CAUSES AND CONSEQUENCES OF VARIATIONS IN EGGSHELLS IN THE LESSER BLACK-BACKED GULL

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Declaration

I declare that the work recorded in this thesis is entirely my own unless otherwise stated, and that it is of my own composition. No part of this thesis has been submitted for any other degree.

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Abstract

Variations in egg size and egg composition between females of the same species, as well as among eggs in the same clutch, have been studied in many avian species. The eggshell serves crucial functions in avian reproduction such as protection of the embryo from mechanical damage and from the invasion of micro-organisms, source of calcium to the embryo, control of gas exchange with the environment and conservation of water. But little attention has been paid to variation in eggshell, especially within-clutch variation. This thesis focuses on variations in eggshell characteristics in relation to laying order in a single species, the lesser black-backed gull (*Larus fuscus*).

In order to evaluate a proper method for measurement of eggshell characteristics, this thesis used more than one technique to measure shell thickness, shell porosity and shell coloration. For the measurement of shell porosity, two techniques for counting pores were validated for the first time in this thesis.

This study found within-clutch variations in shell porosity, mammillary layer contact area and shell coloration but not in shell thickness. The last-laid egg had a larger mammillary layer contact area and often had paler shell colour and streaks on the shell. This study found some relationships between shell structures and shell coloration. A calcium-supplementation experiment was used to investigate whether the shell formation is limited by calcium-availability. This thesis found effect of calcium-limitation on shell thickness, but no effect on shell background colour.

Chapter 1: General Introduction

All birds use eggs to develop their embryo outside the mother's body. The survival of their embryo means that their genes have more chance to be passed to the next generation. So bird eggs have to allow optimal conditions for embryo development, such as to store sufficient resources for the developing embryo, have sufficient space to be able to manage the waste produced by the embryo, control the interior physical environment, and to be an effective shelter for the developing embryo. The eggshell serves many functions. Birds have different forms of eggshell that may serve some different functions according to the necessity of each species. Even within a population, we can find variation in some traits of the eggshell that may influence some functions of the eggshell. I studied the variation in the characteristics of the eggshell of the lesser black-backed gull (*Larus fuscus*), to try to understand the causes and consequences of the observed variation in eggshell characteristics.

In this introduction I will firstly describe the basic structure of bird eggshells, secondly I review the function of the eggshell and finally I explain the research undertaken on the shells of the lesser black-backed gulls.

Structure of the eggshell

For my study I will assume that the true shell consists of five regions; the mammillary layer, the cone layer, the palisade layer, the surface crystal layer and the shell accessory materials (Solomon *et al.*, 1994). Romanoff and Romanoff (1949) showed that the eggshell of the domestic chicken (without shell membrane) consists of about 98.4 % solids and 1.6% water. The solid part contains about 95.1% of

inorganicmatter and 3.3% of organic matter (protein and some traces of lipid). Inorganic matter consists of 97.37 – 98.84 % of calcium hydroxyapatite, 0.44 – 1.88 % of magnesium carbonate, and 0.52 – 0.75 % of tricalcium phosphate. I will describe the different parts of the shell starting from inside towards the outer surface of the shell (Fig. 1.1).

The shell membrane

The shell membrane has two parts, an inner and an outer membrane. They adhere to each other except at the blunt end of the egg, where they separate to form the airspace. These membranes look like a mat of fibres, the inner membrane is in general finer and smaller meshed than the outer membrane. The surface of fibres may be smooth or have buds of variable size (Becking, 1975). The outer membrane has three distinct layers (Romanoff and Romanoff, 1949), whereas the inner membrane has only two layers (Molan and Hale, 1936 cited in Romanoff and Romanoff, 1949). The inner membrane envelopes the albumen. The outer membrane is attached to the true shell (Romanoff and Romanoff, 1949).

The basal cap and cone layer

Mammillary cores are distributed uniformly over the outer surface of the outer shell membrane (Becking, 1975). The mammillary cores consist of organic matter (Simkiss, 1958 cited in Becking, 1975). Abnormalities of mammillary cores can decrease the thickness of the true shell (Solomon, 1991). At the mammillary cores, crystals of calcite attach and grow radially in all directions. Calcite crystals which grow inward and sideways produce the basal caps, and those which grow outward to meet crystals from other centres of crystallization form the cones. The shape of the

cones is irregular and they fit together like a jig-saw pattern which is continued in the columns forming the palisade layer (Becking, 1975). In the domestic chicken egg, the basal cap and cone look like a conical knob (Romanoff and Romanoff, 1949).

The palisade layer

The palisade layer is a thick calcium carbonate layer that is crystallized from the top of the cone layer (Beking, 1975). The palisade layer makes up about two-thirds of the thickness of the calcified eggshell, and is responsible for the main strength of the shell. The thickness of the palisade layer is affected by the density of the mammillary cores. If they are wider apart, neighbouring cones take longer to meet and therefore the palisade layer is thinner (Solomon, 1991). The upper part of the palisade is compact but there are many randomly distributed pits, like swiss cheese, in the lower part (Fig. 1.1). These are larger and more numerous in the eggshells of tropical birds than in the eggshells of temperate birds. The function of these pits is not yet understood (Becking, 1975). Within the palisade layer, there are vertical canals, the pores that connect the inside with the outside environment.

The pore canals originate between the cones, extend radially across the palisade layer and terminate at the outer surface of the true shell. Board *et al.* (1977) and Board and Scott (1980) classified pores according to two criteria: (A) The covering of the pore at the outer surface and (B) the shape of pores:

(A) Covering of pores

1. Simple pore system – a tube opens at both ends

1.1 Unbranched pore canals can be found in the eggshell of the wood pigeon (*Columba palumbus*) and the collared dove (*Streptopelia decaocto*).

1.2 Branched and unbranched pore canals can be found in the eggshell of ostrich (*Struthio camelus*).

2. Occluded pore systems – the outer surface of the shell is coated with unidentified material. Fissures in the material traverse the outer pore orifice.

2.1 Unbranched pore canals can be found in the eggshells of the common gull (*Larus canus*) and the herring gull (*Larus argentatus*).

2.2 Branched and unbranched pore canals – no example

3. Plugged pore systems – the outer orifice contains a plug of organic or inorganic material.

3.1 Unbranched pore canals – no example

3.2 Branched and unbranched pore canals can be found in the eggshells of the greater rhea (*Rhea americana*).

4. Capped pore systems – the outer orifice is covered with a stratum of spheres formed from organic or inorganic material.

4.1 Unbranched pore canals can be found in the eggshells of the gannet (*Sula bassana*) and the king penguin (*Aptenodytes patagonica*).

4.2 Branched and unbranched pore canals can be found in the eggshell of the emperor penguin (*Aptenodytes fosteri*).

5. Reticulate pores – the outer portion of the palisade layer is modified to have small holes in the shell surface.

5.1 Unbranched pore canals can be found in the eggshell of the osprey (*Pandion haliartus*) and the open-billed stork (*Anastomus oscitans*).

5.2 Branched and unbranched pore canals can be found in the eggshell of the cassowary (*Casuarius casuarius*).

(B) Shape of pores

1. The simplest pore shape is a funnel. The channel of the pore is often narrower than the opening at the shell surface outside the shell.

2. Branching. The pore canals of eggs of birds, such as ducks, rheas, cassowary, penguins, ostrich, extinct moas, and *Aepyornis* can have more than one branch (Tyler and Simkiss, 1959; Tyler, 1964 cited in Carey, 1983). The pore canals in the eggshell of most other birds are unbranched. Primitive birds generally have branched pores and modern birds mostly have unbranched pores.

The materials that occlude, plug, cap, or reteculate the pores can be termed generally as "shell accessory materials" (Board and Scott, 1980).

The shell accessory materials

Where the shell accessory material is predominately inorganic or organic, the terms "cover" or "cuticle" are used respectively (Sparks, 1994). Vaterite (one of polymorph of calcium crabonate) is found in most covers (Sparks, 1994). The tranformation of calcite into vaterite during the formation of shell covering is possibly the result of the addition of phosphate to the oviducal fluid during the terminal stage of shell formation (Tullett *et al.*, 1976 cited in Carey, 1983). Board *et al.* (1977 cited in Sparks, 1994) reported that all the eggshells examined from seabirds, except gulls, have a vaterite cover. In domestic chickens, the main chemical components of the

cuticle are glycoproteins (Wedral *et al.*, 1974 cited in Sparks, 1994). Solomon (1991) proposed that the thickness of cuticle varies with age, breed and environment. Ball *et al.* (1975 cited in Sparks,1994) proposed that cuticle quality may be heritable. In the layer of shell accessory material, we can find the pigments that create the colour of the eggshell.

The pigments

Brown eggshells usually have porphyrins whereas blue eggshells usually have biliverdin (a blue-green pigment formed as a by product of hemoglobin breakdown) and maculation is always produced by protoporphyrin (a brown pigment that is a natural metabolite intermediate in the biosynthesis of haem) (Kennedy and Vevers, 1976). But porphyrins are also found in white shells, but in lower concentrations (Solomon, 1991). Eggshells of the herring gull (*Larus argentatus*) and the black-headed gull (*Larus ridibundus*) have protoporphyrin and biliverdin but zinc biliverdin chelate has also been found in the herring gull eggshells (Kennedy and Vevers, 1976). Whether the pigments are derived directly from the blood or are synthesized in the shell gland pouch is still under debate (Solomon, 1991), but is more likely bio-synthesized in the oviduct (With, 1973).

Eggshell colour can vary between eggs laid by the same individual. Mikšík *et al.*(1994) found that in clutches of the red-backed shrike (*Lanius collurio*) the amount of porphyrin decreased through the laying sequence, decreasing towards the last egg. Solomon (1991) reported that in domestic chickens shell colour changed with age, but did not state the direction of change. Stress during egg laying, with its associated hormonal disturbances, is a likely cause of the paleness (Solomon, 1991).

Gosler *et al* (2000) reported that the pigmentation pattern of great tit (*Parus major*) eggs is heritable.

It is generally believed that pigmentation has functions as camouflage (Solomon, 1991). But many hole nesting birds (eg. great tit and treecreeper) still produce maculated eggs. Probably, there may be another function of spots on the eggshell. Nowadays, some researchers have found more functions of shell coloration. Bakken *et al.* (1978) found protoporphyrin in shell colour could reflect light in the near-infrared very well. Moreno *et al.*, (2004) found that the eggshell colours influence paternal care in species with biparental care. Higham and Gosler (2006) reported that eggshell pigment also had a significant effect on water loss in small passerines. Gosler *et al* (2005) found that the pigment on the shell of great tit eggs may not work for camouflage but served a structural function in Ca-limited areas. Solomon (1991) suggested that the structure of porphyrins was similar to phthalocyanine lubricants that are used in solid-state engineering. The porphyrins may act like a cushion between the calcite crystals making the shell more resistant to cracking. Biliverdin is the pigment causes blue-green coloration in eggshells. Moreno *et al.* (2006) found a positive correlation between the intensity of biliverdin and the condition of the laying female in the pied flycatcher (*Ficedula hypoleuca*).

The evolution and origin of avian eggshells

Most reptiles, like all birds, lay eggs, but some characteristics of their eggshells differ from those of birds. Eggshells of reptiles are not coloured and most of them are not hard. A well preserved fossil eggshell of the theropod dinosaur *Troodon formosus* shares some traits with the eggshell of both fossil and recent birds, such as

fibres associated with eisospherites (organic membrane at the innermost of the mammillary layer) attached to the bases of the mammillae (the innermost of the calcified portion of the eggshell); and fine radiating crystals emanating from a central core that forms the spherulite (an ubiquitousform of calcium crystal is characterized by radial growth leading to spherical symmetry) and grades into the coarse, blocky wedge of the mammillae (Zelenitsky *et al.*, 2002). Packard and Packard (1980) suggested that among early reptiles ancestral to birds, more calcified shell membranes improved hatching success because they protected the embryo against attacks from insects and microorganisms in the soil. The eggs of ancestral reptiles, without the calcareous material on the surface of the shell membrane, also needed water from the environment to sustain embryonic development (Gray, 1928; Needham, 1931 cited in Packard and Packard, 1980). Eggs that evolved to collect larger quantities of water and increased the thickness of the shell at oviposition led to reductions in transpirational water loss, and in predation by soil invertebrates and infection by microbes during incubation. This may have pre-adapted avian eggs for incubation in dry environments, independent of a moist environment (Packard and Packard, 1980). As a consequence, birds could colonize new habitats where reptiles were unable to breed.

Formation of the eggshell

Figure 1.2 shows a diagram of the oviduct during egg formation. The process of eggshell formation begins in the isthmus, after the egg receives their albumin from the magnum. The isthmus produces the paired inner and outer shell membrane. These membranes are composed of interlacing protein fibres of variable diameter. The fibres are the product of the gland cells located in the isthmus (Solomon, 1991).

The relatively finer, more compact inner shell membrane, and the loosely woven outer shell membrane, can be easily distinguished (Romanoff and Romanoff, 1949). In the isthmus, carbohydrate and water are added to the albumen to make the membrane taut. The mammillary cores, which are organic matter, are attached to the surface of the outer shell membrane in the distal end of the isthmus (Erben, 1970; Wyburn *et al.*, 1973 cited in Carey, 1983). The first calcium and other mineral crystals deposit onto the mammillary cores, which act as specific nucleation sites in the tubular shell gland. This establishes the mammillary layer of the shell. Before being laid, the egg will spend about 20 hours further shell formation in the shell gland pouch (Solomon, 1991). Calcium crystals continue to be deposited onto the mammillary layer, and rise to form the cone and palisade layers respectively. As a consequence of some calcium crystals not fusing during the stage of palisade formation, numerous pores are generated in the palisade layer (Solomon, 1991).

Different patterns of calcite deposition can be found in eggshell. Simkiss (1964) (cited in Carey,1983) hypothesized that secretion of phosphate into the oviducal fluid could affect further crystal growth. Variation in the chemical composition of the oviducal fluid might also result in the different pattern of calcite deposition that occurs as the palisade layer becomes overlaid by the surface crystalline layer. The shell accessory materials are added onto the surface crystalline layer as the final step prior to laying (Simons, 1971 cited in Carey, 1983). Within this layer the bulk of the pigment is deposited (Solomon, 1991). Solomon (1991) also hypothesized that the variable pH of oviducal fluid between the oviduct epithelium and the calcified shell causes the streaks and patches of pigments on the eggshell.

The functions of eggshell

Eggshell serves 5 crucial functions:

1. **Strength:** The eggshell must be strong enough to support the mass of the egg contents and to avoid damage from the incubating parents or from predation to some extent. At the same time it must be weak enough to allow hatching (Board, 1982; Carey, 1983). Bain (1991) reported that the removal of each layer of the cuticle, vertical crystal layer, and palisade layer significantly decreased the stiffness of the remaining shell of domestic chicken, but the removal of the mammillary layer had no effect on the stiffness.

2. **Mineral source:** Blom and Lilja (2004) reported that the eggshell mainly supplied minerals for the skeletal development of the embryo. Johnston and Comar (1955) found the developing embryo utilized the eggshell as a major calcium source. The mammillary layer which is in contact with the shell membrane functions as a provider of minerals (mainly calcium and magnesium) (Bond *et al.*, 1988; Blom and Lilja, 2004).

3. **Protection against infection:** Pores in the shell can be used by micro-organisms as entrances to the egg content (Cook *et* al., 2003). Without cuticle, the egg appears to be susceptible to the invasion of micro-organisms (Board and Fuller, 1974).

4. **Shape of the egg :** Egg shape may be an adaptation to packing eggs most effectively into the nest or to the nest location. The pear-shaped egg of the guillemot rolls like a top, so it is unlikely to roll off the narrow nest cliff edge where they nest. The pear-shaped eggs of waders can easily pack together in nest and are easy to

incubate (Board, 1982). Barta and Székely (1997) reported that egg shapes seemed to be adapted to meet the efficient use of the brood patch area of the incubating birds.

5. **Gas transport :** Normally, the egg loses weight by releasing H_2O and CO_2 through respiration, but in the same process the egg gains O_2 from the outside. The contribution of CO_2 and O_2 cancel each other out, so that overall the egg loses mass through the loss of water (Rahn and Ar, 1974). So, the shell may be adapted to trade-off between water conservation and gas exchange. Baker and Baker (1992) reported that abnormal porosity affected water vapour and may have affected hatchability of budgerigar eggs.

The effect of the environment on eggshells

Organochlorine residues from pesticides caused the decrease of eggshell thickness in some species of raptors (e.g. Cade *et al.*, 1971; Olsen *et al.*, 1993) some seabirds (e.g. Burger *et al.*, 1995) and four species of thrushes (*Turdus* spp.) in Britain (Green, 1998). Organochlorine residues also affected the eggshell thickness of the herring gull (*Larus argentatus*) at Lake Erie, and eggs with thinner shells were more likely to be crushed during incubation (Wesloh *et al.*, 1990). Lundholm (1997) suggested that DDE may interrupt the process of calcium transportation across the eggshell gland mucosa. Apart from the effect of organochlorine residues, anthropogenic acidification of soil also is an effective cause of decreasing eggshell thickness. Graveland (1996) reported that acid rain caused a decline in snail populations. As snailshells are the main source of calcium for great tits during egg-formation and resulted in an increase of eggshell defects, abnormal pigmentation, and hatching failures in these birds breeding in calcium poor soils in the Netherlands.

The gull eggshell

There have been a few studies on gull eggshell. Board *et al.* (1977) reported that the common gull (*Larus canus*) and the herring gull (*Larus argentatus*) had eggshells with unbranched pore canals and occluded outer pores. Kennedy and Vevers (1976) reported that protoporphyrin and biliverdin were the pigments responsible for the eggshell colour of the herring gull and the black-headed gull (*L.ridibundus*). Ar *et al.* (1979) reported that the shell thickness of the black-headed gull (*L.ridibundus*) was about 231 micrometer. Mänd (1996) found that in eggshells of the black-headed gull (*L.ridibundus*), the pore density of the replacement clutches was higher than that of the initial clutches. Mänd (1996) also found that the number of pores increased during the first seven days of incubation.

The lesser black-backed gull normally lays a three-egg clutch (Royle and Hamer, 1998; Nager *et al.* 2000; Verboven *et al.*, 2003; Muck and Nager, 2006). Earlier studies showed that the third egg is the smallest egg and had the lowest amount of yolk and albumen in the clutch (Bolton *et al.* 1992; Nager *et al.* 2000; Verboven *et al.* 2005), and many other difference in egg composition, such as levels of carotenoids (Blount *et al.*, 2002) and androgen (Verboven *et al.*, 2003). The third or the last-laid eggs hatch later and the chicks have a higher rate of post-hatching mortality than the chicks hatching from the first two eggs in the clutch (Royle and Hamer, 1998). However, the intraclutch variation of egg composition may not be the only causation for the "third-chick disadvantage". Possibly, the within clutch variation of eggshell quality may also be the causation. Muck and Nager (2006) suggested that there may also be strategic changes in egg contents in that the composition of the egg contents may necessitate changes in the structure of the shell

(Massaro and Davis, 2004 and 2005). However, we know little about intraclutch variation in eggshell quality, which is the subject of this thesis.

As well as the structural feature of the shell, the pigmentation may also play a role in shell quality. The lesser black-backed gull is a ground nester having maculated eggs. Blanco and Bertellotti (2002) found that the eggshell colouration may serve as camouflage of the eggs in the South American tern (*Sterna hirundinacea*). But camouflage may not be the only function of the eggshell colour. I have unpublished data from an egg-swapping experiment suggesting that background colour of the lesser black-backed gull eggs were not related to egg survival. Gosler *et al.* (2005) proposed that the pigmentation may be used to strengthen the eggshell in the situation of calcium deficiency. Heaney *et al.* (1998) suggested that, even in a seabird, calcium supply may be limiting and calcium limitation may cause intraclutch variation of eggshell thickness in the common tern (*Sterna hirundo*). However, we lack knowledge of variation of eggshell colour within laying order, and the effect of calcium limitation on eggshell quality in the lesser black-backed gull, and these aspects will also be discussed in this thesis.

Outline of the thesis

In order to evaluate different methods of counting pores in the eggshell of the lesser black-backed gull, **chapter 2** compares two techniques for counting pores, counting pores directly through a light microscope and counting dyed pores through a light microscope. I then compared pore densities to water conductance. The first technique was selected to count pores in this thesis. Earlier studies already found clear differences in composition of egg contents in relation to laying order in this species.

So, here I try to answer whether there were also changes in the eggshell associated with these changes in egg contents. **Chapter 3** investigates whether there is any variation of eggshell structures with laying order by looking at the variation in shell thickness, porosity, and mammillary layer contact area between egg order? **Chapter 4** considers variation of eggshell coloration in relation to laying order. I used two techniques to measure background colour; using digital image and spectral analyses. I also investigated the spot characteristics of the eggshells.

After obtaining some knowledge of eggshell structures and eggshell colorations from the two previous chapters, I tried to relate eggshell colour with eggshell structure as predicted by Gosler *et al.* (2005) in structure-function hypothesis in **Chapter 5.** **Chapter 6** looks at the effects of calcium availability on eggshell characteristics and colour. So, this chapter investigates whether shell formation in lesser black-backed gulls is limited by calcium availability by using a calcium supplementation experiment? **Chapter 7** discusses what has been learned on the within-clutch variation in eggshell characteristics of the lesser black-backed gull.

