted by Thayer (1909) and Cott (1940), background-matching might not be sufficient as a predator deterrent as the animal's outline may make them more detectable, and in turn disruptive colouration might be complimentary to background-matching as it may result in changes in the animal's appearance or shape helping it to merge visually with its background. In my experiment, females preferentially

laid on the brown and black substrates (Fig. 4.6), thus matching egg maculate and substrate colour, and increasing the contrast between egg background and substrate colour. This strategy might help to break up the outline of the egg, which was confirmed by a decreased egg outline visibility ratio, and may diminish the detectability of the egg on the chosen substrate. Thus, my results support the idea that Japanese quail females use disruptive colouration as a camouflage strategy in an artificial environment (Lovell *et al.* 2013).

It has been previously suggested that eggshell maculation significantly varies between females, and that eggshell maculation degree may play a role in a female's decision to choose a specific laying substrate (Lovell *et al.* 2013). Indeed, these authors reported that female quails were contrasting eggshell background colour with the colour of the chosen substrate, only for more maculated eggs (i.e. maculation degree from 34.1 to 66.0 %) compared to the lightly maculated eggs (i.e. maculation degree from 6.3 to 33.9 %) which were using a mixed strategy. My results do not support these findings, as there was no significant effect of egg maculation degree on female choice optimality for the three camouflage variables (i.e. VisRat, Delta E maculation, and Delta E background). Females might still tend to contrast eggshell background colour only for heavily maculated eggs, but because there were eight choices of laying substrates, it might be necessary to repeat the experiment with an increased number of eggs to observe a clear pattern of the effect of eggshell maculation degree on laying choice. An alternative explanation might be that the eggs that I used were generally more maculated (ranging from 16.5 to 83.4 %) than the ones studied by Lovell *et al.* (2013) (ranging from 6.3 to 66.0 %). Thus, the hypothesis that females do not enhance eggshell background/substrate colour contrast for lightly maculated eggs, because there is insufficient maculation to create disruptive colouration, might not be applicable in my focal eggs.

Increasing visual background complexity enhances prey search time (Dimitrova & Merilaita 2010, Dimitrova & Merilaita 2012). In addition, in least killifishes (*Heterandria Formosa*),

displaying body markings that match the heterogeneity of substrate patches may reduce the risk of predation by offering optimal concealment (Kjernsmo & Merilaita 2012). This strategy might also be applicable to eggs, and laying substrate heterogeneity could diminish egg detectability by predators, in particular due to the presence of stones and other materials within the nest area (Colwell *et al.* 2011). Very little is known about the nesting biology of Japanese quails in the wild and females have been observed to use various sites, but preferentially areas with sparse cover. Under semi-captive experimental conditions, Stevens (1961) found that females start nesting in a shallow depression in the ground, and then add some straw or weed stems to the nest after each egg is laid. Thus, not only does substrate colour play a key role in egg camouflage but its pattern and complexity may also help to decrease egg detectability. My preliminary results do not show any benefit of choosing the heterogeneous substrates (i.e. with stones) in term of egg colour matching or outline concealing, except a marginal choice for medium ranks (3-4). In addition, females did not match the size of the stones on the heterogeneous substrates on which they laid, with the size of their egg maculation. This suggests that female quails did not adopt a background-matching strategy (*sensus* Tinbergen 1962) on the heterogeneous substrates.

It is also conceivable that they chose to avoid the heterogeneous substrates as only 34 eggs out of the 128 studied were laid on these substrates. It has been proposed that heterogeneous substrates potentially enhance egg detectability and diminish chances of nest survival (see also Patterson *et al.* 1991), but it is also plausible that because the stones were put on the plain white sand substrate only, the maculation colour was too different from the one of the sand, thus impairing disruptive colouration. It is also possible that females avoided the stones as they may increase the risk of eggshell breakage or it may be an uncomfortable substrate for them to lay. However, I cannot rule out that stones colour and parameters other than substrate colour (e.g. substrate texture) might also be important in female decision, and might explain

why some of the females still chose these particular substrates on which to lay. To note, 27 eggs were laid outside of the experimental arena, which might suggest that the colour and pattern of the floor (blue-green mottled linoleum) conferred some benefit for egg camouflage in some of our focal females, but this might also indicate that some of the females avoided the sand, for the same reasons as they avoided the stones as mentioned above.