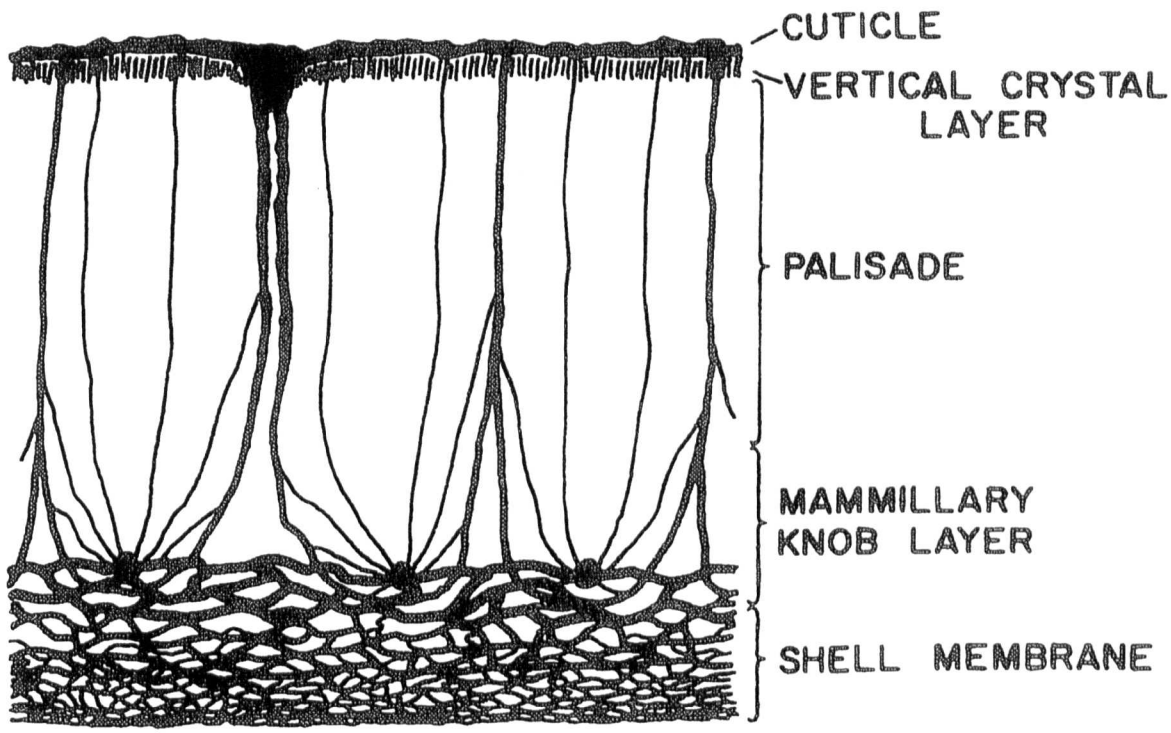

Figure 1.1. Line–drawing of the ultrastructure of the general eggshell. (from Parsons, 1982).

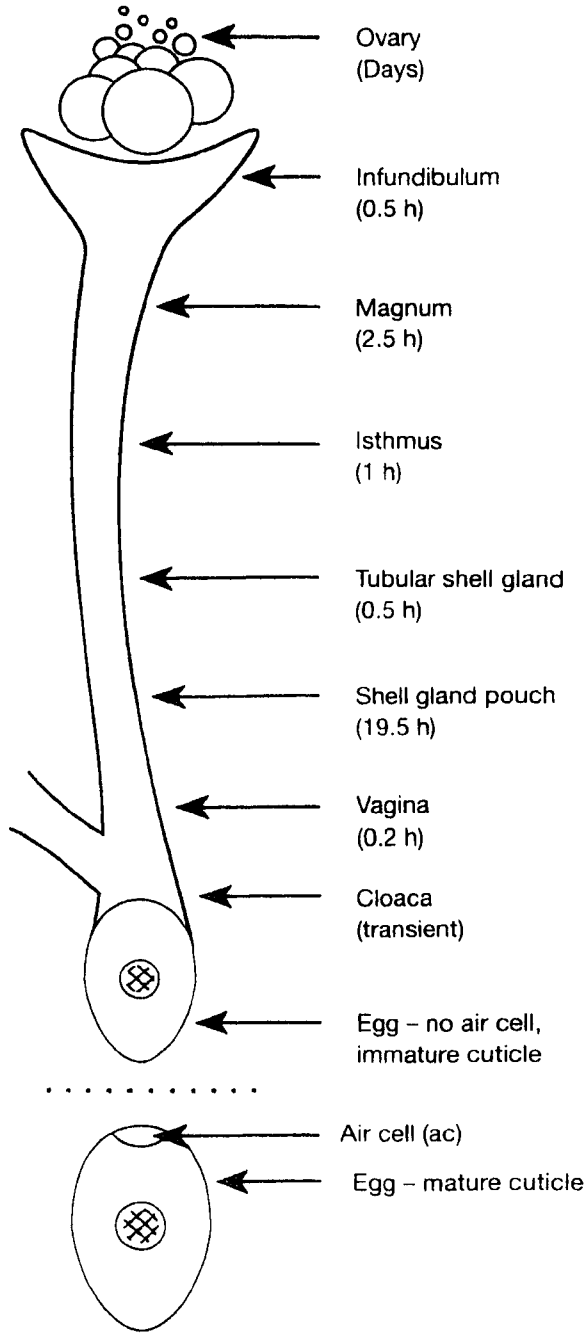

Figure 1.2. A diagram of egg formation in the oviduct (adapted from Board and Fuller, 1994)

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Chapter 2: A validation study of shell porosity measurements

Abstract

During incubation, the egg normally loses weight through loss of water. The rate of water loss should correlate positively with shell porosity. This study uses this relationship to validate two different techniques for counting pores; (A) counting pores directly through a light microscope and (B) counting dyed pores through a light microscope. Technique B seemed to have a problem with counting high pore densities. However, even pore counts from technique A showed only a weak relationship with water loss. Possible reasons for this are discussed.

Introduction

The egg has a tightly controlled water budget during embryonic development. Water is deposited in the albumen and yolk, and during incubation the embryo also produces water as a by-product of its metabolism (Ar and Rahn, 1980). On the other hand, water normally diffuses out of the egg along a water vapour gradient between the inside and the outside of the egg. The water loss may help to expand an air cell for pulmonary respiration of the embryo (Carey *et* al., 1983) and controls the optimal level of water inside the egg (Ar and Rahn, 1980). When there is a lack of water, the embryo may face difficulties in using some essential soluble substances such as proteins and carbohydrates for development (Romanoff and Romanoff, 1949). The suboptimal rates of water loss can cause embryo mortality, but the mechanisms are not clearly known (Carey, 1986).

The water evaporation from the egg is determined by the shell's water conductance. Conductance is the permeability of the shell to water vapour and depends on functional pore area and the length of pore as shown in the equation of Ar *et al.* (1974);

$$M_{H_2O} = c * D_{H_2O} * Ap/L * \Delta P_{H_2O}$$

M_{H_2O} = the rate of weight loss (mg. day^{-1})

ΔP_{H_2O} = water vapour pressure difference across the shell (torr)

D_{H_2O} = diffusion coefficient of water vapour in air (cm^2. sec^{-1})

Ap = total functional pore area (cm^2)

L = length of pores (cm)

c = conversion constant, $155.52 * 10^7 / (R * T)$ where the numerator has the units of sec.mg .day^{-1}. $mole^{-1}$, R = gas constant ($6.24 * 10^4$ cm^3.torr.$mole^{-1}$. $°K^{-1}$) and T = absolute temperature (°K).

Normally, the egg loses weight by releasing H_2O and CO_2 through respiration but in the same process the egg replaces the lost weight of CO_2 with O_2 from the outside. The contribution of CO_2 and O_2 cancel each other out, so that overall the egg loses mass through the loss of water (Rahn and Ar, 1974). If controlled for the water vapour gradient, the water loss from the egg should have a positive relationship with shell conductance, but a negative relationship with the length of pores. The measurement of the mass loss of the egg (controlled for the water vapour gradient) can be used as an indicator of the resistance of the shell to water loss (Ar *et al.* 1974). The pore is a vital part of the gas exchange and the variation in number and structure of pores can affect embryo development.Two techniques are currently available for counting pores in different species of birds; directly counting pores (Mänd, 1996; Massaro & Davis, 2005) and counting dyed pores (Monge *et al.*, 2000; Massaro and

Davis, 2004).Tyler (1953) reported that the merging of dye between closely adjacent pores and possible blocking of pores could result in an underestimation of porosity when using dyes to count pores. We have little knowledge of how the counts of the two techniques relate to each other.

The aim of this study was to validate the two techniques to measure shell porosity of eggshells of the lesser black-backed gull (*Larus fuscus*). For the same eggs, I measured water loss from the egg under controlled conditions and then counted the number of pores by using the two methods. I then validated the pore counts by using the predicted relationship between shell porosity, shell thickness and water loss from the egg. Water loss from the egg should be positively related to pore count and negatively related to shell thickness.

Materials and methods

I collected 30 fresh eggs, 10 A-eggs, 10 B-eggs and 10 C-eggs from 30 different 3 egg clutches at Walney Island during the breeding season of 2004 (under a license from English Nature). I measured their egg weight to the nearest 0.01g using an electronic balance and measured length and width with a calliper to the nearest 0.1 mm on the day the clutch was completed. In order to measure egg weight loss in a standardized way, I put all eggs into the same environmental conditions. I put each egg individually into a home-made desiccator (8 cm x 10 cm x 10 cm) that was filled with 40 g of silica gel at the bottom (according to Rahn and Dawson, 1979). I put the desiccator in an incubator set at a constant temperature of 25°C on the day the clutch was completed. I weighed the eggs after 5 hours in the incubator, and then I weighed the eggs again after 6 days (the silica gel at the bottom of the desiccator

still had the capacity to take up water). I calculated the daily rate of water loss by dividing the weight loss by 6. Ar *et al.* (1974) suggested that using permeability should be more suitable than weight loss to compare the rate of water loss within a species. I therefore calculated the permeability by dividing the daily water loss by surface area of the egg (Carey *et al.*, 1983). The surface area was calculated by using the equation $A = 4.951 \times V^{0.666}$ (V = egg volume in cm^3) (Paganelli *et al.*, 1974); egg volume was calculated by using the equation $V = K_V \times L \times B^2$ (L = length in cm, B = width in cm, K_V (volume coefficient) = 0.4965, as the average from *Larus* species (Hoyt, 1979). I then separated the egg content from the eggshell and left the eggshells to dry at room temperature for a few days. Later, the shells were dried again in an oven at 50°C until they reached a constant weight. I also measured the water content of the egg. The difference between the fresh egg mass and the sum of the dry weights of yolk, albumen and eggshell gave the amount of water in the egg.

Shell thickness

Due to the difficulty of measuring actual pore length, I assumed that shell thickness can provide a convenient index for pore length (following Ar *et al.*, 1974). In order to avoid problems with heterogeneity of the eggshell (Romanoff & Romanoff, 1949; Tyler, 1961; Gosler *et al.*, 2005; Massaro & Davis, 2005), I selected shell pieces from the equatorial zone (figure 3.1) as this is the largest area of the eggshell. Shell thickness was measured by using a Scanning Electron Microscope (Hitachi S-570, E. M. Systems Support, High Peak, UK). A piece of shell ($\approx 1 cm^2$) was cut from the eggshell at the equatorial zone by using a diamond tipped circular saw (Quayle Dental, Worthing, Sussex, UK). This method ensured that the structural integrity of the specimen was retained (M. Bain, pers.comm.). In order to avoid any distortion

effect from the cutting process on shell thickness, the sample was snapped in two and one piece was mounted vertically on a grooved aluminium stub with Plastic Conductive Carbon Cement (Leit C Plast, Gisbourne Microscopy Services, Brocton, Stafford, UK) by placing the snapped size uppermost. The mounted shells were coated with a gold/palladium mixture for 4 minutes in an Emscope sputter coater (SC500, Emitech, Ashford, Kent, UK) and viewed with the scanning electron microscope at 15 kV. The specimen was viewed at a magnification of 200X at a constant working distance of 30 mm. The scanning electron micrograph was saved on a computer and I measured the distance from the tips of the mammillary layer to the bottom end of the cuticle as shell thickness by using the High Resolution Digital Imaging System Version 2.05 software (E. M. Systems Support, High Peak, UK) to the nearest 0.001 μm.

Shell porosity

I measured shell porosity by using 2 techniques, counting pores directly through a light microscope (named technique A) and counting dyed pores through a light microscope (named technique B). I used two samples from each shell, two pieces of $1cm^2$ of shell were cut from the equatorial zone of each egg by using a diamond tipped circular saw. For technique A, the piece of eggshell was flooded with Decalcifier II (Surgipath, Bretton, Peterborough, UK.) for 2 minutes and then put into water for a few seconds to stop the reaction in order to peel off the shell membranes. To make the pores visible, the eggshell was flooded again with Decalcifier II for 5 minutes and then put into water for a few seconds and the remaining shell membrane was cleared using point-tip forceps. The pores in a known

area of a dry eggshell (23.768 mm^2), on the outer side of shell, were counted under a dissection microscope.

After counting the shells were kept in a small plastic bag for using in the second technique. For technique B, the shell was flooded with methylyne blue (100 ml of concentrate methylene blue solution / 1000 ml of distilled water) on the outer side. The methylene blue ran through the pores to the inner side of shell and stained the area around the pores in the forms of tiny dark blue dots. After the dye dried on the outer shell, the tiny dark blue dots in a known area of the inner shell surface (23.768 mm^2) were counted under a dissection microscope. Both techniques were applied on two pieces of shell for each egg. The correlations of the porosity count from the same egg were $r_{28} = 0.712$, $P < 0.001$ (technique A) and $r_{28} = 0.193$, $P = 0.306$ (technique B).

Statistical analysis

I used a broken stick model (Huizingh, 1994) to relate the data from the two pore counting techniques. I carefully selected the threshold point from the model with the lowest residual square value. All tests were two-tailed and $P < 0.05$ was considered significant. Mean values ± S.E. were reported.

Results

There was a weak, but significant positive correlation between the pore counts from the two techniques (Spearman, $r_s = 0.379$, df = 28, $P = 0.039$) (Fig. 2.1). Technique A gave a higher pore density (45.30 ± 3.93 pores / cm^2, n = 30) than using the technique B (36.46 ± 3.38 pores / cm^2, n = 30; pair t-test: $t_{29} = 2.08$, $P = 0.047$). Figure 2.1 suggested that possibly, the two techniques may not relate linearly to each

other. It might be that technique B underestimated the pore count at high pore densities, because at high pore density the chance that the dye of two adjacent pores to merge increased. So, I checked if I could improve the relationship between the two techniques by allowing for a threshold value above which counts for technique B no longer increased despite higher pore counts by technique A. To do so I used a broken stick model with different thresholds and selected the model with the lowest residual square value (table 2.1 & figure 2.1). Without the three extreme values, pore counts from technique A were not significantly different from technique B (paired t-test: $t_{23} = 1.05$, $P = 0.303$).

In order to avoid the merging effect in technique B at high pore density, I used the result from technique A for the analysis of relationships with permeability, water content and shell thickness. Permeability tended to have a positive relationship with pore density (Spearman, $r_s = 0.339$, df = 28, $P = 0.066$), but had no relationships with water content (Spearman, $r_s = 0.093$, df = 28, $P = 0.624$) and shell thickness (Spearman, $r_s = -0.147$, df = 28, $P = 0.677$). Shell thickness had no significant relationship with pore density (Spearman, $r_s = -0.201$, df = 28, $P = 0.286$),

I compared the pore density between A, B and C-eggs using the results of the two techniques. Using data from technique A, there was no significant difference in pore density within the laying order ($F_{2,27} = 2.42$, $P = 0.108$) (Fig. 2.2), but when using data from technique B, A-eggs had the lowest pore density within the laying order ($F_{2,27} = 7.01$, $P = 0.004$) (Fig. 2.2). There was no significant difference in permeability within the laying order ($F_{2,27} = 1.10$, $P = 0.349$).

Table 2.1

Model	No. of deleted extremes	Sum of residual squares
1	0 (full model)	8851.62
2	2 (extremes > 80)	8815.24
3	3(extremes > 70)	8583.94*
4	4(extremes > 66)	8692.56
5	6(extremes > 65)	9488.92
6	7(extremes > 61)	9058.51

The results of the "broken stick" model describing the relationship between the two pore count techniques. 2-7 values for highest pore counts from technique A were assumed to show no relationship between technique A & B. Model with the least sum of square (*) was selected ($F_{1,28} = 21.48$, $P < 0.01$).

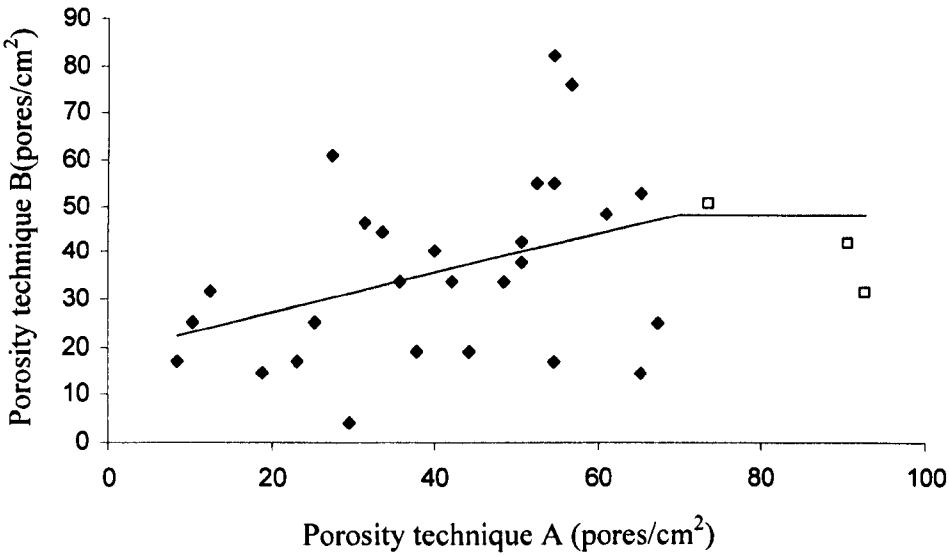

Figure 2.1. Relationship between shell porosity from technique A and technique B. All are shown, ♦ increase linearly (B = 18.607 + (0.424 x A) ($F_{1,28}$ = 21.48, P < 0.01) whereas the relationship seems to level off for data above 70 pores / cm^2 (technique A) (□).

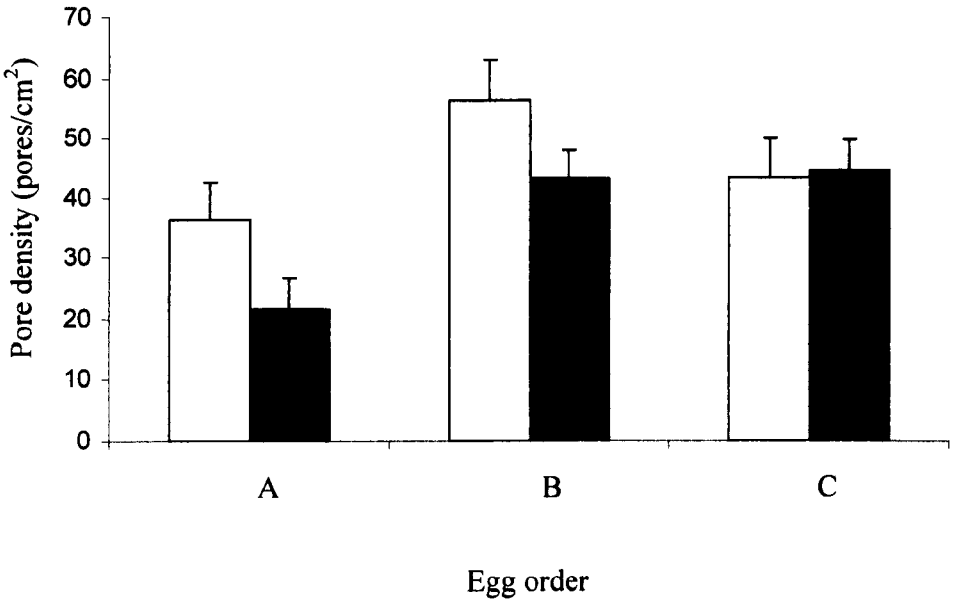

Figure 2.2. Shell porosity from technique A (white bars) and technique B (black bars) of A, B and C-eggs

Discussion

The aim of this study was to compare two different techniques to count the number of pores in the eggshells of lesser black-backed gulls. I further looked for relationships between pore count and permeability based on the predicted relationship between shell porosity, shell thickness and water loss. Technique B seemed not to allow accurate pore counting in the shell with high pore densities. Merging of dye between closely adjacent pores may be the cause (Tyler, 1953). There was a weak relationship between permeability and pore counts from technique A suggesting this to be the most appropriate technique for counting pores in lesser black-backed gulls' eggs.

According to technique A, A, B and C-eggs seemed to have similar pore density at the equatorial zone, but the A-egg had a lower pore count than the B- or C-eggs in technique B. The fact that technique B gave a lower pore density for A-eggs than for B- or C-eggs may give a clue to the pattern of pore distribution. A-eggs may have a different pattern of pore distribution from B and C-eggs. Possibly, pores of A-eggs were distributed in a more clumped pattern, so that the merging effect could happen more easily. Further study of pore distribution may clarify the clump pattern in A-egg. Rahn & Dawson (1979) reported pore densities (derived from water vapour conductance and shell thickness) of two species of gulls, Heermann's gull (*Larus heermanni*) (134 pores / cm^2) and western gull (*Larus occidentalis livens*) (233 pores / cm^2), both species had higher pore densities than the lesser black-backed gull. However, Rahn & Dawson (1979) did not directly count the pores. In his study Mänd (1996) showed that black-headed gull (*Larus ridibundus*) eggs had higher pore counts (151±26.4 pores / cm^2) than lesser black-backed gull eggs in this study. But,

Mänd (1996) found that pore count changed with increasing duration of incubation. Possibly, incubation activity may reveal non-functional pores. So, the number of pore counts in this study may include non-functional pores that were later revealed by decalcifying the shell. Interestingly, Massaro & Davis (2004 & 2005) found differences in pore densities between eggs of the same clutch in yellow-eyed penguin (*Megadyptes antipodes*) and snares penguin (*Eudyptes robustus*). Both species lay two eggs, the first species had a lower pore density in second-laid eggs but vice versa in the latter species. Snares penguins seemed to have a higher pore density than yellow-eyed penguins. Even two relatively closely related species seemed to differ in their patterns of pore density with laying order and in pore density itself. These differences may reflect difference in the species' life history.

There was a weak relationship between pore density and permeability. So far, we still know little about the ultra structure of the pores in the lesser black-backed gull's egg. Probably, the size of pore may also affect permeability. More information from further study about the dimension of pores could clarify this weak relationship.

Interestingly, great northern divers (*Gavia immer*) normally nest in damp areas and produced eggs with very high pore densities (307 pores / cm^2) (Tullett & Board, 1977). The lesser black-backed gulls which nest in dry areas and produce eggs with far lower pore densities (45.30 pores / cm^2). Pores in the shell normally serve as channels for gas exchange (Board, 1982), but at the same time they may allow entry by micro-organisms to the egg (Cook *et* al., 2003). Great northern divers may not need to conserve water in the damp environment, so they may use this advantage to produce eggs with a high capacity for gas exchange, but they have to trade-off this

with the increased risk of micro-organism invasion. This scenario may be the reverse for the lesser black-backed gull that bred in a colony where there is probably a higher abundance of potential microbes, and eggs may lose water more easily (windy climate at the sea coast).

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Chapter 3: Variation of eggshell structures with laying order

Abstract

Variations in egg size and egg composition between females of the same species, as well as among eggs in the same clutch, have been found in many avian species. Some researchers found variation in egg composition can affect offspring fitness. Variation in egg size and egg content has received most attention and little attention has been paid to the eggshell, particularly variation within the clutch. Eggshell serves some crucial functions in avian reproduction such as protecting of the embryo from mechanical damage, as a calcium provider to the embryo, gas exchange with the environment and conservation of water. In this study, I investigated variations in three eggshell characteristics, shell thickness, shell porosity and mammillary layer contact area, in relation to laying order. This study found within-clutch variations in shell porosity and mammillary layer contact area but not in shell thickness.

Introduction

There is a large variation in egg size and egg composition between females of the same species as well as between eggs of the same clutch (Carey, 1996; Christians, 2002; Williams, 2005). At laying, all the nutrients required for successful development of the embryo must be deposited into the egg in sufficient quantity (Carey, 1996) and variation in egg composition can influence offspring fitness (Williams, 1994; Nager *et al.*, 2000). Although variation in egg size, and the content of albumen and yolk, have received much attention, little attention has been paid to variation in the eggshell. Egg production in birds is generally recognized as a demanding process (Monaghan and Nager, 1997; Williams, 2005; Nager in press).

Egg formation is generally believed to be costly because of the biosynthesis and deposition of resources into the yolk (Williams, 2005). Recently, however,Williams and Ames (2004) have suggested that functioning of the oviduct may also have significant energetic costs and the female's ability to deposit non-yolk components of the egg (albumen and / or shell) may also affect egg quality.

The functions of the eggshell include protection of the embryo from mechanical damage, calcium provision to the growing embryo, gas exchange with the environment, conservation of water (Board, 1982; Carey, 1996) and concealment of the egg from predators (Underwood and Sealy, 2002). The eggshell must be strong enough to support the mass of the egg contents and to avoid damage from the incubating parents. At the same time it must be weak enough to allow the embryo to hatch from the egg (Board, 1982; Carey, 1983). Generally, the structure of the avian eggshell is composed of four layers; cuticle layer (outer surface of an eggshell), vertical crystal layer (below the cuticle), palisade layer (below the vertical crystal layer) and mammillary layer (the innermost of the calcified part below the palisade layer) (Parsons, 1982). Bain (1991) reported that the removal of the cuticle, vertical crystal layer, and palisade layer each significantly decreased the stiffness of the remaining shell of the domestic chicken, but that the removal of the mammillary layer had no effect on shell stiffness. So, the effective shell thickness (excluding shell membranes and mammillary layer) is the best indicator of eggshell strength (Bain, 1991). Johnston and Comar (1955) found that the developing embryo utilized the eggshell as a major calcium source. Blom and Lilja (2004) reported that the eggshell mainly supplied minerals for the skeletal development of the embryo. The mammillary layer, which is in contact with the shell membrane, functions as a

provider of minerals (mainly calcium and magnesium) (Bond *et al.*, 1988; Blom and Lilja, 2004). So, the contact area between the tips of the mammillary layer and the shell membrane is of interest as it affects the potential for the embryo to take up minerals from the eggshell. Shell porosity also affects the development of the embryo. Baker and Baker (1992) reported that abnormally low or high shell porosity affected water loss, which affected the hatchability of budgerigar (*Melopsittacus undulatus*) eggs. Birds breeding at high altitude, where the water vapour diffusion gradient is strong, lay eggs with shells that have a lower pore density than do members of the same species breeding at lower altitude. This is presumed to be an adaptation to reduce water loss from the eggs (Carey *et al.*, 1983; Monge *et al.*, 2000). Pollution can also affect eggshell quality. Birds breeding in areas of high acid rain or organochlorine residues have been found to lay eggs with thinner shells and to have lower reproductive success compared to the same species in unpolluted areas (*e.g.* Drent and Woldendorp, 1989; Findholt, 1984). Drent and Woldendorp (1989) suggested that the embryo in thin-shelled eggs dried out during incubation because of excessive evaporation. However, we know little about the variation in eggshell structure within a clutch.

In this study, I investigated whether there was any variation in eggshell characteristics in relation to laying order in the lesser black-backed gull (*Larus fuscus*), a species that normally lays three eggs in a clutch. In this study, I refer to the first egg as A-egg, the second egg as B-egg and the last egg as C-egg. Earlier studies have shown clear differences in egg composition in relation to laying order in this species (Bolton *et al.*, 1992; Royle *et* al., 1999; Nager *et al.*, 2000; Blount *et al.*, 2002; Verboven *et al.*, 2003 & 2005). I measured three characteristics of the eggshell,

which relate to three of the main functions of the eggshell; shell thickness (for protection of the embryo), shell porosity (for gas and water exchange services) and the mammillary layer contact area (calcium provider). In order to find whether there was variation in the eggshell characteristics among different areas of the same eggshell, I measured the eggshell characteristics at three different areas on the shell; the blunt end, equatorial zone and pointed end (Fig. 3.1). In order to investigate whether females laying at different times differed in eggshell characteristics, the laying date (the date that the female laid the first egg) was included as a covariate in the statistical analyses.

Materials and methods

Fresh eggs from 32 three-egg clutches of lesser black-backed gulls were collected from the central part of the colony at South Walney Nature Reserve, Walney Island, northwest England, UK, during the breeding season in 2002. The collected clutches were initiated between late April and late May 2002. The eggs were collected on the day of laying and replaced by dummy eggs in order not to disturb normal laying behaviour. Eggs were collected under a licence from English Nature. On the day of laying, fresh eggs were weighed to the nearest 0.01g using an electronic balance and maximum length and width measured with a calliper to the nearest 0.1 mm. Eggs were then frozen until analysis. The frozen eggs were cut longitudinally in half by using a scalpel. The two-half shells (with shell membrane) were separated from the egg content. The eggshell was dried in an oven at 50°C until it reached a constant dry weight. The dry weight of the eggshell was measured by using an electronic balance and weighed to the nearest 0.001 g.

The eggshells were investigated for three characteristics; shell thickness, shell porosity and mammillary layer contact area. To avoid the effect of heterogeneity of eggshell characteristics across different areas of the eggshell (Romanoff and Romanoff, 1949; Tyler, 1961; Gosler *et al.*, 2005), I measured eggshell characteristics at three areas of the egg: at the blunt end, equatorial zone and pointed end. I took specimen pieces as close as possible to the broad and narrow apices (blunt and pointed end, respectively). Specimens of the equatorial zone were taken from the area where the egg was widest (figure 3.1).

Shell thickness

In this study I also took the opportunity to compare three different methods of measuring shell thickness. Firstly I measured the eggshell thickness index (ETI). For this egg volume V was calculated by using the equation $V = K_V \times L \times B^2$ (L = length in cm, B = width in cm, K_V (volume coefficient) = 0.4965, as the average from *Larus* species (Hoyt, 1979). The surface area A was calculated by using the equation $A = 4.951 \times V^{0.666}$ (V = egg volume in cm^3) (Paganelli *et al.*, 1974). I calculated ETI by dividing shell dry weight by the surface area of the egg (Green, 1998). Secondly, I measured shell thickness at five different places within the equatorial zone and three different places at the blunt end and pointed end to the nearest 0.005 mm using a micrometer (Draper PM 025). The micrometer was modified with rounded tips to fit to the curvature of the eggshell. The repeatabilities (calculation of the repeatability after Lessells and Boag (1987)) of the thickness measures were, $r = 0.822$ ($F_{95, 192} = 14.82$, $P < 0.001$) for the blunt end, $r = 0.842$ ($F_{95, 384} = 27.68$, $P < 0.001$) for the equatorial zone and $r = 0.847$ ($F_{95, 192} = 17.66$, $P < 0.001$) for the pointed end. Measurements from the same shell area were averaged for use in subsequent

analyses. Thirdly, I measured the eggshell thickness using a scanning electron microscope (Hitashi S-570 supported by E. M. Systems Support, High Peak, UK). Two pieces of shell ($\approx 1 cm^2$) were carefully cut out from the eggshell at the target area by using a diamond tipped circular saw (Quayle Dental, Worthing, Sussex, UK), which ensured that the structural integrity of the shell was retained. In order to get the precise measurement of shell thickness, the samples were snapped in two and one piece was mounted vertically on a grooved aluminium stub with Plastic Conductive Carbon Cement (Leit C Plast, Gisbourne Microscopy Services, Brocton, Stafford, UK) by placing the snapped size uppermost. The mounted shells were coated with gold / palladium for 4 minutes in an Emscope sputter coater SC500 (Emitech Limited, Ashford, Kent, UK). These specimens were viewed in the scanning electron microscope at 15 kV with a magnification of 200X at a constant working distance of 30 mm. The pictures were stored and then later measured. In order to use the SEM to measure shell thickness as an indicator of the strength of the eggshell, the measured area should not include the mammillary layer and the shell membranes (Bain, 1991). But, from the scanning electron micrographs, the mammillary layer of the lesser black-backed gull's eggs were in confluent forms and the thickness of cuticle layer was not even (Fig. 3.2). So, I chose to measure the distance from the tips of mamillary layer to the inner surface of the cuticle as shell thickness, hence not including shell membranes and cuticle (Fig 3.2). The measurements were made from the recorded pictures to the nearest 0.001 μm using High Resolution Digital Imaging System software, version 2.05 (E. M. Systems Support, High Peak, UK). Two specimens were used per area. One measurement was taken from each specimen. The correlations between the two thickness measurements were $r_{94} = 0.77$ at the blunt end, 0.70 at the equatorial zone and 0.80 at the pointed end (all $N = 96$, $P < 0.001$).

Measurements from the same shell area were averaged for use in subsequent analyses.

Shell pore density

Due to a shortage of shell material, I only used 31, 27 and 27 complete clutches for measuring shell pore density at the blunt end, equatorial zone and pointed end, respectively. The pore density of eggshells was measured once or twice for each of the three different shell areas for each egg. A piece of eggshell ($\approx$ 1 cm^2) cut as above was flooded with Decalcifier II (Surgipath, Bretton, Peterborough, UK.) for 2 minutes and then put into water for a few seconds to stop the reaction in order to peel off the shell membranes. To make the pores visible, the eggshell was flooded again with Decalcifier II for 5 minutes and then again put into water for a few seconds to stop the reaction. The remaining shell membrane was cleared by using point-tip forceps. Under a dissection microscope, the pores were clearly visible and from a known surface area of shell (23.768 mm^2) I counted the number of pores. The correlations of the porosity count from the same egg were $r_{88} = 0.47$ ($P < 0.001$) at the blunt end, $r_{65} = 0.25$ ($P = 0.04$) at the equatorial zone and $r_{68} = 0.51$ ($P < 0.001$) at the pointed end. I did not have two measurements for every specimen. Measurements from the same shell and area were averaged for use in subsequent analyses.

Mammillary layer contact area

The contact area between the tips of the mammillary layer and shell membranes is of interest as it affects the potential for the embryo to take up minerals from the eggshell. I chose a random subsample of 16 three-egg clutches for measuring the

mammillary layer contact area (Fig. 3.3). The laying date, egg size and shell thickness of the eggs included did not differ from the rest of the sample (laying date: $t_{30} = 0.93$, P = 0.360; fresh mass of A-egg: $t_{30} = 0.17$, P = 0.864; fresh mass of B-egg: $t_{30} = 0.98$, P = 0.335; fresh mass of C-egg: $t_{30} = 0.19$, P = 0.849; ETI of A-egg: $t_{30} = 0.77$, P = 0.447; ETI of B-egg: $t_{30} = 0.13$, P = 0.899; ETI of C-egg: $t_{30} = 1.26$, P = 0.217). I measured the mammillary layer contact area for three shell areas (blunt end, equatorial zone and pointed end). One piece of 1 cm^2 from each shell area was cut as above and was soaked in distilled water for 2 – 3 days in order to soften the shell membrane. As much shellmembrane as possible was removed manually by using point-tip forceps. The remaining shell membrane was removed using plasma etching, a non-destructive technique of removing organic material. The Nanotech 100 Plasma Chemistry Unit used low temperature activated plasma to remove the rest of the tightly attached membrane from the inner surface without damaging the underlying mineral structure (Reid, 1983). The specimen was placed with the inner surface uppermost in an atmosphere of oxygen gas at 133.3 Pascals that was made reactive by applying a radio frequency of 100 ohms. The organic eggshell membrane was volatilised and any residual ash was dusted off with a jet pressure duster. The membrane-free shell was prepared as above for scanning electron microscopy at 250X magnification at a constant working distance of 30 mm. Two different places on each piece of eggshell were scanned. I measured the mammillary layer contact area instead of counting the mammillary tips per area. In order to avoid the distortion part on the left side on the print of the scanning electron micrograph, I selected an area of 0.2215 mm^2 on the top right corner of the micrograph by tracing the tips of the mammillary layer on an acetate sheet and measured the area from those traces in the following way. The drawings on the acetate sheets were scanned into a digital file

and the mammillary layer contact area was determined using Leica Q-Win software (Leica Microsystems Limited, Milton Keynes, UK). In order to get highly accurate of the measurements, I highlighted the outline of the traced area as thinly as possible by selecting white value at 150 and black value at 0 in "Grey detect" and the width value of line at 1 in "Binary edit" within the software. Two areas from the same specimen were scanned, the correlations of the two independent measurements of mammillary layer contact area from the same area and shell was $r = 0.662$ ($df = 139$, $P < 0.001$) (due to damage occurring during the preparation process, eggshells at the pointed end from three eggs were not included in the measurement). Then, I calculated the percentage of mammillary layer contact area within the selected measurement area. Measurements from the same shell and area were averaged and used in subsequent analyses.

Statistical analysis

All the data were normally distributed, except the data on the density of mammillary layer contact area (data from the blunt end of A and C-eggs and data from the equatorial zone of B-egg), which I corrected using arcsine transformation. If I found a significant interaction between laying date (the date that the female laid the first egg of the clutch) and the laying order or shell area, I would carry out separate analyses for early laying and late laying birds. I used the mean laying date to separate the two laying groups. In order to investigate the difference of the measurements of the eggshell characteristics between A, B and C-eggs of the same clutch and measures from different areas within an eggshell, I used repeated-measures analyses with egg number and shell area as the repeated measures in SPSS (Version 13). If the

assumption of the sphericity test was violated, I used the Greenhouse-Geisser correction. For interactions, I reported only the significant ones in the results. All tests are two-tailed and $P < 0.05$ was considered significant. Mean values ± S.E. are reported.

Results

Firstly, I analysed the data of fresh egg mass, shell dry mass and shell surface area (Table 3.1). Only shell dry mass showed a significant difference within the laying order, the post hoc test suggested that shell dry mass of A- and B-eggs did not differ, but C-eggs had lower shell dry mass.

Secondly, I analysed the data for the shell thickness. There were three methods of measurements for shell thickness in this study; ETI, using the micrometer and using the SEM. None of these methods showed a significant difference in shell thickness with laying order (Table 3.2). However, there was variation in shell thickness between the different shell areas, and the post hoc test suggested that the shell at the blunt end was thinner than at the other areas (Table 3.2). Eggs laid later in the season had thinner eggshells, but this effect was only significant when using the SEM measurement (Table 3.2).

Overall, the two measurements of shell thickness from the modified micrometer and SEM were positively correlated, but the SEM gave a consistently lower average shell thickness than the micrometer measurement (table 3.3). The difference between the two techniques was largest at the pointed end for all laying orders and for the blunt

end of C-eggs. Both measurements of shell thickness at three different areas of A, B and C-eggs positively correlated with ETI ($r \geq 0.528$, $P \leq 0.002$).

Thirdly, I analysed the data of the shell pore density. There was a significant interaction between laying order and laying date on shell pore density (Table 3.2). So, I separated the data into two groups (the mean of the laying date = 40.90 ± 1.34, range of the early laying date: 26-39; range of the late laying date: 41-54) and I then analysed them separately. In the early laying group, there was no significant interaction between laying order and shell area on pore density ($F_{4,6} = 0.71$, $P = 0.612$). There was no significant difference in pore density across the laying orders and between the three different shell areas (laying order: $F_{2,8} = 1.13$, $P = 0.369$; shell area: $F_{2,8} = 2.55$, $P = 0.139$) (Fig. 3.4a). In the late laying group, there was no significant interaction between laying order and shell area on the pore density ($F_{4,9} = 2.33$, $P = 0.134$). The laying order had a significant effect on pore density ($F_{2,11} = 8.14$, $P = 0.007$). The post hoc-tests suggested that there was no significant difference in pore density between A-and B-eggs ($F_{1,12} = 3.87$, $P = 0.073$) and between A-and C-eggs ($F_{1,12} = 0.37$, $P = 0.557$), but the B-egg had a higher pore density than the C-egg in the late laying group($F_{1,12} = 17.69$, $P = 0.001$), but from Figure 3.4b the B-egg seemed to have higher pore density than the A- or C-egg. There was no significant difference in pore density between the three shell areas of A-, B- and C-eggs in the late laying group ($F_{2,11} = 1.61$, $P = 0.244$).

Finally, I analysed the data of the density of mammillary layer contact area. Generally, from the scanning electron micrographs, the mammillary layer of the lesser black-backed gull's eggs were in confluent forms. There was a significant

interaction between laying order and laying date on the density of mammillary layer contact area (Table 3.2). So, I separated the data into two groups (the mean laying date = 39.50 ± 1.85, range of the early laying date: 27-39; range of the late laying date: 41-54) and I then analysed them separately. In the early laying group, there was no significant interaction between laying order and shell area on density of mammillary layer contact area ($F_{4,3} = 2.83$, $P = 0.210$). There was a tendency towards a difference in the density of mammillary layer contact area within the laying order ($F_{2,5} = 5.14$, $P = 0.061$) and between the three different shell areas ($F_{2,5} = 18.14$, $P = 0.005$) (Fig. 3.5a). The post- hoc tests suggested that C-eggs had a higher density of mammillary layer contact area than A-and B-eggs. The pointed end had lowest density of mammillary layer contact area than the other two areas. Among the eggs of late laying birds, laying order had no significant effect on the density of mammillary layer contact area ($F_{2,5} = 0.86$, $P = 0.477$) but there was the same change across shell areas as among eggs of early laying birds ($F_{2,5} = 24.12$, $P = 0.003$) (Fig. 3.5b).

Shell thickness (SEM), pore density and the density of mammillary layer contact area were generally unrelated to each other. I found statistically significant relationships only between shell thickness and pore density at the pointed end of B- and C-eggs and at the blunt end of the C-egg (Table 3.4), but with this number of correlations I would expect 1 or 2 to appear significant by chance.

Table 3.1

	A-egg	B-egg	C-egg	Repeated-measures Anova
Fresh egg mass (g)	78.40±1.18	74.88±1.27	73.27±1.56	Egg order: $F_{2,29} = 2.87$, $P = 0.073$ Laying date: $F_{1,30} = 0.58$, $P = 0.452$ Interaction: $F_{2,29} = 2.20$, $P = 0.129$
Dry shell weight (g)	5.24±0.08	5.20±0.08	4.88±0.09	Egg order: $F_{1.67,50.07} = 3.43$, $P = 0.048$ Laying date: $F_{1,30} = 2.91$, $P = 0.099$ Interaction: $F_{1.67,54.43} = 1.17$, $P = 0.313$
Shell surface area (cm^2)	88.12±0.96	86.98±1.04	82.01±0.98	Egg order: $F_{1.62,48.49} = 1.70$, $P = 0.192$ Laying date: $F_{1,30} = 0.49$, $P = 0.491$ Interaction: $F_{1.62,48.49} = 0.03$, $P = 0.972$

Average (± S.E.) fresh egg mass, dry shell weight and shell surface area of A, B and C-eggs.

Table 3.2

	ETI	Thickness (micrometer)	Thickness (SEM)	Porosity	Mammillary layer
Egg order x shell area x laying date		$F_{2.76,82.75} = 0.21^{G}$	$F_{4,27} = 2.28$	$F_{4,18} = 0.36$	$F_{4,9} = 1.29$
Egg order x laying date	$F_{2,29} = 1.60$	$F_{2,29} = 1.54$	$F_{2,29} = 1.10$	$F_{2,20} = 4.22^{*}$	$F_{2,11} = 6.20^{*}$
Shell area x laying date		$F_{2,29} = 1.75$	$F_{2,29} = 0.09$	$F_{2,20} = 0.06$	$F_{2,11} = 0.30$
Egg order x shell area		$F_{2.76,82.75} = 0.26^{G}$	$F_{4,27} = 1.59$	$F_{4,18} = 0.28$	$F_{4,9} = 1.33$
Egg order	$F_{2,29} = 1.74$	$F_{2,29} = 1.68$	$F_{2,29} = 1.70$	$F_{2,20} = 3.66^{*}$	$F_{2,11} = 8.22^{*}$
Shell area		$F_{2,29} = 11.24^{**}$	$F_{2,29} = 3.57^{*}$	$F_{2,20} = 0.23$	$F_{2,11} = 3.13$
Laying date	$F_{1,30} = 3.80$	$F_{1,30} = 2.89$	$F_{1,30} = 5.28^{*}$	$F_{1,21} = 0.16$	$F_{1,12} = 1.64$

The results of repeated measurement analysis with laying date as a covariate (^{G} shows Greenhouse-Geisser correction, * shows $P \leq 0.050$ and ** shows $P < 0.001$).

Table 3.3

	Micrometer vs. SEM		
	A-egg	B-egg	C-egg
Shell thickness at blunt end	$t_{31} = 19.00$, 0.041 ± 0.002 $r = 0.80$	$t_{31} = 11.49$, 0.041 ± 0.004 $r = 0.55$	$t_{31} = 16.88$ 0.050 ± 0.003 $r = 0.63$
Shell thickness at equatorial zone	$t_{31} = 14.71$, 0.040 ± 0.003 $r = 0.75$	$t_{31} = 20.25$, 0.042 ± 0.002 $r = 0.79$	$t_{31} = 22.27$, 0.041 ± 0.002 $r = 0.82$
Shell thickness at pointed end	$t_{31} = 16.20$, 0.052 ± 0.003 $r = 0.75$	$t_{31} = 15.25$, 0.056 ± 0.004 $r = 0.72$	$t_{31} = 17.48$, 0.055 ± 0.003 $r = 0.74$

The relationship of shell thickness between measurements using a micrometer and SEM. Differences between techniques were tested using a paired *t*-test, the micrometer technique gave higher values for shell thickness; means paired differences (± S.E.) given in mm are shown underneath the paired *t*-test values. The correlation coefficients (*r*) between the two techniques are also shown in this table. All tests had significant values at $P \leq 0.001$.