In conclusion, my results mainly support previous findings (Lovell *et al.* 2013). Japanese quails adopt a disruptive colouration strategy rather than substrate background-matching to maximise the camouflage of their eggs within the range of substrates that are available to them. I have shown that eggshell colouration is a key component that drives female choice of laying substrate. I used different coloured and patterned substrates which highlighted that their laying choices are repeatable, and that egg camouflage is as an anti-predator strategy even in a species that has been domesticated for a long time. In addition, contrary to animals that adopt behavioural responses to environmental changes and change their body appearance to maximise background matching, quail have to make the choice of nesting area before laying the egg, which suggests that they make laying decision based upon their perceived knowledge of their egg's appearance to maximise clutch survival. This might underline some perceptual and cognitive abilities specific to ground-laying species in the context of egg camouflage and may require further studies to investigate inter-individual abilities of camouflage optimization.

4.6. Chapter Four - Summary and perspectives

In this chapter, I experimentally investigated the choice of laying substrate in a ground-laying species, the Japanese quail. The study of optimality of choice between eight differently coloured and patterned substrates showed that females laid on the substrate that best matched the colour of eggshell maculation and concealed egg outline, but contrasted with eggshell background colouration. However, there was no strong effect of maculation degree on laying choices, and laying on heterogeneous substrates did not maximise egg camouflage. My findings suggest that quails have prior perceptual knowledge of their eggs that allow them to choose the most camouflaging substrate for laying, out of the availability of substrate types. Female quails seem to use substrate colour rather than heterogeneity as a cue for optimizing egg camouflage via disruptive colouration rather than background-matching.

I encourage further studies that investigate not only colour matching but also texture matching between eggs and laying substrates, which may help to understand whether the presence of stones potentially increases the degree of texture matching between eggs and substrate. In addition, eggs are laid in a clutch and when parents leave the nest to forage or for self-maintenance behaviours, it is conceivable that not only individual eggs but also the whole clutch needs to be undetected by predators. Thus, additional experiments investigating clutch visibility on the same substrates may increase our knowledge of the benefits of being laid in a clutch for clutch camouflage.

Chapter Five

GENERAL DISCUSSION

5.1. Thesis summary and implications

Offspring survival is the primary goal for parents to optimise their fitness, and two strategies have evolved in vertebrates to insure embryonic protection: developing inside the mother's body in viviparous species, or laying eggs in a safe environment in oviparous species. In birds, eggs are exposed to both biotic (e.g. bacteria, predators) and abiotic threats (e.g. temperature changes), and one strategy that may help to protect them against predation is to lay a cryptic clutch (reviewed in Kilner 2006). In many species, parents build a dome and use nest materials to conceal and hide the eggs (McCrimmon 1980, Collias & Collias 1984). However, in most ground laying species, matching the colour of the eggs with the laying area background may be the best strategy to decrease egg visibility by predators (Solís & de Lope 1995, Šálek & Cepáková 2006, Mayer *et al.* 2009). Thus, egg crypsis is one of the earliest hypotheses proposed to explain the adaptive role of eggshell pigmentation in birds (Wallace 1889). Besides optimizing egg camouflage, eggshell pigmentation may help achieve egg mimicry and defence against brood parasitism (Moksnes & Røskaft 1995, Stokke *et al.* 2002b), reinforce eggshell structure (Gosler *et al.* 2005), enhance embryo protection (reviewed in Maurer *et al.* 2011) and act as a signal of female immuno-competence towards male (Moreno & Osorno 2003, Hanley *et al.* 2010). Eggshell pigmentation may also be strongly related to female physiological condition due to the physiological properties of biliverdin and protoporphyrin. Many correlative and empirical studies have attempted to investigate the relationship between female condition and eggshell pigmentation, however a large number of unanswered questions remain, especially in species where maintaining a constant eggshell appearance may be crucial for egg camouflage such as ground-laying species like the Japanese quail.

My thesis addressed some of these unanswered questions using an experimental approach to test the effects of changes in the maternal environment such as food availability restriction or exposure to stress hormones, on female physiology, eggshell appearance and pigment concentrations. In addition, I have provided support for preliminary information on the potential role of eggshell patterning in egg camouflage in my study species by experimentally testing to what extent females make specific choices of laying substrates that maximise the crypsis of their eggs.

Both biliverdin and protoporphyrin possess opposite physiological properties, the former being antioxidant and the latter pro-oxidant (Vanore & Batlle 1999, McDonagh 2001). Thus, their deposition into the eggshell may strongly depend on female condition and immuno-competence at the time of laying, and variations in eggshell pigmentation strategy may directly result into changes in eggshell colouration and maculation pattern in spotted eggs. One particular environmental condition that may change during reproduction is the abundance of resource available to the female (Stearns 1992). In Chapter Two, I examined the effect of food quantity restriction on female body condition and eggshell pigmentation. I showed that eggshell pigment deposition strongly depended on female body condition, and that maintaining eggshell reflectance, via manipulation of eggshell maculation, may be a strategy adopted by better females to maximise the camouflage of their eggs. In that context, other studies have experimentally manipulated female environmental conditions via food, calcium or carotenoids supplementation (Moreno *et al.* 2006, Morales *et al.* 2011, Dearborn *et al.* 2012, Hargitai *et al.* 2013) in species laying either blue or brown-spotted eggs. However, I stressed two major limits of these studies: 1) the absence of eggshell pigments quantification speculating that eggshell colour is a direct proxy of its pigment content (see Cassey *et al.* 2012a), and 2) the supplementation of individuals that do not require more nutrients, which might explain the inconsistency of results obtained. Using food restriction as