Table 3.4

		Pore density	Mammillary layer contact area
A-egg			
Blunt end	Thickness	r = -0.201, df = 27, P = 0.277	r = 0.039, df = 14, P = 0.887
	Pore density	-	r = -0.208, df = 14, P = 0.384
Equatorial zone	Thickness	r = -0.244, df = 27, P = 0.203	r = -0.208, df = 14, P = 0.438
	Pore density	-	r = 0.139, df = 14, P = 0.608
Pointed end	Thickness	r = -0.168, df = 29, P = 0.365	r = -0.324, df = 14, P = 0.141
	Pore density	-	r = -0.036, df = 14, P = 0.894
B-egg			
Blunt end	Thickness	r = -0.012, df = 29, P = 0.948	r = -0.052, df = 14, P = 0.848
	Pore density	-	r = 0.103, df = 14, P = 0.704
Equatorial zone	Thickness	r = -0.307, df = 26, P = 0.113	r = -0.150, df = 14, P = 0.580
	Pore density	-	r = 0.309, df = 14, P = 0.245
Pointed end	Thickness	r = -0.533, df = 26, P = 0.003	r = 0.257, df = 13, P = 0.356
	Pore density	-	r = 0.009, df = 13, P = 0.975
C-egg			
Blunt end	Thickness	r = -0.388, df = 29, P = 0.031	r = -0.374, df = 14, P = 0.154
	Pore density	-	r = 0.114, df = 14, P = 0.675
Equatorial zone	Thickness	r = -0.235, df = 28, P = 0.212	r = -0.324, df = 14, P = 0.222
	Pore density	-	r = -0.357, df = 14, P = 0.174
Pointed end	Thickness	r = -0.447, df = 28, P = 0.013	r = -0.008, df = 12, P = 0.978
	Pore density	-	r = 0.305, df = 12, P = 0.298

Relationships (Pearson) between shell thickness (SEM), pore density and mammillary layer contact area.

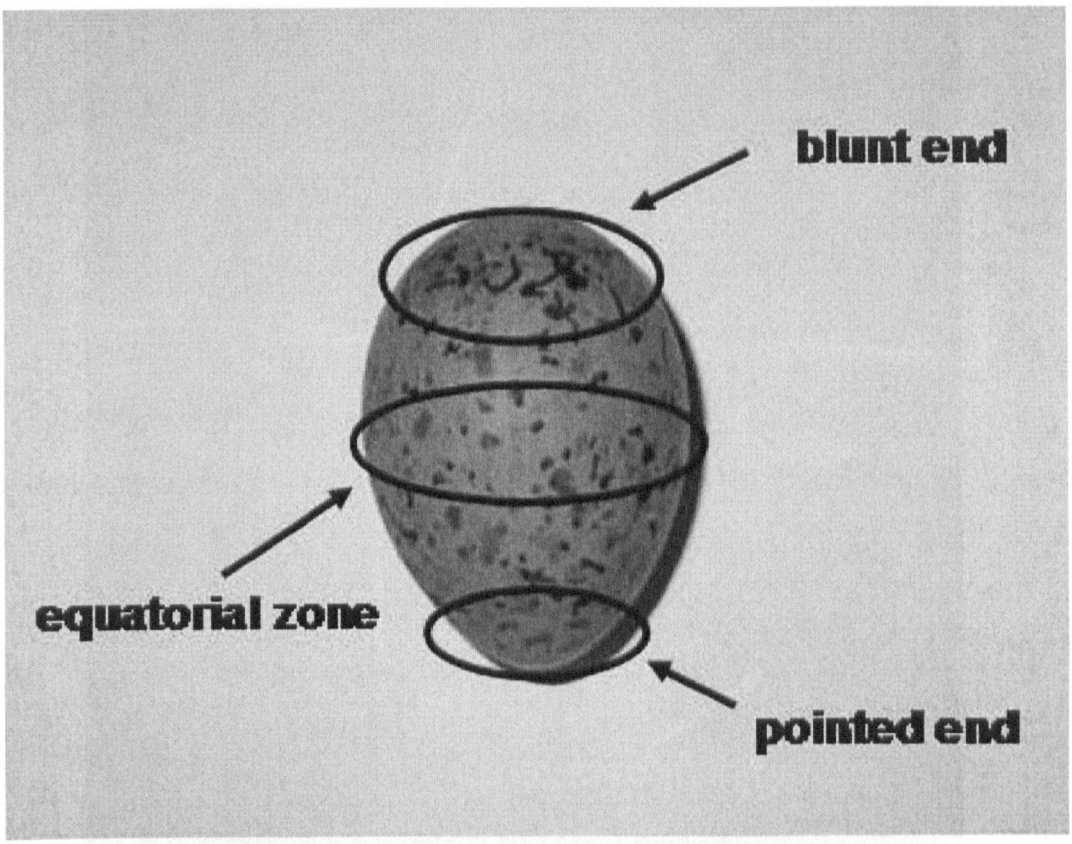

Figure 3.1. The three areas of the eggshell where eggshell characteristics were measured. The blunt end was the area as close as possible to the broad apex. The equatorial zone was the widest part of the egg. The pointed end was the area as close as possible to the narrow apex.

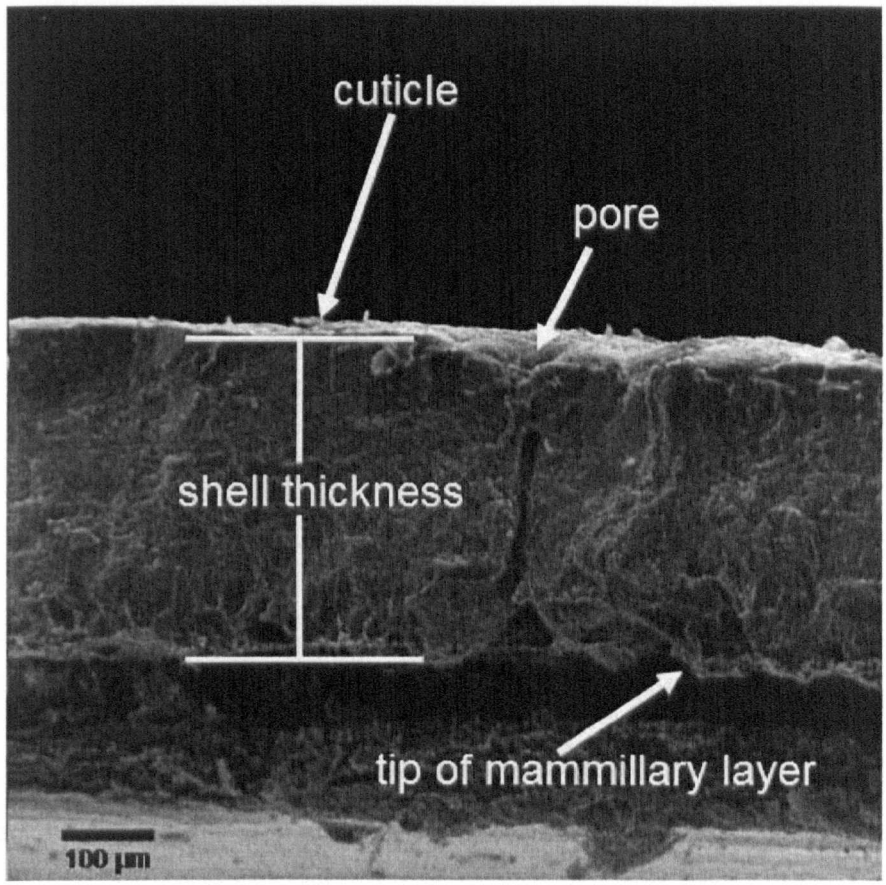

Figure 3.2. Scanning electron micrograph (200X) of the cross-section of an eggshell to measure shell thickness. The outer surface of the eggshell lays at the top of the photograph. This figure shows cuticle (the top layer of the outer shell), an unbranched pore forming a vertical tube through the eggshell and tips of the mammillary layer where shell membranes form a strong bond with the shell.

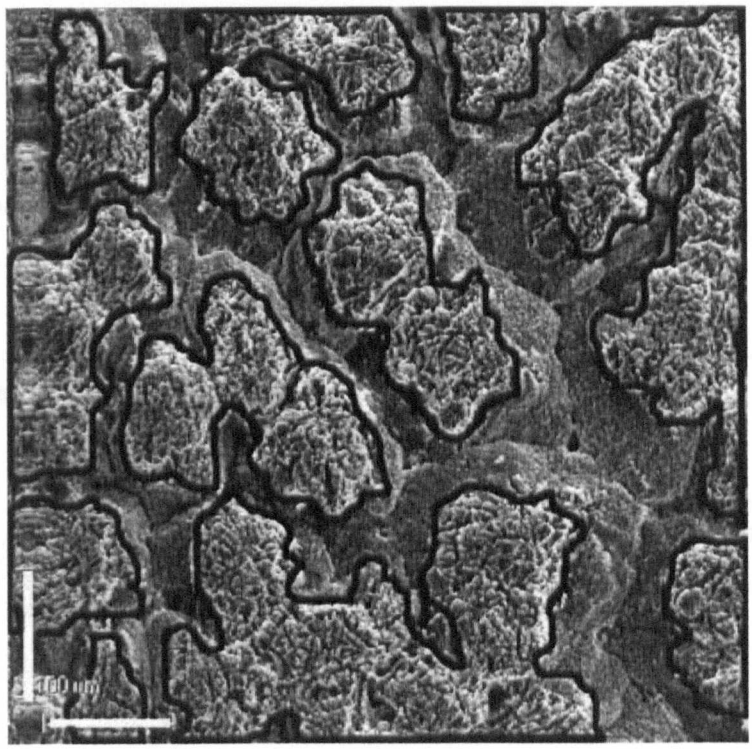

Figure 3.3. Scanning electron micrograph (250X) of the inner eggshell without shellmembranes. A white bar shows the length of 100 μm and dark lines tracing around the mammillary layer contact areas.

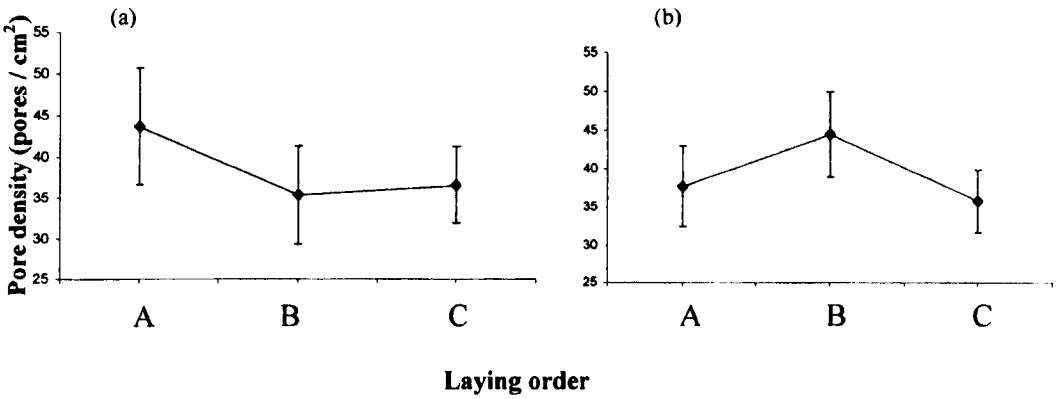

Figure 3.4. Pore density (± SE) of A, B and C-eggs from early laying birds (a) and late laying birds (b).

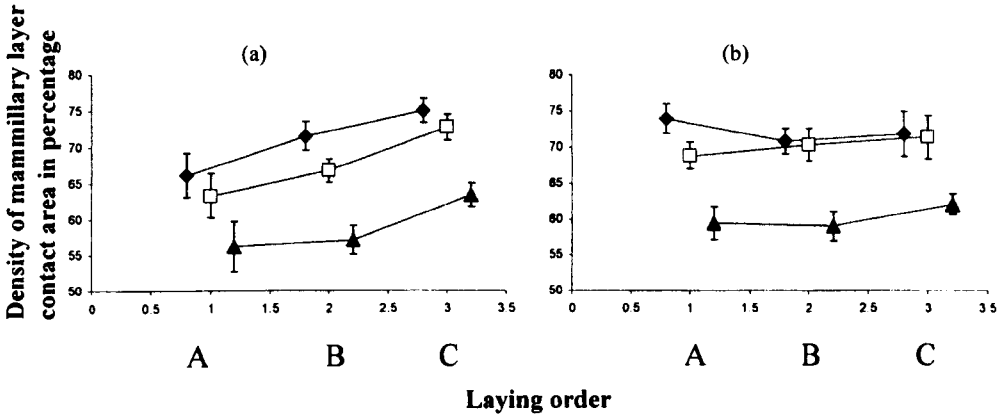

Figure 3.5. Density of mammillary layer contact area (± SE) of A, B and C-eggs at the blunt end (♦), equatorial zone (□) and pointed end (▲) from early laying birds (a) and late laying birds (b).

Discussion

There was within-clutch variation of shell pore density in late laying birds. There was a tendency for variation in density of the mammillary layer through the laying order in early laying birds. There was a difference in shell thickness between shell areas, but this pattern did not depend on egg order or laying date.

The measurements using a modified micrometer gave greater shell thickness than the measurement using the SEM, but there was a strong positive relationship between these two measurements. The eggshells still contained shell membranes and cuticle when they were measured using a modified micrometer, whereas in the SEM, I measured only the distance from the inside of the cuticle to the tip of mammillary layer. Therefore, the higher value of the shell thickness as measured by the micrometer compared to the SEM may be because it included shell membrane and cuticle. Both techniques showed the same within-shell pattern of shell thickness with the blunt end being thinner than the other two areas. Interestingly, the difference in these two types of measurements was quite high at the pointed end, possibly because of the effect of the high curvature at the pointed end. So, to save the cost and time, measuring shell thickness at the equatorial zone using the micrometer technique may be the better method for the measurement of shell thickness.

The space beneath the blunt end is occupied by the air cell (Romanoff and Romanoff, 1949). So, the thin shell at the blunt end may shorten the distance for gas diffusion to support gas exchange for the metabolic processes of the embryo. But the egg may have to trade off increased O_2 intake with increased water loss. During the hatching period, the embryo generally starts cracking the shell in the area around the blunt end

or shoulder of the egg (Gosler *et al.*, 2005), so it may not be too difficult for the lesser black-backed gull's chick to start breaking out through the thin shell.

We know very little about variation in shell thickness within eggs of wild birds. The pattern of shell thickness within eggs of the lesser black-backed gull was opposite to that in great tits, *Parus major* (Gosler *et al.*, 2005) and snare penguins, *Eudyptes robustus* (Massaro and Davis, 2005) that had the thickest part of the shell at the blunt end. In the domestic chicken, the shell thickness was more variable, but thicker at the blunt end and pointed end than at the equatorial zone (Romanoff and Romanoff, 1949; Tyler, 1961). So, the pattern of shell thickness within eggs may not be the same for all species.

B-eggs tended to have higher pore densities than A- or C-eggs in the late laying group. It was quite difficult to explain this within-clutch variation. Further study of the dimension of pores may clarify this variation. In this study, the pore density was 33.10 ± 1.81 pores / cm^2 (at equatorial zone). Mänd (1996) studied the shell porosity of the black-headed gull and counted 151 ± 26.4 pores/cm^2 by using a different technique and his eggs were partially incubated, which increases the number of pores. For further study, the variation in the size of pores may help to get a clearer explanation of gas exchange and water conservation of the eggs in this species. In order to know the actual size of pore, the researcher should avoid any technique, especially the decalcifying technique,that could change the integrity of pores. Recently, researchers have found variation in shell porosity through the laying order in two species of penguins. Massaro and Davis (2004) found that A-eggs had a higher pore density than B-eggs in the same clutch of yellow-eyed penguins

(*Megadyptes antipodes*). The opposite result was found for snares penguins (*Eudyptes robustus*) which produced second-laid eggs with higher pore counts than first-laid eggs in the same clutch (Massaro and Davis, 2005). In domestic chickens, the shell porosity is fairly constant between the eggs of individual hens (Almquist and Holst, 1931; Romanoff, 1943 cited in Romanoff and Romanoff, 1949). This study found no difference in pore density between different areas of the shell as it was also found for snares penguins in the study of Massaro and Davis (2005).

From the scanning electron micrographs, the mammillary layer of the lesser black-backed gulls' eggs were generally in confluent forms, not in isolated tip forms like domestic chickens' eggs (Romanoff and Romanoff, 1949).The results showed that C-eggs in the early-laying group tended to have the highest density of mammillary layer contact area within the clutch. The mammillary layer contact area is where the embryo obtains some of the minerals (mainly Ca & Mg) that it requires for successful development (Blom and Lilja, 2004). Usually C-eggs have a smaller overall shell surface, and so C-egg may obtain compensation by increased mammillary layer contact area to maintain similar mineral uptake from the shell. Having a lower density of mammillary layer contact area than the equatorial zone, the pointed end may be less important in providing calcium for the embryo.

So far, variation in eggshell structure within a clutch in relation to the laying order has been found in some species of wild birds (Heaney *et al.*, 1998; Massaro and Davis, 2004 & 2005). Heaney *et* al., (1998) found that the experimentally induced fourth-laid egg from manipulated clutches of common terns (*Sterna hirundo*) had thinner shell than the last-laid eggs (third egg) from unmanipulated control clutches

and suggested that a nutritional constraint may have caused the reduced thickness in the additional egg. Massaro and Davis (2005) found that second-laid eggs had a higher pore density and shorter incubating period than the first-laid eggs in Snares penguins. This study also found within-clutch variation in shell porosity, but only in the late-laying group. This study did not find the effect of laying order on shell thickness in the lesser black-backed gull, and in this respect it was similar to black-headed gull (*Larus ridibundus*) in the study of Mänd (1996).

For the future, it will be interesting to discover whether the eggshell variation in the lesser black-backed gull is generated by manipulation by the females or by nutritional constraint.

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Chapter 4: Variation in eggshell coloration in relation to laying order

Abstract

Eggshell coloration varies among avian species. The variation may have specific functions which depend on the life history of the species. Birds also produce eggs that differ in eggshell colour within the clutch. The last-laid egg is often paler than the other eggs in the clutch. This study describes the variation in eggshell coloration in relation to laying order in lesser black-backed gull (*Larus fuscus*). Digital image analysis and spectral analysis were used to measure eggshell colour in this study. Variation of shell coloration within the clutch was found in this species. The last-laid egg often had paler shell colour and streaks on the shell.

Introduction

Eggshell coloration varies among species of wild birds. This variation may have specific functions which depend on the life history of the species. The principal pigments in the eggshell are protoporphyrin that provides brown colour and biliverdin that provides blue-green colour (Kennedy and Vevers, 1976). Underwood and Sealy (2002) reviewed the hypotheses on the function of eggshell coloration, such as camouflage, egg recognition and mimicry, filtering solar radiation, eggshell strength and aposematism. Moreno and Osorno (2003) proposed a new hypothesis that the eggshell colours may indicate the fitness of females and may influence paternal care in species with biparental care. Gosler *et al.* (2005) added another hypothesis that more pigments may be deposited to the shell in areas of limited

calcium resource, in order to maintain the strength of eggshell. A few studies have tested these hypotheses with a focus on variation within species.

Birds also produce eggs that differ in eggshell colour within the clutch (Underwood and Sealy, 2002). There are some possible functions of intraclutch variation in egg colour. Firstly, the last-laid egg of the clutch has a distinctive coloration to advertise clutch completion and the start of incubation to brood parasites; so the brood parasites would not waste their egg production in the complete clutch of hosts (Yom-Tov, 1980). Secondly, Hockey (1982) proposed that the variation in colour among the eggs of the clutch may improve the efficiency of crypsis; this hypothesis was supported by the study of Lloyd *et* al., (2000) on Namaqua sandgrouse (*Pterocles namaqua*). Finally, Verbeek (1988) suggested that the pale colour of the last-laid egg may attract predator interest to the egg with the lowest fledging rate in the clutch. Interestingly, the last egg is often paler than the other eggs in the clutch (Kilner, 2006). Some researchers suggested that running out of pigment before completing the clutch may cause the pale colour in the last-laid egg (reviewed in Underwood and Sealy, 2002). Soh and Koga (1994) proposed that sex steroid hormones (progesterone) may influence the pigmentation process. Solomon (1991) suggested that the changes of eggshell colour within one clutch may depend on the age of the female. The paleness of the eggshell may also be caused by stress associated with hormonal disturbances (Solomon, 1991).

In this study, I describe the variation in eggshell coloration in relation to laying order in the lesser black-backed gull (*Larus fuscus*). The eggshell colour of this gull is brown to light green with dark brown markings on the egg. I measured background

colour of eggshells with two techniques, digital imaging analysis and spectral analysis. I also visually scored the characteristics of markings on the eggshells.

Materials and methods

I collected 32 complete three-egg clutches of the lesser black-backed gull (*Larus fuscus*) from a large colony at South Walney Nature Reserve, Walney Island, northwest England, UK, during the breeding season in 2002. The eggs were collected on the day of laying and replaced by dummy eggs in order not to disturb normal laying behaviour. Each egg was labelled according to its position in the laying sequence (A is the first-laid egg, B is the second-laid egg and C is the last-laid egg). Eggs were then frozen until analysis. The frozen egg was cut longitudinally into two halves using a scalpel; the two-half shells were separated from the egg content. I measured background colour of the eggshell with two different methods; digital imaging analysis and spectral analysis. I used simple visual scores to quantify the characteristics of markings.

Digital imaging analysis

I took digital photographs of 96 eggs with a Nikon Coolpix 4500 (at 2272 x1704 pixels) through the camera-sized hole (8 cm x 8 cm) on the top of 1ft x 1ft x 1ft gray wooden box (without bottom). To control the standard of the light in all images, a flashlight was used in all images. The eggshell was put on white paper. In every image there were the same three colour chips as a colour reference and a measuring scale reference. I made the background colour measurement from the digital images by using the method of Villafuerte & Negro (1998). Red, green and blue values of the background colour from digital images were measured by using Adobe

Photoshop version 7.0. I made measurements of background colour on three different randomly chosen spots within the area around the reflectance of flashlight. I standardized all measurements by using the three colour chips as a colour standard. The repeatability value (calculation of the repeatability value after Lessells and Boag (1987)) of the standardized measurements of red, green and blue value from the same egg were $r = 0.854$ ($F_{95,192} = 18.53$, $P < 0.001$), $r = 0.904$ ($F_{95,192} = 29.10$, $P < 0.001$) and r = 0.903 ($F_{95,192} = 28.81$, $P < 0.001$), respectively. The three standardized measurements from the same shell were averaged and used in the subsequent analysis. To simplify the analysis of colour, I derived a single variable by entering the red, green and blue values into a principal components analysis (Bortolotti *et al.*, 2003).

Spectral analysis

I placed a sensor over a mask with a 4 mm diameter hole (area around the hole was painted in black), while holding a piece of eggshell without markings under the hole in order to measure only eggshell background coloration. Reflectance was measured from 300 to 700 nm (at 0.3 nm intervals) using a spectrophotometer (Ocean Optics S2000). As one egg had too small background area to use the spectrophotometer, only 31 complete clutches from the same collection as above were used in spectral analyses.

Three different areas were measured for each egg. Each measurement (spectrum) comprised 1153 data points. In order to manage the large amount of data points practically, I averaged the data points for every 20 nm interval between 300-700 nm. To do this, I derived a series of 20 values for each egg from the original data. The

repeatability values of the measurements from the same egg of each value ranged from 0.661 to 0.837, all $P < 0.001$. The measurements from the same shell were therefore averaged to use in the subsequent analysis. I standardized the data by subtracting the mean reflectance over all 20 values per egg from the value of each wavelength class of that egg (Cuthill *et al.*, 1999). To simplify the analysis of the spectra, I reduced the variables by using a principal components analysis. To avoid extreme multicollinearity, I eliminated some wavelength-classes that were highly correlated with each other during the process of analysis until I obtained a determinant value of *R*-matrix > 0.00001 (Field, 2005). As a result, the wavelengths from 520 to 700 nm were selected for principal components analysis. I measured the brightness of the background colour by totalling the reflectances between 300-700 nm ($R_{300\text{-}700}$) of each egg. I used the wavelength (between 300-700 nm) that had maximum reflectance as hue (Endler, 1990). I measured blue-green chroma by dividing the reflectance of the blue-green region ($R_{400\text{-}570}$) by the total reflectance ($R_{300\text{-}700}$) (Moreno *et al*, 2006; Siefferman *et al.*, 2006). I used blue-green chroma as an index of biliverdin concentration (Moreno *et al.*, 2006).

Visual score for background colour and characteristics of markings

I scored background colour visually by categorizing background colour of eggshells into 3 scores: dark brown, light brown and light green. I scored them from digital images of eggshell. The percentage of disagreement of scoring between successive assessment of the same egg for the A, B and C-egg were 34.38%, 21.89% and 15.63%, respectively. In order to measure the characteristics of markings on the eggshell, I scored the distribution of spots visually by using the following scores.

Score 1 = clumped distribution: there was some aggregation of spots at the blunt pole

but some other spots distributed equally on the remaining parts of the shell.

Score 2 = even distribution: all the spots distributed equally all over the eggshell.

I scored the average size of spots visually by using the following scores. The appearance of streaks was also observed during scoring.

For average size of spots

Score 1 = small spot (diameter ≤ 2 mm)

Score 2 = large spot (diameter > 2 mm)

For the appearance of streak

Score 1 = have streaks

Score 2 = have no streaks

Examples of scores are displayed in figure 4.1. I scored each egg three times on different days. The percentage of disagreement of visual scores for the distribution of spots for A, B and C-eggs were 6.25%, 12.5% and 18.75%, respectively, for average size of spots for A, B and C-eggs were 18.75%, 18.75% and 34.38%, respectively and for appearance of streaks for A, B and C-eggs were 12.50%, 15.63% and 0% , respectively.

Statistical analysis

If there was an interaction between laying order and laying date, I separated the analysis into two groups, early laying birds and late laying birds. I used the mean laying date to separate the two laying groups. In order to investigate the difference of the measurements of the background colour of eggshells between A, B and C-eggs of the same clutch, I used repeated-measures analyses in SPSS (Version 11.5). If the assumption of sphericity was violated, I used the Greenhouse-Geisser correction. All tests are two-tailed and $P < 0.05$ is considered significant. Mean values with ± S.E.

are reported. For the analysis of the data on spot characteristics, I used chi-square tests.

Results

Firstly, from the visual scores for shell background colour, A-eggs had a high frequency of dark brown eggs, B-eggs had a high frequency of light brown egg and C-eggs had a high frequency of light brown and light green eggs ($\chi^2 = 9.77$, df = 4, $P = 0.045$) (Table 4.1).

Digital imaging analysis

Only one component (PC1) was extracted. PC-RGB represented 93.63 % of the variance and had a positive relationship with the visual score of background colour; $r_s = 0.680$, df = 94, $P < 0.001$(Fig. 2). I interpreted the low PC1 scores as dark brown and the high PC1 scores as light green.

There was a significant effect of the interaction between the variability colour with laying order and laying date on PC-RGB values ($F_{2, 29} = 5.82$, $P = 0.007$). So, I analysed the data of early laying and late laying birds separately. There was a difference in PC1 score between laying orders among early laying birds ($F_{2, 13} = 4.84$, $P = 0.027$). The post-hoc test suggested that C-eggs had higher PC-RGB scores than A- & B-eggs, which means A- & B-eggs were browner and C-eggs greener (Fig. 4.3a). There was no difference in PC-RGB scores with laying order in late laying birds ($F_{2, 15} = 1.36$, $P = 0.286$), the C-eggs seemed to remain of a similar brown colour as the two first-laid eggs (Fig. 4.3a).

Spectral analysis

The mean reflectance in wavelength 300-700 nm of dark brown egg, light brown egg and light green egg are displayed in figure 4.4a. The mean reflectances of background colour spectrum (300-700 nm) of A-, B-and C-eggs are shown in Fig. 4.4b. The principal component analysis extracted two variables (PC1 & PC2) from the spectral data. PC1 and PC2 presented 47.16% and 32.75% of the variation in the spectra, respectively. The PC1 covered 520 to 619 nm (from green to orange colour in spectrum) and the coefficient values of PC1 from principal component analysis were positive at relatively short wavelength (green colour) and negative at relatively long wavelength (orange colour) (Fig. 4.5). The PC2 covered 619 to 700 nm (from orange to red colour in spectrum) and the coefficient values of PC2 from principal component analysis were negative at relatively short wavelength (orange colour) and positive at relatively long wavelength (red colour) (Fig. 4.5). Hence, I interpreted the low PC1 scores as orange and the high PC1 scores as green and low PC2 scores as orange and the high PC2 scores as red. PC1 (r = -0.339, df = 91, P = 0.001) and PC2 (r = -0.341, df = 91, P = 0.001) score from the spectral analysis were negatively correlated with PC-RGB.

There was a significant interaction between laying order and laying date on PC1 score ($F_{2,28}$ = 4.15, P = 0.026) and PC2 score ($F_{2,28}$ = 3.46, P = 0.046). So, I analysed the data of early laying and late laying birds separately. There was a difference in PC1 score with laying order among early laying birds ($F_{2,13}$ = 5.41, P = 0.020). The post-hoc test indicated that C-eggs had lower PC1 scores than A- & B-eggs (Fig. 4.3b). This suggested that C-eggs had a higher orange component than A & B-eggs. There was no difference in PC1 score with laying order among late laying birds ($F_{2,14}$

= 0.18, $P = 0.836$) (Fig. 4.3b). There was a difference in PC2 score with laying order among early laying birds ($F_{2,13} = 7.21$, $P = 0.008$). The post-hoc test suggested that C-eggs had a lower score than A- & B-eggs (Fig. 4.3c). This suggested that C-eggs had a lower red component than A & B-eggs. There was no difference in PC2 score with laying order among late laying birds ($F_{2,14} = 0.94$, $P = 0.412$) (Fig. 4.3c).

As there was a significant interaction between laying order and laying date on brightness ($F_{2,28} = 6.78$, $P = 0.004$). So, I analysed the data of early laying and late laying birds separately. There was a difference in brightness with laying order among early laying birds ($F_{2,13} = 7.86$, $P = 0.006$). The post-hoc test indicated that C-eggs were brighter than A-and B-eggs, whereas B-egg was brighter than A-egg (Fig. 4.6a). There was no difference in brightness with laying order among late laying birds ($F_{2,14} = 1.06$, P = 0.371) (Fig. 4.6a).

There was a marginally significant interaction between laying order and laying date on hue ($F_{2,28} = 3.30$, $P = 0.052$). The post-hoc test suggested that C-eggs had a lower hue than A and B-eggs ($F_{2,29} = 8.35$, $P = 0.001$) (Fig. 4.6b) There was no significant interaction between laying order and laying date on blue-green chroma ($F_{2,28} = 0.403$, $P = 0.672$). There was no difference in blue-green chroma between A, B and C-eggs ($F_{2,29} = 0.327$, $P = 0.724$) (Fig. 4.6c). PC-RGB was positively correlated with blue-green chroma ($r = 0.467$, df = 91, $P < 0.001$).

As there was a significant interaction between laying order and laying date on UV-chroma ($F_{2,28} = 8.90$, P = 0.001) I analysed the data of early laying and late laying birds separately. There was a difference in UV-chroma with laying order among

early laying birds ($F_{2, 13} = 20.27$, $P < 0.001$). The post-hoc test indicated that C-eggs had more UV-chroma than A- and B-eggs. There was no difference in UV-chroma with laying order among late laying birds (Greenhouse-Geisser correction: $F_{1.33, 20.02} = 1.60$, $P = 0.226$) (Fig. 4.6d).

A-, B- & C- eggs had a similar pattern of spot distribution ($\chi^2 = 5.26$, df = 2, $P = 0.072$). A- & B- eggs had larger spots than C-eggs ($\chi^2 = 25.44$, df = 2, $P < 0.001$). I rarely found streaks on A-& B-eggs (3.13% & 18.75%, respectively), but often found them on C eggs (81.25%) ($\chi^2 = 48.49$, df = 2, $P < 0.001$). The contingency table score of characteristics of markings on eggshells is shown in Table 4.2.

Table 4.1

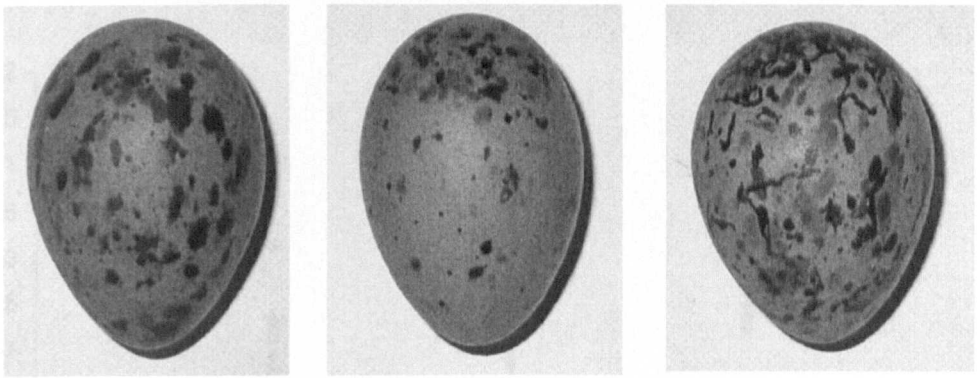
	Dark brown	Light brown	Light green
A	10	20	2
B	6	19	7
C	2	21	9

Visual scores for shell background colour for A-, B- and C-eggs.

Table 4.2

	Distribution of spots		Average size of spots		Appearance of streaks	
	Clumped	Even	Small	Large	No	Yes
A-egg	6	26	6	26	31	1
B-egg	13	19	8	24	26	6
C-egg	14	18	24	8	6	26

Contingency table score of characteristics of markings on eggshell



a b c

Figure 4.1. Egg with an even distribution of large spots without streaks (a), egg with a clumped distribution of small spots without streaks (b), and egg with an even distribution of small spots with streaks (c).

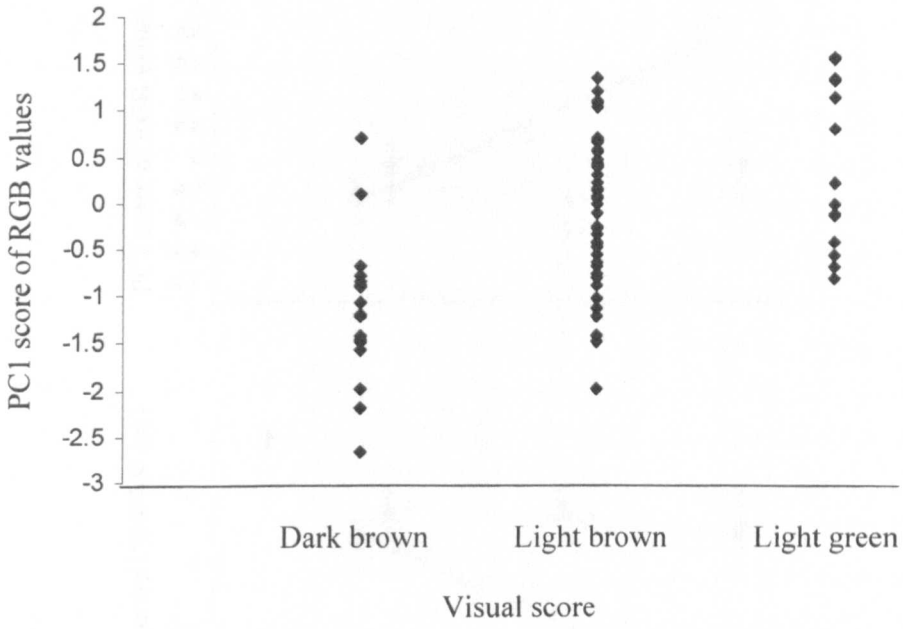


Figure 4.2. Relationship between visual colour score and PC-RGB values of the eggshell background colour

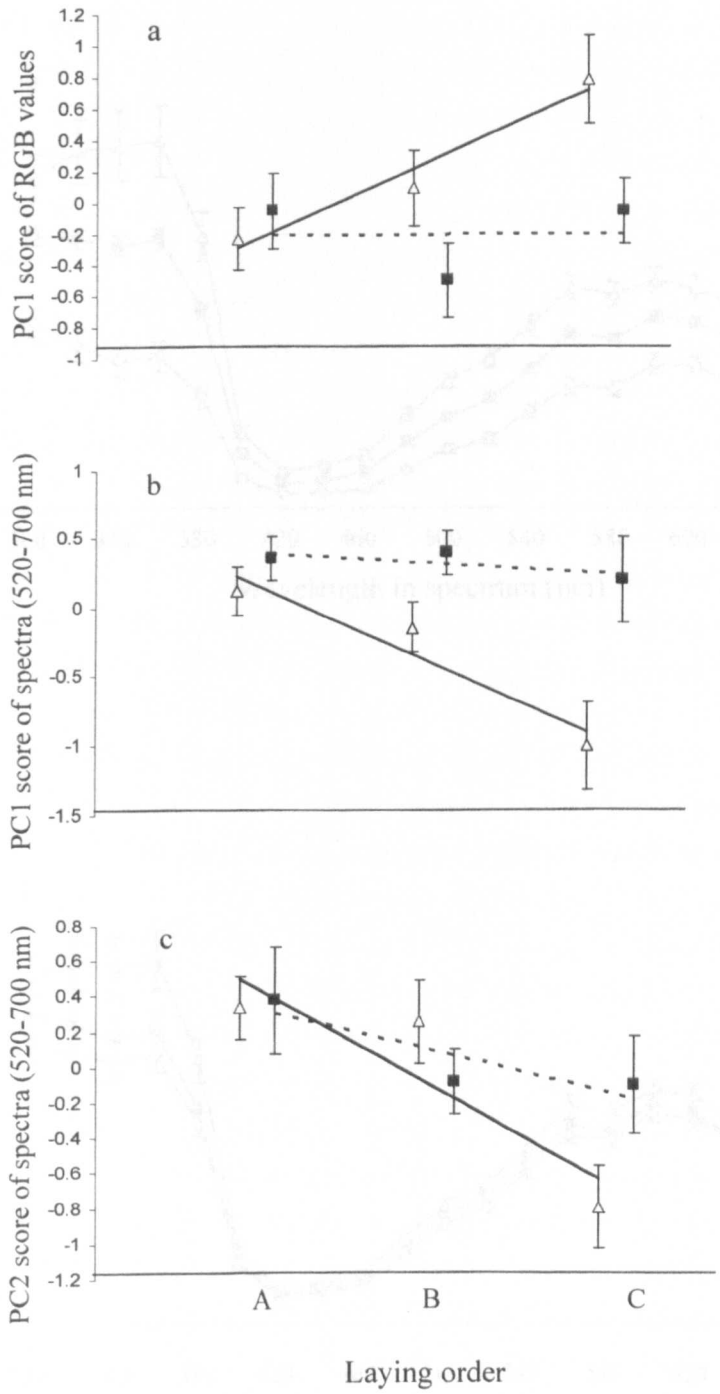
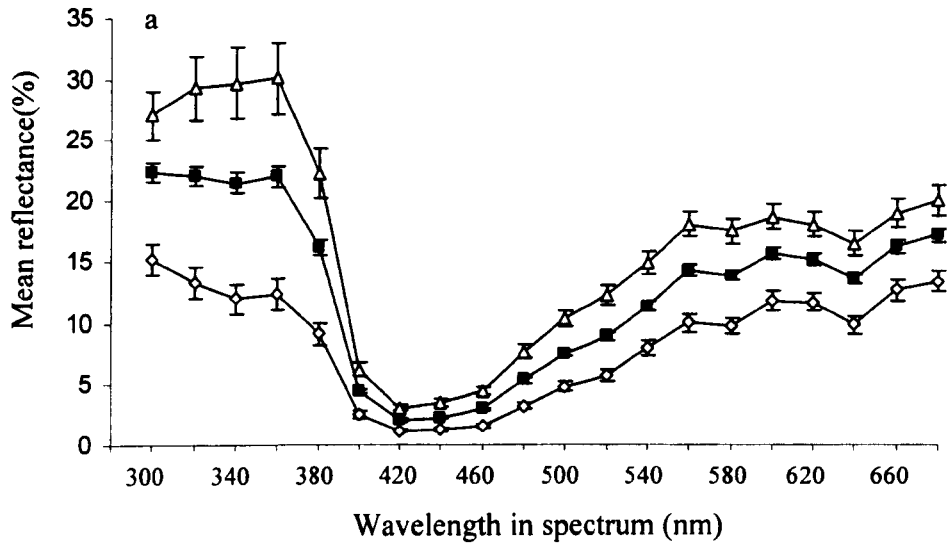


Figure 4.3. PC-RGB values of the eggshell background colour (a), PC1 score (b) and PC2 (c) score of spectra 520-700 nm of background colour of A-, B- and C-eggs from early laying birds (Δ) and late laying birds (■).



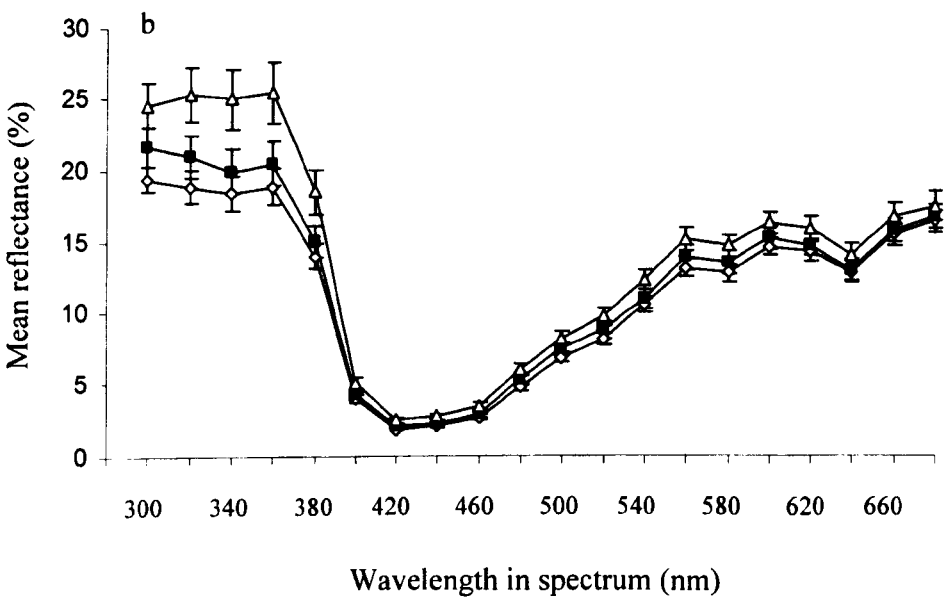


Figure 4.4. (a) Mean reflectance of background colour spectrum (300-700 nm) of dark brown egg (◊), light brown egg (■) and light green egg (Δ). (b) Means reflectance of background colour spectrum (300-700 nm) of A (◊), B (■) and C-eggs (Δ).

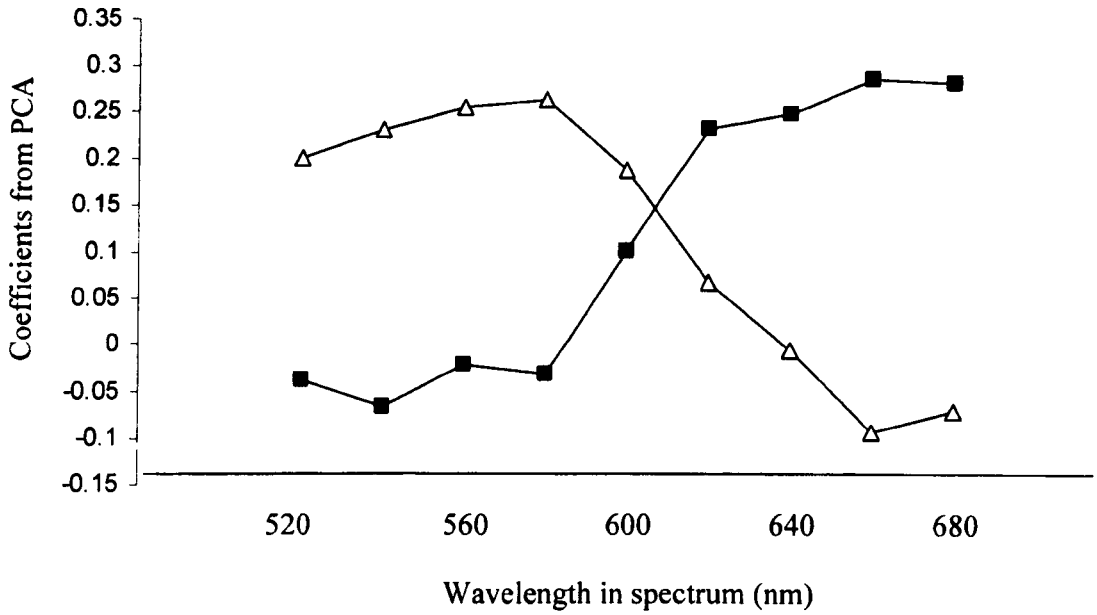


Figure 4.5. Coefficients of PC1 (Δ) and PC2 (■) from a principal components analysis on the spectra (520-700 nm) of the eggshell background colour of the eggs.