an experimental manipulation allowed me to induce an energetic challenge in females, which induced a decreased body condition. I proposed that females might face a trade-off between fighting against oxidative stress, while preserving the appearance of the egg to keep it cryptic. The potential relationship between female immuno-competence and the crypsis of their eggs is a novel idea that has never been investigated to date.

Unpredictable or restricted access to food during reproduction can have negative impact on the immune system (Alonso-Alvarez & Tella 2001) and affect individual physiology, inducing weight loss or increased plasma stress hormones, such as corticosterone in birds (Lynn *et al.* 2010). If both biliverdin and protoporphyrin deposition strongly depend on maternal immunocompetence before clutch formation, stress might be one factor that influences the quantity of pigments deposited in the eggshell. In Chapter Three, I experimentally supplemented females with corticosterone and investigated the effects of stress exposure on eggshell appearance and pigments concentrations. I found that eggshell reflectance varied in all individuals, and in particular in stressed females which laid brighter eggshells, but eggshell pigment concentrations remained unchanged. I hypothesised that stress might affect the assimilation of some nutrients such as calcium, modifying eggshell structure and appearance independently from eggshell pigment allocation. Only one recent study has found that females showing a higher stress level (e.g. heat shock proteins concentration) lay more maculated eggs (Martínez-de la Puente *et al.* 2007). Thus it is conceivable that females may face a trade-off between maintaining their egg cryptic while coping with their own physiological stress, at the risk of suffering from long-term effects of stress later in life.

Stress experienced during development may also influence female reproductive performances (Lindström 1999), either via affecting their immune system such as antioxidant capacities (Marasco *et al.* 2013) or potentially affecting the development of reproductive organs and

their function (Hull *et al.* 2007) such as pigment deposition via the shell gland. In addition, an alternative hypothesis proposed that early life stress may be beneficial to the developing embryo, as it may shape its phenotype to be able to cope in a similar hostile environment (Gluckman *et al.* 2007, Monaghan 2008, Mangel 2008). In Chapter Three, I also measured eggshell pigmentation of eggs laid by females that had experienced developmental stress or undisturbed development, and bred under stressful or control conditions. I found that biliverdin and protoporphyrin were differentially affected by developmental stress, depending on the stage of life at which it occurred. I proposed that pre-natal stress may shape eggshell characteristics that play a major role in egg camouflage in Japanese quail, such as protoporphyrin concentration and eggshell maculation. Experiencing pre-natal stress may improve female resistance to stress during reproduction, in particular oxidative stress, thus they would be able to cope better with higher concentrations of protoporphyrin and keep constant eggshell protoporphyrin concentration and maculation, insuring egg crypsis in such stressful breeding conditions. However sustaining high concentrations of protoporphyrin would induce oxidative stress when accumulated in the liver (Afonso *et al.* 1999). Thus, my results provide an evidence for a potential adaptive role of pre-natal stress, but at the risk of suffering from delayed negative effects associated to an increased oxidative stress later in life. In addition, post-natal stress only influenced biliverdin deposition and eggshell reflectance. I proposed that post-natal stress may influence a mother's decision to allocate the antioxidant pigment into the eggshell, at the cost of her own antioxidant response and at the risk of long-term consequences. This assumes that biliverdin could confer benefits such as antibacterial or solar protection properties to the offspring under hostile conditions (reviewed in Maurer *et al.* 2011). Alternatively, biliverdin deposition could be a passive process that depends on female antioxidant capacity and on the circulating pigment concentration. Thus, female with enhanced antioxidant capacity may not need to use biliverdin as a main

antioxidant, would possess higher circulating concentration of the pigment, and thus would passively deposit higher amounts into the eggshell.