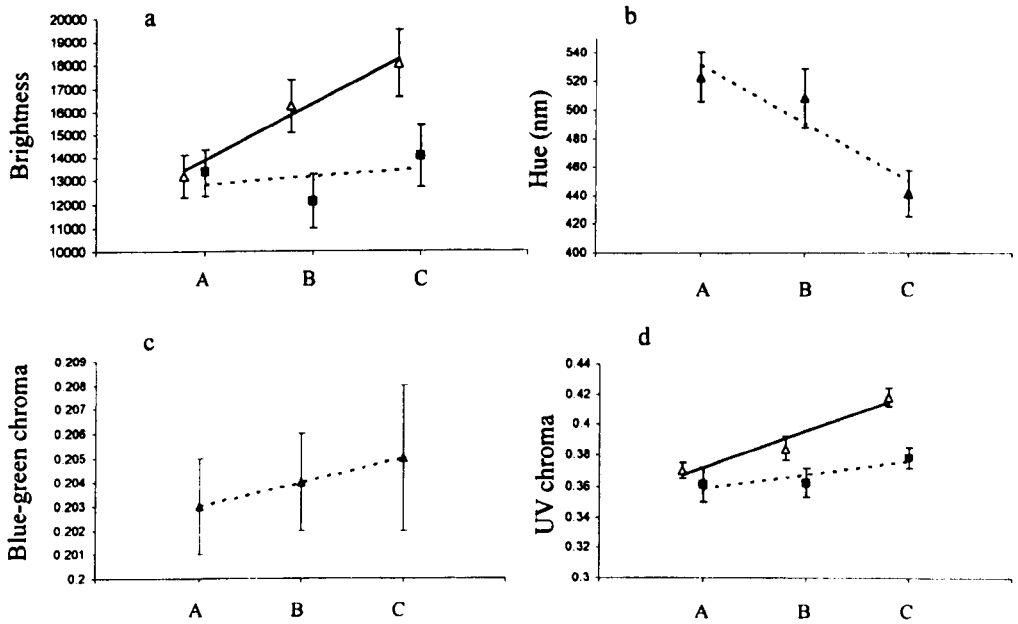


Figure 4.6. (a) Brightness of background colour of A-, B- and C-eggs from early laying birds (Δ) and late laying birds (■). (b) Hue of background colour of A-, B- and C-eggs.(c) Blue-green chroma of background colour of A-, B- and C-eggs. (d) UV chroma of background colour of A-, B- and C-eggs from early laying birds (Δ) and late laying birds (■).

Discussion

The aim of this study was to describe variation in eggshell coloration in relation to the laying order. Overall, the coloration of C-eggs differed from A and B-eggs, but only among early-laying birds and not among late-laying birds. Digital imaging and visual scores gave the impression that C-eggs were greener than A- and B-eggs. But spectral analysis revealed no difference in blue-green chroma among laying orders. This blue-green chroma is well correlated with biliverdin (Moreno *et al.*, 2006). So A-, B- and C-eggs were unlikely to differ in biliverdin content.

Porphyrin pigments produce brown coloured shell and reflect in the orange to red wavelengths (Solomon, 1991). So, this study interpreted the influence of protoporphyrin from the PC2 score of spectral analysis that covered the orange to red wavelength range. Spectral analysis showed that C-eggs differed from A- and B-eggs in red and orange hues, so porphyrins may be responsible for this colour change. Mikšík *et al.* (1994) studied eggs of red-backed shrike (*Lanius collurio*) and found that the amount of porphyrin decreased over the laying sequence, decreasing toward the last egg. This pattern may be similar to that of the lesser black-backed gull.

Early-laying females may put less effort into porphyrin deposition in the last egg than in the first two eggs of the clutch. Possibly, females run out of the pigment during the production of the last egg in the clutch (reviewed in Underwood and Sealy, 2002), but then it would be unclear why late-laying birds did not show the same pattern. Early laying females might be older than the late laying birds; and age difference may affect their physiological condition that may be relevant to pigmentation (Solomon, 1991). Alternatively, the early laying birds might be more

stressed, a condition associated with hormonal disturbances, which affects pigmentation (Solomon, 1991). So far, there is insufficient information on the costs of egg pigment production and whether pigment deposition is limited by resource or manipulated by laying birds.

By being brighter and having higher UV-chroma, C-eggs of early laying birds may stand out more from their surroundings than A- and B-eggs. This distinctive C-egg may attract a predator's interest to the egg with the lowest fledging rate in the clutch (Verbeek, 1988). But from my preliminary analysis on eggshell colour and risk of predation, the eggshell background colour seemed not to be a factor (green and brown eggs had the same survival rate). In the lesser black-backed gull, C-egg are less likely to fledge (Royle and Hammer, 1998). Alternatively, increasing colour variation of the clutch may improve camouflage of the clutch (Hockey, 1982). Moreno *et al.*, (2006) also found UV-reflectance on the blue-green eggshell of pied flycatchers (*Ficedula hypoleuca*).

Visual scoring for shell coloration was quite poor on repeatability in this study, especially for shell background colour and average size of spot. However, we can use digital image and spectrophotometer to measure background colour. For future work, measuring density of area of spot using image analyser should be more effective, and we may be able to use this measurement as an indicator for the amount of pigment in the shell.

Baerends and Hogan-Warburg (1982) studied eggs of herring gulls (*Larus argentatus*) and found that C-eggs were paler and more often streaked than A and B-eggs, similar

to the lesser black-backed gull in this study. But they did not mention any relationship with laying date. Kilpi and Byholm (1995) also studied herring gull eggs, but they often found similar background colour in all eggs in the same clutch. Glaucous-winged gulls (*Larus glaucescens*) also lay a distinctive, pale C-egg (Verbeek, 1988). Possibly the distinctive C-egg may be a typical trait of Laridae.

So far, this study found variation in shell coloration within laying order. According to Gosler *et al.* (2005)'s structure function hypothesis, it would be interesting to investigate whether shell colour correlates with shell structure in the lesser black-backed gull.

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Chapter 5: Relationship of eggshell colour and eggshell structure

Abstract

According to the structure-function hypothesis of Gosler *et al.* (2005) birds may deposit more protoporphyrin pigment into eggshell where calcium is limited in order to maintain the strength of the shell. This predicts that there should be a relationship between the eggshell structure and egg coloration. This study tests whether there are any correlations between shell pigmentation and shell thickness, shell porosity and density of mammillary layer contact area in lesser black-backed gull (*Larus fuscus*). This study found just some relationships between shell structures and shell coloration.

Introduction

Generally, crypsis has been believed to be the main function of shell coloration. But many species of birds (e.g. swallow, nuthatch, treecreeper and great tit) that build nests in closed environments without being seen by predators still produce maculated eggs. This suggests that there is another function for markings on the eggshell. Bakken *et al.* (1978) found protoporphyrin (brown pigment) in eggshells could reflect light in the near-infrared very well. Moreno *et al.*, (2004) suggested that the biliverdin-based eggshell colours may influence paternal care in species with biparental care. Gosler *et al.* (2005) proposed the structure-function hypothesis; the birds may deposit more pigment into the shell to maintain the strength of the shell where calcium is limited source. Kennedy and Vevers (1976) reported that maculated eggs always had protoporphyrin. Solomon (1991) suggested that the structure of porphyrins was similar to phthalocyanine lubricants that are used in solid-state engineering. The porphyrins may act like a cushion between the calcite crystals

making the shell more resistant to cracking. Biliverdin is the pigment that can be found in blue-green eggshells, Moreno *et al.* (2006) found a positive correlation between intensity of biliverdin and body condition of laying female of pied flycatchers (*Ficedula hypoleuca*).

From Gosler *et al*'s hypothesis (2005) it follows that there should be relationships between the eggshell structure and eggshell coloration. In this study, I test whether there are any correlations between shell thickness, shell porosity and density of mammillary layer contact area and eggshell coloration in the lesser black-backed gull (*Larus fuscus*).

Materials and methods

Fresh eggs from 32 three-egg clutches of the lesser black-backed gull (*Larus fuscus*) (the same eggs as in chapter 3 and 4) were collected from a large colony at South Walney Nature Reserve, Walney Island, northwest England, UK, during the breeding season in 2002. On the day of laying fresh eggs were weighed to the nearest 0.01g using an electronic balance and length and width measured with a calliper to an accuracy of 0.1 mm. Eggs were then frozen until analysis. The frozen egg was cut longitudinally by using a scalpel into two halves; the two-half shells (with shell membranes) were separated from egg content. The eggshell was dried in an oven at 50°C until it reached a constant dry weight. The dry weight of eggshell was measured by using an electronic balance to the nearest 0.001 g.

Egg volume was calculated by using the equation $V = K_V \times LB^2$ (L = length in cm, B = width in cm, K_V (volume coefficient) = 0.4965, as the average from *Larus*

species (Hoyt, 1979). The surface area was calculated by using the equation A = 4.951 . $V^{0.666}$ (V = egg volume in cm^3) (Paganelli *et al.*, 1974). An eggshell thickness index (ETI) was calculated by dividing eggshell mass by the surface area of the egg (Green, 1998).

I measured the thickness of eggshells from all 32 three-egg clutches at three different areas; blunt end, equatorial zone and pointed end, by using a scanning electron microscope (see detail in chapter 3). I measured eggshell thickness index (ETI) of 31 three-egg clutches (see detail in chapter 3). For the comparison of shell thickness between in spot area and non-spot area, shells from 29 eggs were randomly selected from the same population as above. Then, I measured shell thickness at two areas; the spot area and non-spot area (adjacent to the spot) of these 29 eggs (with no specific area of egg selected) to the nearest 0.005 mm by using a modified micrometer (Draper PM 025). The micrometer was modified with rounded tips to fit to the curvature of the eggshell. I measured shell porosity for 23 three-egg clutches at the blunt end, the equatorial zone and the pointed end (see detail of method in chapter 3). I measured density of mammillary layer contact area for 14 three-egg clutches at the blunt end, the equatorial zone and the pointed end (see detail of method in chapter 3).

I measured background eggshell colour for 32 three-egg clutches by using digital image analysis (principal components analysis; see detail of analysis in chapter 4), I interpreted the low PC-RGB scores as dark brown and the high PC-RGB scores as light green, so PC-RGB can be used as an indicator for porphyrins. I also measured background eggshell colour for 31 clutches by using a spectrophotometer (due to one

clutch having an egg that had too small a background area to use with the spectrophotometer, so only 31 clutches were used in the spectral analyses) (see detail of spectral analysis in chapter 4). I interpreted the low PC1 scores as orange and the high PC1 scores as green. I interpreted the low PC2 scores as orange and the high PC2 scores as red. So, PC1 and PC2 together can suggest the degree of porphyrins in eggshell. I used blue-green chroma as an index of biliverdin concentration (Moreno *et al.*, 2006).

Statistical treatment

I checked the data, for normal distribution, and used arcsin-transformation for the data of density of mammillary layer contact area. If I found interactions between egg order and shell colour on eggshell structure (I report only statistically significant interactions), I then analysed the relationship between shell structure and shell colour for A, B and C-eggs separately. SPSS Version 11.5 was used to analyse the data. All tests are two-tailed and $P < 0.05$ is significant.

Results

The area of the spot had thinner shell than adjacent non-spot area (paired t-test: t_{28} = 3.73, P = 0.001; Fig. 5.1). There was a significant interaction between egg order and PC-RGB ($F_{3,92} = 3.75$, $P = 0.014$) on ETI. Only ETI of A-egg had a significant relationship with PC-RGB (A-egg: $r = -0.513$, df = 30, $P = 0.003$; Fig. 5.2). ETI was not related to PC1, PC2, brightness and blue-green chroma (all $P \geq 0.146$).

Shell thickness at the blunt end, was significantly influenced by an interaction between egg order and blue-green chroma ($F_{3,89} = 3.50$, $P = 0.019$). Only shell

thickness at the blunt end of the A-egg was significantly correlated with blue-green chroma (r = -0.402, df = 29, P = 0.025; Fig. 5.3a). Shell thickness at the blunt end did not correlate with PC1, PC2 and brightness (all $P \geq 0.057$), had a weak negative correlation with PC-RGB (r = -0.195, df = 94, P = 0.057; Fig. 5.3b) (Shell thickness at the equatorial zone was significantly influenced by an interaction between egg order and PC-RGB ($F_{3,92}$ = 3.08, P = 0.031). Again, only shell thickness at the equatorial zone of A-egg was significantly correlated with PC-RGB (r = -0.439, df = 30, P = 0.012; Fig. 5.3c). Shell thickness at the equatorial zone did not correlate with PC1, PC2, blue-green chroma and brightness (all $P \geq 0.204$). Shell thickness at the pointed end did not correlate with PC-RGB, PC1, PC2, blue-green chroma and brightness (all $P \geq 0.159$).

Pore density at the blunt end was significantly affected by the interaction between egg order and blue-green chroma ($F_{3,62}$ = 3.83, P = 0.014). Only pore density at the blunt end of A-eggs correlated with blue-green chroma (r = 0.473, df = 20, P = 0.026; Fig. 5.4), but not with PC-RGB, PC1, PC2 and brightness (all $P \geq 0.343$). Pore densities at the equatorial zone and the pointed end did not correlate with PC-RGB, PC1, PC2, blue-green chroma and brightness (all $P \geq 0.084$).

Mammillary layer contact area at the blunt end was significantly affected by the interaction between egg order and PC1 ($F_{3,38}$ = 3.35, P = 0.029). Only the mammillary layer contact area of A-eggs was significantly correlated with PC1 (r = -0.532, df = 12, P = 0.050; Fig. 5.5), but not with PC-RGB, PC2, blue-green chroma and brightness (all $P \geq 0.184$). The mammillary layer contact area at the equatorial zone was also influenced significantly by the interactions between egg order and PC2

($F_{3,38}$ = 4.07, P = 0.013) and between egg order and brightness ($F_{3,38}$ = 3.05, P = 0.040). I found, however, no significant correlation between mammillary layer contact area and PC2 or brightness for any egg order. The density of mammillary layer contact area at the equatorial zone did not correlate with PC-RGB, PC1 or blue-green chroma. Overall, the density of mammillary layer contact area at the pointed end was unrelated to any shell colour characteristics.

For comparisons of shell thickness, porosity and mammillary layer contact area between spot characteristics at three different shell areas, all of them had no significant differences (Table 5.1), except the eggs with large spots had thicker shell than the eggs with small spots at the blunt end, the eggs with small spots had higher density of mammillary layer contact area than the eggs with large spots at the pointed end and the eggs with clumped distribution of spots had lower density of mammillary layer contact area than the egg with even distribution of spots (Table 5.1).

Table 5.1

	Spot distribution pattern (clump vs even)	Average spot size (small vs large)	Appearance of streaks (streaks vs no streaks)
Thickness			
Blunt end	$t_{94} = 1.48$, $P = 0.144$	$t_{94} = 2.49$, $P = 0.015$	$t_{94} = 1.81$, $P = 0.074$
Equatorial zone	$t_{94} = 1.36$, $P = 0.177$	$t_{94} = 1.21$, $P = 0.228$	$t_{94} = 0.13$, $P = 0.900$
Pointed end	$t_{94} = 0.63$, $P = 0.531$	$t_{94} = 0.57$, $P = 0.569$	$t_{94} = 0.68$, $P = 0.496$
Porosity			
Blunt end	$t_{67} = 0.53$, $P = 0.597$	$t_{67} = 0.30$, $P = 0.763$	$t_{67} = 1.16$, $P = 0.251$
Equatorial zone	$t_{67} = 0.34$, $P = 0.732$	$t_{67} = 1.13$, $P = 0.262$	$t_{67} = 0.86$, $P = 0.392$
Pointed end	$t_{67} = 0.43$, $P = 0.672$	$t_{67} = 0.23$, $P = 0.819$	$t_{67} = 0.33$, $P = 0.741$
Mammillary layer contact area			
Blunt end	$t_{40} = 1.47$, $P = 0.149$	$t_{40} = 0.97$, $P = 0.339$	$t_{40} = 0.80$, $P = 0.429$
Equatorial zone	$t_{40} = 0.46$, $P = 0.650$	$t_{40} = 0.03$, $P = 0.976$	$t_{40} = 1.54$, $P = 0.131$
Pointed end	$t_{40} = 2.63$, $P = 0.012$	$t_{40} = 3.45$, $P = 0.001$	$t_{40} = 1.71$, $P = 0.096$

Comparisons of egg shell characteristics in three spot characteristics at three different shell area.

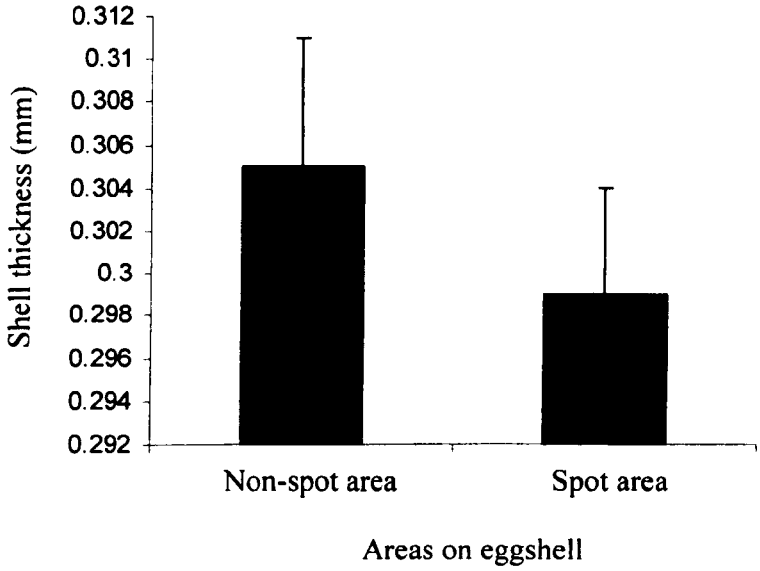

Figure 5.1. Shell thickness at area of the spot and adjacent non-spot area

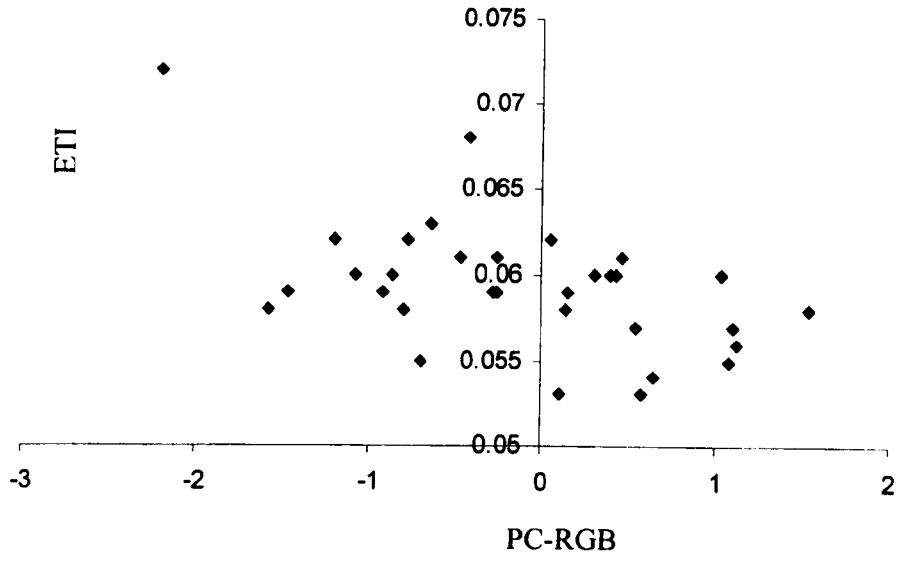

Figure 5.2. Correlation between ETI and PC-RGB of A-eggs.

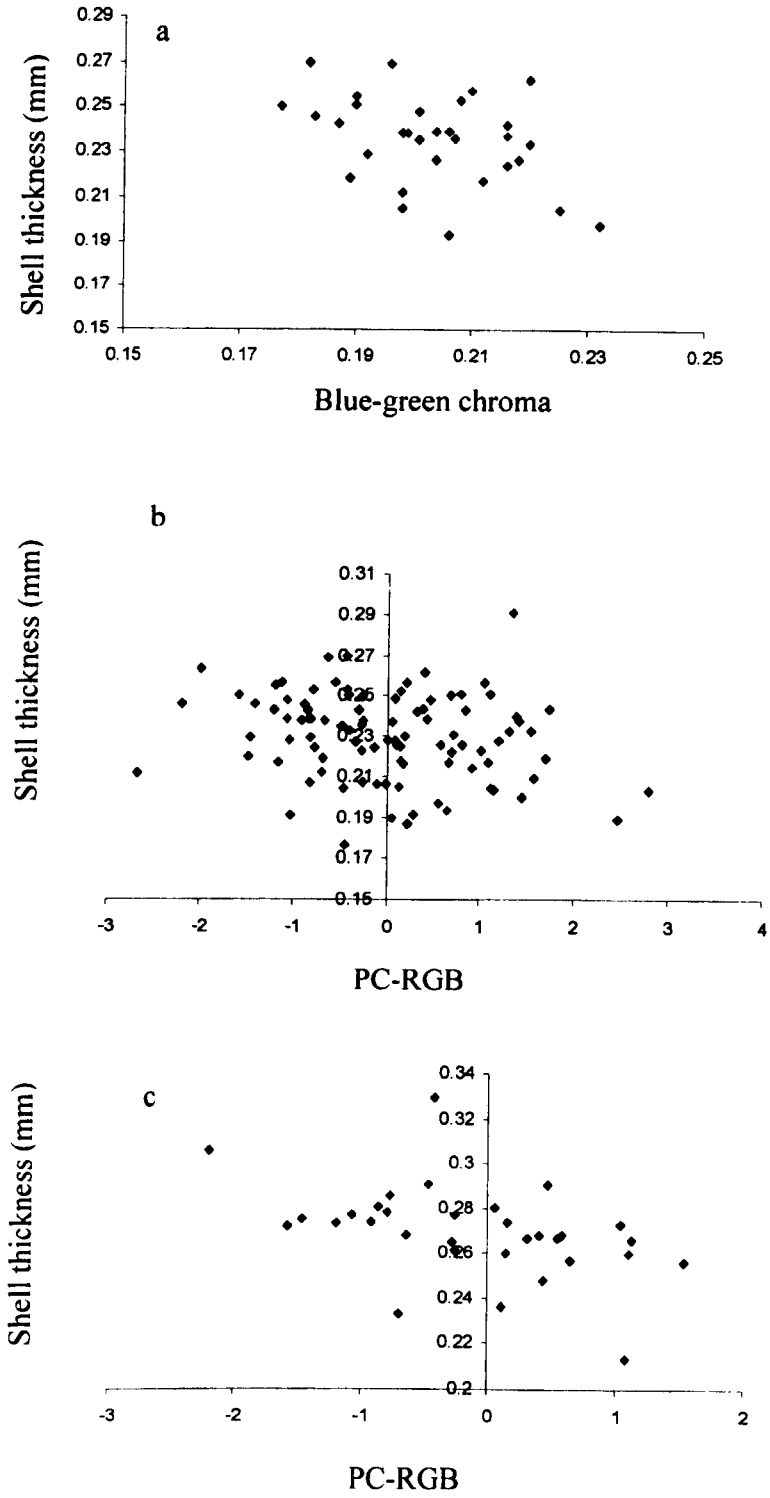

Figure 5.3. Correlation between shell thickness at the blunt end of A-egg and blue-green chroma (a). Correlation between shell thickness at the blunt end and PC-RGB (b). Correlation between shell thickness at the equatorial zone of A-egg and PC-RGB (c).