If eggshell pigmentation strongly depends on maternal condition in Japanese quail, any change in a mothers breeding environment may be vital for clutch survival in such a ground-laying species in which laying camouflaged eggs might be the only strategy to hide them from predators. But what parameters are used by female Japanese quails to choose the laying substrate that will diminish the detectability of their eggs? Lovell *et al.* (2013) recently gave the first insights into egg camouflage strategies in quail, via an experimental demonstration of laying substrate choice, and showed that females use disruptive colouration strategy to maximise egg camouflage. However, not only the colour but also the heterogeneity of the substrate might play a key role in female laying choices and enhance egg/nest-background matching, hence maximising egg crypsis (Colwell *et al.* 2011). In Chapter Four, I provided female Japanese quails the choice between 8 laying substrates, plain or patterned (i.e. heterogeneous) with differently coloured and sized stones. I found that females laid on substrates that matched the colour of eggshell spots, contrasted eggshell background colour and concealed egg outline, independently of eggshell maculation degree. In addition, females did not choose the heterogeneous substrates in order to match stones size with eggshell spots size, according to the specific model used. I proposed that disruptive colouration have evolved preferentially as strategy to maximise egg camouflage in quails.

5.2. Study limitations and areas of future research

My study has shown that eggshell pigmentation in Japanese quail strongly depends on the environmental parameters that influence the condition of the breeding female, and the relationship between eggshell pigment concentrations and its appearance is complex. This has

vital importance, particularly in species where eggshell appearance is involved in offspring survival via egg camouflage. My results suggest that both eggshell maculation pattern and reflectance are keys factors of egg crypsis in quails, and may be the result of trade-offs between resource allocation to immune response and eggshell pigmentation. However, a few limitations to my study can be noted and require further investigation.

One major assumption in eggshell colouration studies is the physiological role of both protoporphyrin and biliverdin. However, no study has yet investigated experimentally the effect of both biliverdin and protoporphyrin on female oxidative stress and antioxidant capacities, and has related female pigments concentrations to the one of the eggshell. In addition, further studies should restrict maternal dietary antioxidant and measure pigments in both their plasma and eggshell, to help to elucidate the relationship between eggshell colouration and female oxidative stress in both blue and brown-spotted eggshells layers.

A second limitation is related to the methodology, in particular the quantification of pigments in the whole eggshell. As my results showed, the relationship between eggshell maculation, its reflectance, and its pigments concentrations is complex. Eggshell colouration in Japanese quail cannot be used as a direct proxy of its pigment content, which questions other studies that based their main findings on eggshell colouration with no pigment quantification. Additional studies should quantify pigments allocation in different zones of the eggshell independently (i.e. eggshell spots and background) in both blue and brown-spotted eggshells, to allow to clarify which proportion of colour variation is due to actual pigment quantity changes.

The main assumption of my study is that maintaining eggs that are cryptic to predators may be the main role of eggshell pigmentation in Japanese quail, and that they try to maximise it even under stressful breeding conditions. It is now known that Japanese quails can recognize

their eggs (Pike 2011) and that they 'know' the maculation characteristics of their eggs enough to be able to choose the laying substrate that is optimal for egg camouflage (Lovell *et al.* 2013). However, whether eggs that are laid on optimal substrates are visually less detectable by a predator visual model remains to be tested. Further studies should use artificial predators to assess whether the chosen substrates actually decrease egg detectability, taking into account predator vision. In addition, not only individual eggs are detected but also the whole clutch once clutch completion is reached. Thus it is possible that laying a clutch containing eggs of a similar appearance might enhance crypsis, similarly to the strategy used by host species to recognise brood-parasite eggs (reviewed in Kilner 2006). In species such as the Japanese quail where intra-female variability is low in terms of egg patterning (Chapter Two, section 2.4) and colouration (Duval *et al.* 2013), it is possible that clutch background rather than nest background plays an important role in individual egg detectability. As more eggs are laid the nest area may look less like the laying substrate and more like a collection of eggs (Fontaine & Martin 2006). Alternatively, laying similarly patterned and coloured eggs close together might disrupt the detectability of the edge of each egg, and potentially that of the entire clutch. Thus, an additional experimental study on clutch detectability would help to clarify if there is a benefit of being laid in a clutch in term of predator avoidance.

5.3. Conclusion

In conclusion, I have experimentally demonstrated in a ground-laying species that eggshell pigmentation is a dynamic trait, and that both eggshell colour and maculation are independently affected by female environmental conditions changes. Eggshell patterning might have evolved to maximise egg camouflage in heterogeneous habitats in quails. If egg crypsis relies on such fluctuating characteristics, any change in eggshell appearance during breeding may impair egg crypsis, and may be perceived by conspecifics and in particular by

the male. Further interdisciplinary research should investigate egg crypsis in the context of sexual selection. Indeed, egg crypsis may be an honest indicator of female health that males may use as a post-mating sexual signal. To maximise fitness, a male may increase his paternal effort, such as nest defence, with a female that is able to best camouflage their eggs, maximising the survival of the clutch. This hypothesis has never been tested so far and my results open a new research area, requiring further experimental manipulation of female camouflage ability and male behaviour in response to the degree of camouflage of the clutch and the predation risk within the nesting area.

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