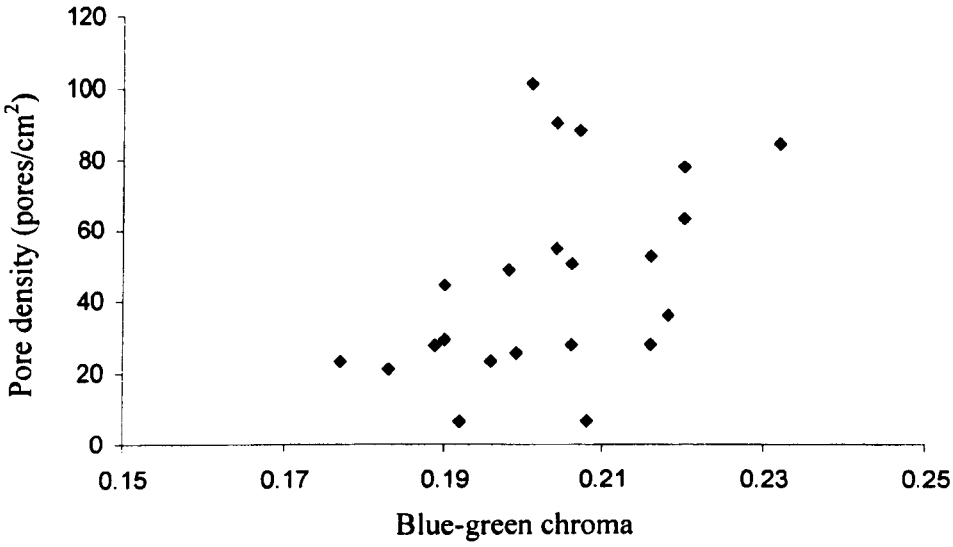

Figure 5.4. Correlation between pore density at the blunt end of A-eggs and blue-green chroma.

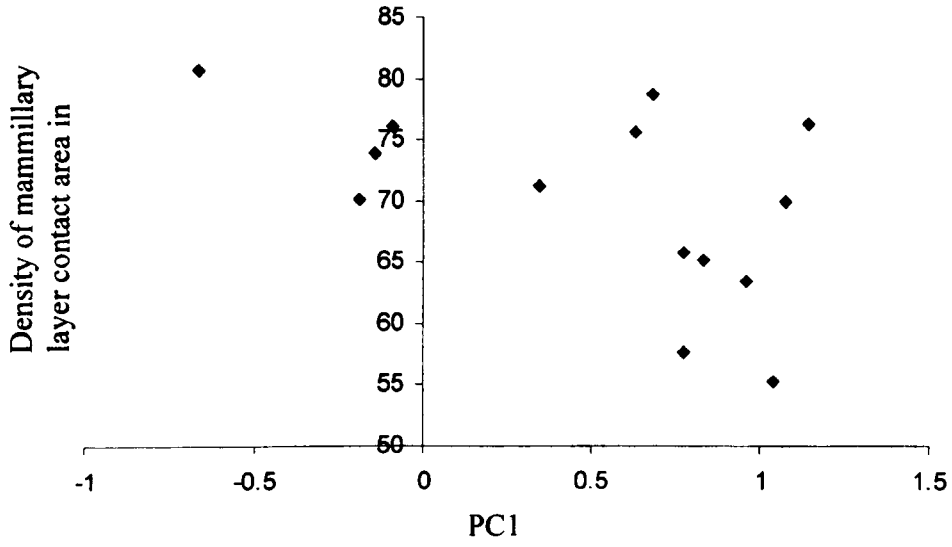

Figure 5.5. Correlation between density of mammillary layer contact area at the blunt end of A-eggs and PC1.

Discussion

This study found relationships between shell structures and shell coloration just in A-eggs, most of them appeared at the blunt end. Even non-significant but weak negative relationship with PC-RGB may indicate that thicker shell at the blunt end seemed to have more protoporphyrin pigment (brown colour), it supported a negative relationship between shell thickness at the blunt end of A-egg and blue-green chroma (indicator for biliverdin concentration), so shell background colour may be used as an indicator for shell thickness at the blunt end of A-eggs. Shell thickness at the equatorial zone of A-egg had a negative relationship with PC-RGB, but shell thickness at the equatorial zone generally had no relationship with blue-green chroma, so it was still unclear about the relationship between pigmentation and shell thickness at the equatorial zone of A-eggs. For shell porosity, the biliverdin concentration had a positive relationship with pore density at the blunt end, but without variation of pore density within the shell (in chapter 3), it was difficult to interpret this relationship. The density of mammillary layer contact area at the blunt end of A-egg had a positive relationship with PC1, but without any relationship with PC2 or PC-RGB, again it was difficult to interpret this relationship. However, the shell background colour measurements in this study were not measured on three different areas on the shell, because generally the shell did not have proper size of non-spot area for the sensor of spectrophotometer through out the shell. For further study, by using the proper sensor size of spectrophotometer (a modified one) for colour measurement on the three different areas of the shell may clarify the relationships between shell coloration and shell structures (with variations within shell).

For the comparison of shell thickness between high contrast coloration areas, it was quite clear that the area of the spots (dark colour area) had thinner shell than the adjacent non-spot areas (light colour area), this result was quite similar to the work of Gosler *et al.* (2005) in great tits.

For comparisons of shell structure characteristics between the spot characteristics, the spot characteristics had no effect on shell porosity, but spot size had effects on shell thickness at the blunt end and on density of mammillary layer contact area at the pointed end and the distribution of spot had an effect on density of mammillary layer contact area at the blunt end. With limited sample sizes, these results were from pooled data of A, B & C-eggs, so it was still unclear about the effect of laying order.

This study rarely found a relationship between shell structure and shell background coloration. So, the structure-function hypothesis (Gosler *et* al., 2005) may not generally work for shell background colour in the lesser black-backed gulls.

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Chapter 6: Is the shell formation in lesser black-backed gulls limited by Ca availability?

Abstract

Calcium is essential for skeletal development in the embryo. Many researchers have found avian reproduction to be limited by Ca-availability. Generally, there are two strategies for managing calcium for egg production; storing calcium in bone long before egg laying and foraging for calcium while forming the eggs. Ca-specific foraging has been reported in some marine birds. Some researchers already found eggshell quality declined with laying order. This study used a Ca-supplementation experiment to investigate whether the shell formation in lesser black-backed gulls (*Larus fuscus*) is limited by Ca-availability. Ca-limitation had an effect on shell thickness, but no effect on shell background colour.

Introduction

Some researchers have reported calcium-limited reproduction in avian species in Ca-poor areas and most evidence shows that females in Ca-poor areas produce thin-shelled eggs (Reynolds *et al.*, 2004). Normally, eggshell serves some crucial roles in reproductive processes; protection of the embryo from mechanical damage, calcium provider to the growing embryo, gas exchange with the environment and conservation of water (Board, 1982; Carey, 1996). Thin shell potentially affects eggshell functions, and limits reproduction of birds in Ca-poor habitats.

There are two strategies for managing calcium for egg production; storing calcium long before egg laying and foraging for calcium while forming the eggs. Graveland & Berends (1997) reported that a small passerine, the great tit (*Parus major*) did not store calcium, but produced eggshells on calcium that was consumed just shortly before and during egg laying. From high-resolution radiography, Pahl *et al.* (1997) also found no evidence of calcium storage prior to egg laying in the leg bones in three species of small passerines. On the other hand, Piersma *et al.* (1996) found evidence of calcium storage in the skeleton of the red knot (*Calidris canutus*) long before egg laying.

So far, we still have little knowledge about strategies for managing calcium resources in marine birds. However, there are two reports of mollusc shell feeding prior to egg laying in Sandwich tern (*Sterna sandvicensis*) (Brenninkmeijer *et al.*, 1997) and common tern (*Sterna hirundo*) (Nisbet, 1997). Generally, for piscivorous marine birds, it has been assumed that they would obtain enough calcium for egg production from their regular diet, but the evidence of Ca-specific foraging in two terns indicate that marine birds may need extra calcium for egg production. By manipulating female common terns to lay an additional egg, Heaney *et al.* (1998) found that the additional egg had thinner shell than the last egg of the regular clutch. They suggested that the female may run out of calcium as the laying sequence progresses.

In order to find out if there is a calcium deficiency, calcium-supplementation experiments have been used widely in passerines (Reynolds *et al.*, 2004). With its high sensitivity to calcium availability, eggshell thickness was often used as a

response variable in many calcium supplementation experiments (Reynolds *et al.*, 2004).

In this study, I investigated whether shell formation in lesser black-backed gulls (*Larus fuscus*) is limited by Ca-availability. In order to set the situation of limited calcium resource, I manipulated the female birds to produce additional eggs. In order to clarify whether the female manipulated the shell quality, a Ca-supplementation experiment was used in this study. From the structural-function hypothesis of Gosler *et al.* (2005), in shortage of calcium the laying female may deposit more pigment to maintain the strength of the shell. So, apart from study on shell thickness, this study also took an opportunity to observe effect of Ca-limitation on shell coloration.

Materials and methods

I carried out a Ca-supplementation experiment in the central area of the mixed breeding colony of lesser black-backed gulls (*Larus fuscus*) and Herring gulls (*Larus argentatus*) at South Walney Nature Reserve, Walney Island, northwest England, UK, in 2003. I randomly selected 80 nests and provided them with calcium supplement. To avoid an unintentional effect of the Ca-supplementation, control nests were not directly adjoining supplemented nests. The calcium supplement contained equal amounts of fragmented chicken's eggshells and oystershell grit mixed together. Nests were supplemented as soon as I found the bird making a scrape (the mean date when supplementation started = 25.97±1.05; range = 18-37; day 1 = 1st April 2003). Each evening, I added 25 g of calcium supplement on a place close to the nest until the last egg was laid {the mean duration of supplementation period (until they start laying) = 18.43±1.55; range = 2-39 days}. I did not provide the calcium supplement to nests

that still had a lot of remaining calcium supplement, so I tried to maintain about the same amount of Ca-supplement at each nest.

For about half of control and supplemented nests, I experimentally increased the females'egg production effort. *Larus* gulls normally lay a clutch of three eggs, removal of the first-laid eggs within 12 h of laying would induce them to lay a fourth egg (following Heaney *et* al., 1998; Nager *et al.*, 2000), this created three-egg and four-egg clutches. For all nests, I collected first- and last-laid eggs. For three-egg clutches, I collected first-laid eggs (A_3-eggs) and replaced them with dummy eggs on the day they were laid and the last-laid eggs (C-eggs) I collected on the day they were laid without replacing them. For four-egg clutches, I collected first-laid eggs (A_4 eggs) without replacing. For the control groups, I collected the first and third eggs for 18 nests, and the first and fourth eggs for 11 nests. For the Ca-supplemented groups, I collected the first and third eggs for 17 nests, and the first and fourth eggs for 18 nests. So there were two types of clutch sizes, 3-egg clutch and 4-egg clutch, in the control and Ca-supplemented groups. On the day eggs were laid I weighed their fresh mass to the nearest 0.01g using an electronic balance and measured maximum length and width to the nearest 0.1 mm, using a calliper. I recorded laying dates, laying sequence and laying intervals.

I separated the eggshell from the egg contents.The eggshell was then dried in an oven at 50°C until it reached a constant dry weight. I measured the dry weight of eggshells to the nearest 0.001 g using an electronic balance.

I measured the background colour of eggshell by using digital image analysis. This yield R, G and B values that were combined using a principal components analysis

(see detail in chapter 4). I investigated the distribution of spots in this study. Apart from the distribution of spots, I also looked at the average size of spots and appearance of streaks (see detail in chapter 4). I categorized the distribution of spots into two categories: clumped distribution where there was some aggregation of spots around the blunt end and even distribution where all the spots are distributed equally all over the eggshell.

I calculated an eggshell thickness index (ETI) by dividing eggshell mass by the surface area of the egg (Green, 1998). Egg volume was calculated by using the equation $V = K_V \times LB^2$ (L = length in cm, B = width in cm, K_V (volume coefficient) = 0.4965, as the average from *Larus* species (Hoyt, 1979). The surface area was calculated by using the equation $A = 4.951 \times V^{0.666}$ (V = egg volume in cm^3) (Paganelli *et al.*, 1974). In order to investigate the effect of Ca-supplementation on shell thickness, I measured shell thickness at three different areas of the shell, the blunt end, the equatorial zone and the pointed end (see detail in chapter 3), to the nearest 0.005 mm using a micrometer (Draper PM 025). The micrometer was modified with rounded tips to fit to the curvature of the eggshell.

Statistical treatment

In this study I analysed ETI, shell thickness and shell background colour in relation to the number of eggs laid and Ca-supplementation. Since each nest contributed two eggs, I used repeated-measures analyses in SPSS (Version 11.5) to compare related sample. If the assumption of sphericity was violated, I used Greenhouse-Geisser correction. To analyse the data on maculation, likelihood of laying D-egg and the laying interval between C and D-eggs, I used chi-square tests. All tests are two-

tailed and $P < 0.05$ is considered to be significant. Mean values with ± S.E. are reported.

Results

There was no significant difference in the laying date of A-egg between control and Ca-supplemented groups (U = 388.50, control = 29, Ca-supplement = 35, P = 0.107). For nests, where I removed the A-egg as soon as it was laid, there was no significant difference in their likelihood of laying a fourth egg between control and Ca-supplemented groups ($\chi^2 = 0.19$, df = 1, $P = 0.665$; Table 6.1). For birds that laid a fourth egg after their first egg was removed, there was no significant difference in laying interval between C and D-eggs between control and Ca supplemented groups ($\chi^2 = 0.12$, df = 1, $P = 0.728$; Table 6.2).

Laying date and duration of the supplementation period had no effects on eggshell characteristics in the control and Ca-supplemented groups, respectively (Table 6.3). Therefore there was no need to consider these two variables in the subsequent analyses. Then I analysed the data of egg mass, shell dry weight, ETI, shell thickness and shell coloration.

There was a significant effect of egg order on egg mass, and none of the interactions were significant (Table 6.4), the first-laid egg was heavier than the last-laid egg.

There was a significant effect of egg order on dry shell weight but there was a significant interaction between egg order and Ca-supplementation (Table 6.4). The statistical analysis showed no significant difference in dry shell weight between the

first-laid eggs of control group and Ca-supplemented group ($t_{62} = 0.78$, $P = 0.436$) and also between the last-laid eggs of control group and Ca-supplemented group ($t_{62} = 0.86$, $P = 0.392$), but the interaction graph (Fig. 6.1) showed the last-laid egg of Ca-supplemented group seemed to have higher dry shell weight than the last-laid egg of control group.

There was a significant effect of egg order on ETI and also there was a significant interaction between laying order and Ca-supplementation (Table 6.4). Then I analysed the data of control and Ca-supplemented groups separately. The first-laid egg had higher ETI than the last-laid egg in control group (paired t-test: $t_{28} = 4.94$, $P < 0.001$; Fig. 6.2). In Ca-supplemeted group, there was no significance difference in ETI between the first-and last-laid eggs (paired t-test: $t_{34} = 1.14$, $P = 0.263$; Fig. 6.2). Having a significant effect of clutch size on ETI (Table 6.4), the eggs in 3-egg clutch had higher ETI than the eggs in 4-egg clutch ($t_{126} = 2.78$, $P = 0.006$).

For the shell thickness, I started with an exploration in interactions between egg order, shell area, Ca-supplementation and clutch size (Table 6.5). There were two significant interactions, between egg order and Ca-supplementation, and between egg order, shell area and clutch size (Table 6.5). Other interactions were non-significant ($P \geq 0.230$). Having a significant effect of shell area on shell thickness (Table 6.5) and shell area also had a significant interaction with egg order and clutch size, so I analysed the data in each shell area separately.

At the blunt end, there was a significant effect of egg order, but none of the interactions were significant (Table 6.6).The first egg had thicker shell than the last egg at the blunt end.

At the equatorial zone, there was a significant effect of egg order, but there was also a significant interaction between egg order and Ca-supplementation, and all other interactions were not significant (Table 6.6). Then, I analysed the data in control and Ca-supplemented groups separately. In control groups, the first-laid egg had thicker shell than the last-laid egg (paired t-test: $t_{28} = 4.73$, $P < 0.001$; Fig 6.3). In Ca-supplemented group, there was no significant difference in shell thickness between the first- and last-laid eggs (paired t-test: $t_{34} = 2.02$, $P = 0.052$; Fig. 6.3). Having a significant effect of clutch size on shell thickness at the equatorial zone (Table 6.6), the eggs in 3-egg clutch had thicker shell than the eggs in 4-egg clutch ($t_{126} = 2.65$, $P = 0.009$).

At the pointed end, there was a significant of egg order but there were interactions between egg order and Ca-supplementation and between egg order and clutch size and all other interactions were not significant (Table 6.6). Then, I analysed the data in three- and four-clutches separately in the control and the Ca-supplemented groups. In control groups, there was no significant difference in shell thickness between the first- and last-laid eggs in three-egg clutch (paired t-test: $t_{17} = 1.78$, $P = 0.093$; Fig. 6.4a), but the first-laid egg had thicker shell than the last-laid egg in four-egg clutch (paired t-test: $t_{10} = 3.29$, $P = 0.008$; Fig. 6.4a). In Ca-supplemented groups, there were no significant differences in shell thickness between the first-laid and last-laid

eggs in three-egg (paired t-test: $t_{16} = 0.58$, $P = 0.569$; Fig. 6.4b) and four-egg clutches (paired t-test: $t_{17} = 1.55$, $P = 0.140$; Fig. 6.4b).

For background colour of the shell, there was a significant effect of egg order, but there was a significant interaction between egg order and clutch size (Table 6.4). Then, I analysed the data in three- and four-clutches separately. The statistical analysis showed no significant difference in shell background colour between the first-laid egg of 3-egg clutch and 4-egg clutch ($t_{61} = 1.86$, $P = 0.068$) and also between the last-laid egg of 3-egg clutch and 4-egg clutch ($t_{61} = 1.50$, $P = 0.138$), but the interaction graph (figure 6.5) showed the first-laid egg of 4-egg clutch seemed to have darker shell than the first-laid egg of 3-egg clutch and the last-laid egg of 4-egg clutch seemed to have paler shell than the last-laid egg of 3-egg clutch.

For spot characteristics, I scored distribution of spots, average size of spots and appearance of streaks. Overall, the last-laid eggs had a high frequency of clump distribution ($\chi^2 = 15.32$, df = 1, $P < 0.001$) and the appearance of streaks ($\chi^2 = 53.59$, df = 1, $P < 0.001$), but the first-laid egg had a high frequency of large spots ($\chi^2 = 23.96$, df = 1, $P < 0.001$). For comparisons of the first-laid eggs between control and Ca-supplemented groups, the first-laid eggs of control group had high frequency of clumped distribution ($\chi^2 = 3.80$, df = 1, $P = 0.050$), but there were no significant difference in spot size ($\chi^2 = 0.41$, df = 1, $P = 0.523$) and the first-laid eggs of these two groups rarely had streaks on the shell ($\chi^2 = 1.03$, df = 1, $P = 0.310$). For comparisons of the last-laid eggs between control and Ca-supplemented groups, there were no significant difference in frequency of clumped distribution ($\chi^2 = 0.331$, df =

1, $P = 0.565$), small spots ($\chi^2 = 1.09$, df = 1, $P = 0.296$) and both groups often had streaks on the shell ($\chi^2 = 3.05$, df = 1, $P = 0.081$).

For comparisons of the last-laid eggs in control group between 3-egg and 4-egg clutches, D-egg had high frequency of even distribution of spots ($\chi^2 = 6.75$, df = 1, $P = 0.009$), C- and D-eggs had high frequency of small spots ($\chi^2 = 1.25$, df = 1, $P = 0.264$) and often had streaks on the shell ($\chi^2 = 0.63$, df = 1, $P = 0.426$). For comparisons of the last-laid eggs in Ca-supplemented group between 3-egg and 4-egg clutches, there was no significant difference in frequency of clumped distribution ($\chi^2 = 2.39$, df = 1, $P = 0.122$), C- and D-eggs had high frequency of small spots ($\chi^2 = 0.31$, df = 1, $P = 0.581$) and D-egg had high frequency of non-appearance of streaks ($\chi^2 = 6.84$, df = 1, $P = 0.009$).

For comparisons of the last-laid eggs in 3-egg clutch between control and Ca-supplemented groups, both groups had no significant difference in frequency of clumped distribution ($\chi^2 = 0.024$, df = 1, $P = 0.877$), both groups had high frequency of small spots ($\chi^2 = 0.31$, df = 1, $P = 0.581$) and high frequency of appearance of streaks ($\chi^2 = 0.97$, df = 1, $P = 0.324$). For comparisons of the last-laid eggs in 4-egg clutch between control and Ca-supplemented groups, both groups had no significant difference in frequency of clumped distribution ($\chi^2 = 0.284$, df = 1, $P = 0.092$), both groups had high frequency of small spots ($\chi^2 = 1.25$, df = 1, $P = 0.264$) but the last-laid egg in Ca-supplemented group had high frequency of non-appearance of streaks ($\chi^2 = 4.62$, df = 1, $P = 0.032$).

Table 6.1

	Ca supplemented	Control
Not laying D-egg	6	6
Laying D-egg	18	11

The females were forced to lay an additional egg (D-egg). This table show frequencies of laying and not laying D-egg in control and Ca-supplemented groups.

Table 6.2

	Two days	More than two days
Control	6	5
Ca supplemented	11	7

The females in control and Ca-supplemented groups were forced to lay an additional egg (D-egg). This table shows frequencies of two days and more than two days of laying interval between C- and D-eggs in control and Ca-supplemented groups.

Table 6.3

	ETI	Thickness at blunt	Thickness at equatorial	Thickness at point	Shell colour
First-laid eggs					
Laying date (control)	-0.099	-0.026	0.020	-0.005	-0.193
Supplemented period (exp.)	-0.089	-0.197	-0.167	-0.236	0.157
C-eggs					
Laying date (control)	-0.018	0.057	0.055	-0.096	0.123
Supplemented period (exp.)	-0.068	0.122	-0.061	0.096	0.416
D-eggs					
Laying date (control)	-0.082	-0.101	-0.289	-0.199	-0.700
Supplemented period (exp.)	-0.301	-0.359	-0.235	0.045	-0.026

The spearman correlation values between the laying date and five eggshell characteristics in the control group and between the supplemented period and five eggshell characteristics in the experiment group. By applying Bonferroni correction $P = 0.05 / 5$, hence only $P < 0.01$ was considered significant, but none was statistically significant.

Table 6.4

	Egg mass	Dry shell weight	ETI	Shell background colour
Egg order x Ca-supplement x clutch size	$F_{1,60} = 0.89$	$F_{1,60} = 1.82$	$F_{1,60} = 0.96$	$F_{1,59} = 0.14$
Egg order x Ca-supplement	$F_{1,60} = 0.002$	$F_{1,60} = 4.62^{*}$	$F_{1,60} = 11.42^{**}$	$F_{1,59} = 0.49$
Egg order x clutch size	$F_{1,60} = 0.24$	$F_{1,60} = 0.94$	$F_{1,60} = 0.97$	$F_{1,59} = 10.84^{*}$
Ca-supplement x clutch size	$F_{1,60} = 0.05$	$F_{1,60} = 0.69$	$F_{1,60} = 1.29$	$F_{1,59} = 0.12$
Egg order	$F_{1,60} = 129.18^{**}$	$F_{1,60} = 127.54^{**}$	$F_{1,60} = 22.90^{**}$	$F_{1,59} = 45.99^{**}$
Ca-supplement	$F_{1,60} = 0.02$	$F_{1,60} = 0.06$	$F_{1,60} = 0.02$	$F_{1,59} = 1.20$
Clutch size	$F_{1,60} = 0.09$	$F_{1,60} = 2.93$	$F_{1,60} = 4.46^{*}$	$F_{1,59} = 0.11$

The results of repeated measurement analysis of Ca-supplementation experiment. All results use Greenhouse-Geisser correction (* shows $P \leq 0.050$ and ** shows $P \leq 0.001$).

Table 6.5

	Shell thickness
Egg order x shell area x Ca-supplement x clutch size	$F_{2,59} = 0.33, P = 0.719$
Egg order x Ca-supplement x clutch size	$F_{1,60} = 1.47, P = 0.230^{G}$
Egg order x shell area x Ca-supplement	$F_{2,59} = 0.76, P = 0.472$
Egg order x shell area x clutch size	$F_{2,59} = 6.31, P = 0.003$
Shell area x Ca-supplement x clutch size	$F_{2,59} = 0.28, P = 0.754$
Egg order x Ca-supplement	$F_{1,60} = 6.51, P = 0.013^{G}$
Egg order x clutch size	$F_{1,60} = 0.81, P = 0.372^{G}$
Egg order x shell area	$F_{2,59} = 2.03, P = 0.141$
Shell area x Ca-supplement	$F_{2,59} = 0.04, P = 0.958$
Shell area x clutch size	$F_{2,59} = 0.63, P = 0.539$
Ca-supplement x clutch size	$F_{1,60} = 0.94, P = 0.337$
Egg order	$F_{1,60} = 22.57, P < 0.001^{G}$
Shell area	$F_{2,59} = 516.14, P < 0.001$
Ca-supplement	$F_{1,60} < 0.001, P = 0.992$
Clutch size	$F_{1,60} = 3.67, P = 0.060$

The results of repeated measurement analysis of Ca-supplementation on shell thickness (^{G} shows Greenhouse-Geisser correction).

Table 6.6

	Blunt end	Equatorial zone	Pointed end
Egg order x Ca-supplement x clutch size	$F_{1,60} = 2.24$	$F_{1,60} = 0.87$	$F_{1,60} = 0.52$
Egg order x Ca-supplement	$F_{1,60} = 2.43$	$F_{1,60} = 5.72^{*}$	$F_{1,60} = 6.96^{*}$
Egg order x clutch size	$F_{1,60} = 0.19$	$F_{1,60} = 0.01$	$F_{1,60} = 6.96^{*}$
Ca-supplement x clutch size	$F_{1,60} = 0.61$	$F_{1,60} = 1.75$	$F_{1,60} = 0.41$
Egg order	$F_{1,60} = 16.88^{**}$	$F_{1,60} = 24.63^{**}$	$F_{1,60} = 10.51^{*}$
Ca-supplement	$F_{1,60} = 0.01$	$F_{1,60} < 0.001$	$F_{1,60} = 0.01$
Clutch size	$F_{1,60} = 1.92$	$F_{1,60} = 4.28^{*}$	$F_{1,60} = 3.42$

The results of repeated measurement analysis of Ca-supplementation experiment on shell thickness at three different shell areas. All results use Greenhouse-Geisser correction (* shows $P \leq 0.050$ and ** shows $P \leq 0.001$).

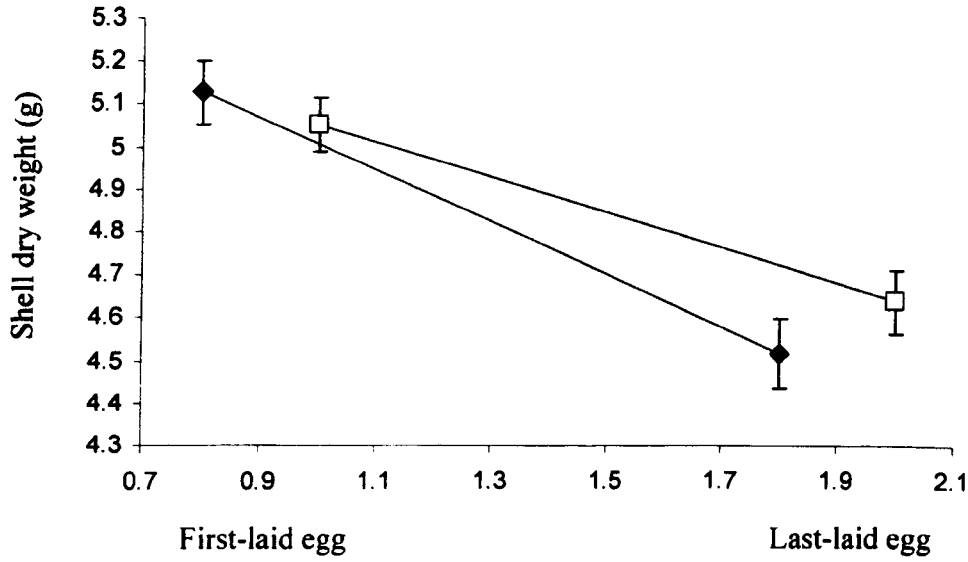

Figure 6,1. Shell dry weight (g) of first- and last-laid eggs in control (♦) and Ca supplemented groups (□).

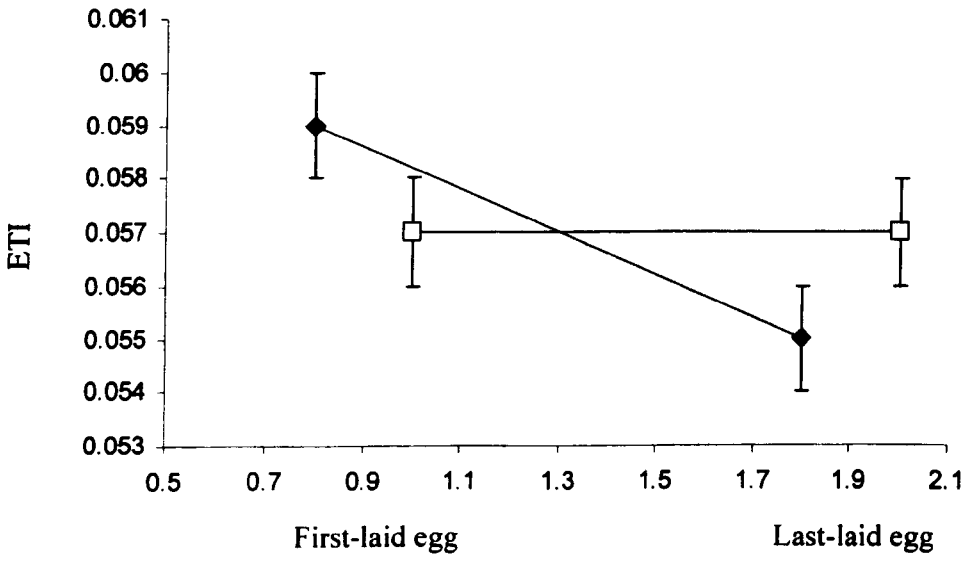

Figure 6.2. ETI of first- and last-laid eggs in control (♦) and Ca-supplemented groups (□).

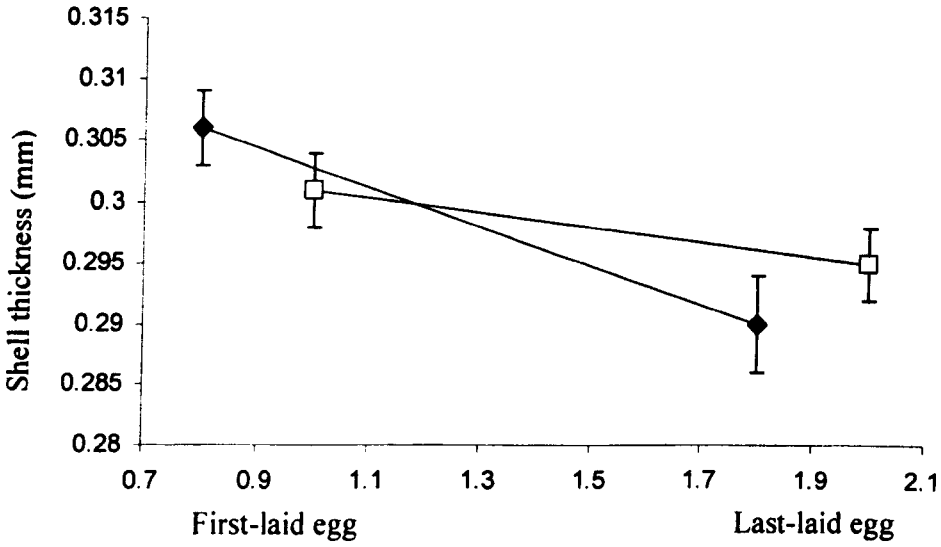

Figure 6.3. Shell thickness at the equatorial zone of first- and last-laid eggs in control (♦) and Ca-supplemented groups (□).

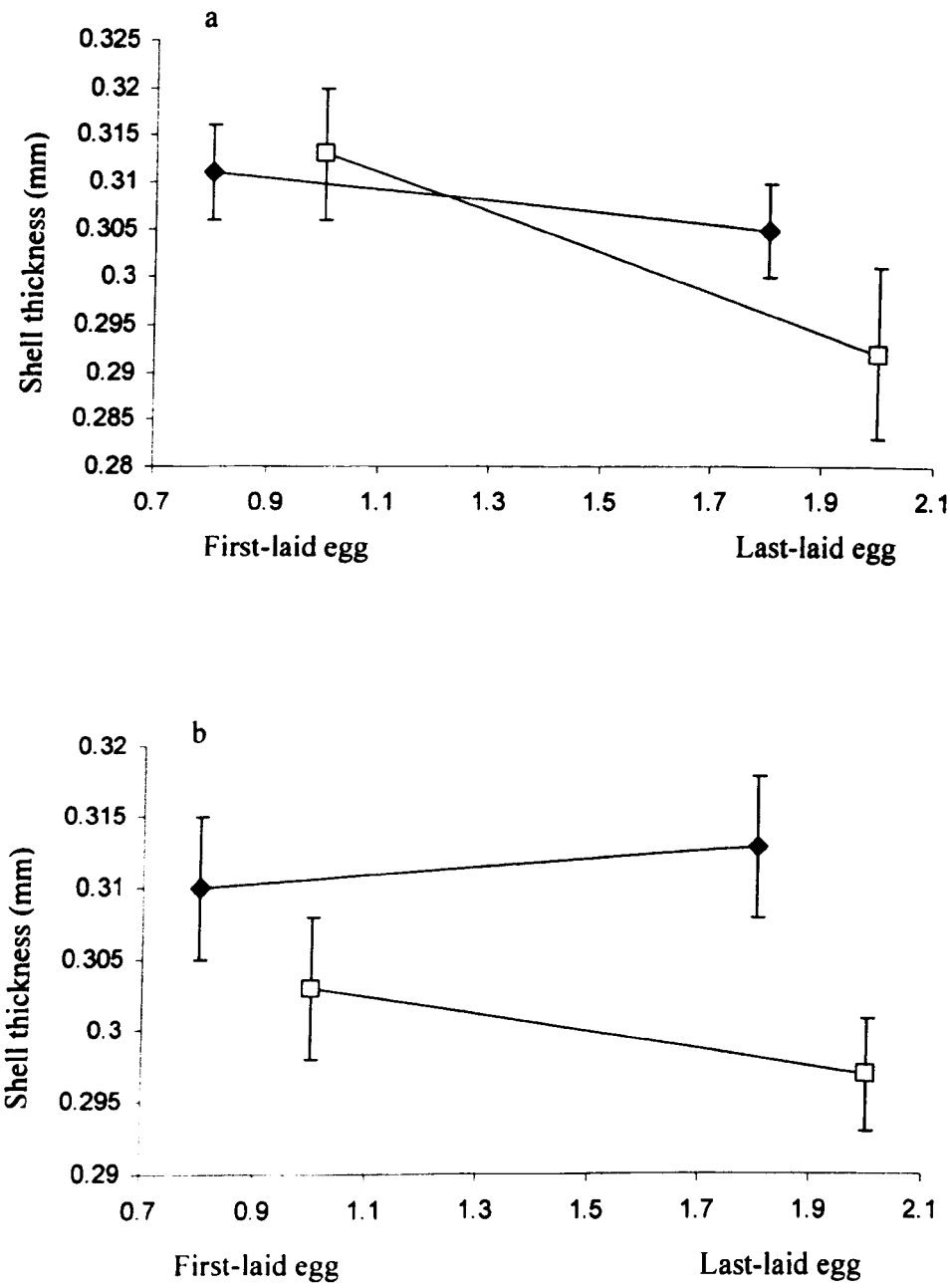

Figure 6.4. Shell thickness at the pointed end of first- and last-laid eggs in 3-egg clutch (♦) and 4-egg clutch (□) of control group (a) and Ca-supplemented group (b).

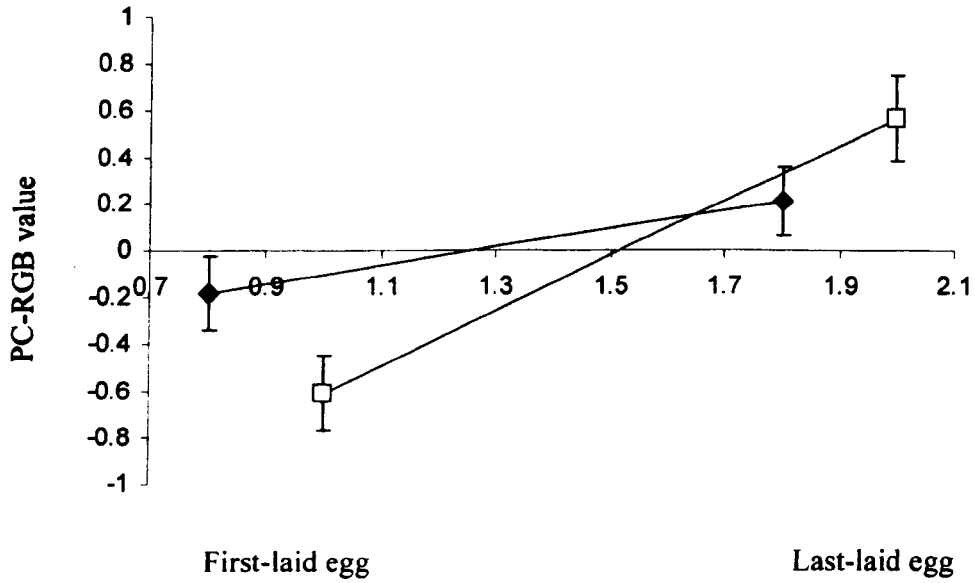

Figure 6.5. PC-RGB value of shell background colour of first- and last-laid eggs in 3-egg clutch (♦) and 4-egg clutch (□).

Discussion

Overall, the Ca-supplements had effects on ETI and shell thickness, but no effect on laying date, egg mass, likelihood of laying an additional egg and shell coloration.

Interestingly, even having a Ca-supplement, the females did not advance the laying date. It seemed that the female just used Ca-supplement to improve eggshell quality. Both control and Ca-supplementation groups showed similar likelihood of laying an additional egg, this indicated that Ca-supplementation did not influence the female to lay an additional egg.

By comparison of eggshell characteristics between the first-and last-laid eggs in the same clutch, the Ca-supplementation affected the shell formation in lesser black-backed gulls when females were forced to produce an additional egg, females that received Ca-supplementation produced shells of similar thickness for first- and last-laid eggs of their clutch at the equatorial zone and pointed end. However, the calcium supplementation did not affect the shell thickness at the blunt end. The last-laid eggs still had thinner shells than the first-laid eggs at the blunt end. Even with additional calcium, the female still produced thinner shell at the blunt end. This specific character may still be useful for the species. In the case of last-laid eggs, having a thinner shell at the blunt end than first-laid eggs, this property may help the embryo to increase its gas exchange when close to hatching and therefore may have a higher metabolism and then can hatch out faster than the first two eggs of the clutch (Muck and Nager, 2006). Chicks from last-laid eggs may expend less effort in hatching than their siblings, because of the thinner shell at the blunt end close to where pipping occurs. In common tern, Heaney *et al.* (1998) found the effect of increasing effort on

egg production, the additional egg had thinner shell at the equatorial zone. This study confirmed Heaney *et al.* (1998) and Ca-supplementation experiment suggested that the shell thickness at the equatorial zone was limited by calcium availability.

Ca-supplementation did not have any effect on shell background colour and spot characteristics. So, this study did not confirm the structural-function hypothesis of Gosler *et al.* (2005). But, the result from this study showed D-eggs had low frequency of appearance of streaks in Ca-supplemented group. It seemed like Ca-supplement had an effect on the appearance of streak on the additional eggs. So far, it is still not clear how the females deposit pigment in the shell.

Even having an increasing effort on egg production, the control bird did not prolong the laying interval between C-and D-egg, the Ca-availabilty may be limited by the attempt to control the laying interval. Possibly, if the females prolong the laying interval between C-and D-eggs and forage for additional calcium to produce a high quality shell for the D-egg, B-and C-eggs may be left in its nest too long. Without the proper incubation time, the embryos in B-and C-eggs may develop poorly. Being in the open nest longer, B-and C-eggs may have a better chance of being predated. So, the effect of Ca-limitation on reproduction might happen in the area without calcium limitation.

It was quite clear that Ca-supplementation in the period immediately before laying affected shell formation, but it does not tell us yet whether females store calcium long before egg laying or not or whether foraging for calcium just shortly before egg laying is the main source of calcium for egg formation in gulls. Probably,

comparisons of bone density of breeding females before and after egg laying may clarify the strategy of using calcium. From this study, the last-laid egg still had thinner shell than the first-laid egg at the blunt end in the Ca-supplemented group, this may suggested that both an adaptive strategies and a constraint may be the causes of this within-clutch variation in the shell thickness of the lesser black-backed gulls.

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Chapter 7: General Discussion

Techniques on measuring eggshell characteristics

In this thesis, I studied variation of eggshell characteristics; shell thickness, shell porosity, shell coloration and Ca-availability. In order to find proper methods, some shell characteristics were measured by using more than one technique. I used three different techniques to measure shell thickness. ETI was a simple technique (chapter 3), just divide dry shell weight by surface area, so it was very low cost. But, ETI can not give measurements of thickness at different shell areas and the thickness In order to measure shell strength, the effective shell thickness (not including shell membranes and mammillary layer) is the best indicator of eggshell strength (Bain, 1991). But in ETI, we can remove shell membranes but can not avoid measuring mammillary layer, so this technique may be not suitable to be an indicator of shell strength. However, SEMs show mammillary layer of lesser black-backed was generally confluent, so it may be possible to use ETI as an indicator of shell strength in lesser black-backed gulls or any species with confluent mammillary layer. A micrometer with rounded tips has been used widely for measuring shell thickness. Using a modified micrometer is more useful than ETI. The micrometer technique can measure shell thickness at different area of shell, but it may have a problem in measuring thickness at the curved part of the shell, because the rounded tips of the micrometer may not properly touch the surface of the curved shell. A micrometer with pin tips may manage the problem of the curved shell. SEM is a sophisticated technique for measuring shell thickness (chapter 3). We can select to measure any layer of the shell, so this technique is the most effective method to measure shell thickness. We can also use the scanning electron micrographs to observe the

character of shell layer. But the SEM technique is quite expensive and takes some time to prepare the specimens. The micrometer and SEM technique gave similar results in chapter 3, so in order to save money, the micrometer should be used for measuring shell thickness in future works.

I used two different techniques for counting pores in this thesis: directly counting pores and counting dyed pores (chapter 3). These two techniques were validated for the first time in this thesis (chapter 2). The directly counting pores technique is quite convenient and low cost. After removal of shell membranes, the pores were still not visible. To make pores visible, I had to enlarge pores by decalcifying the shell. During the decalcifying process, some non-functional pores (pores that end somewhere in the shell) may be revealed. If the shell is immersed in Decalcifier too long, the shell may be too thin to be handled and may reveal more non-functional pores. So, researchers should standardize the decalcifying process before using this technique. For the second technique, I dropped methylene blue on the outer shell after removal of shell membranes, but methylyne blue did not run through the pores. So, I had to use the shell with enlarged pore from the first technique. The dots of methylene blue appeared on the inner shell. But the dye from closely adjacent pores may merge together, causing a problem for shells that had high pore density or clumped distribution of pores. So far, direct counting of pores seems to be more effective than counting dyed pores.

Three different techniques were used to measure shell background colour in this thesis (chapter 4). Visual score was not used for the main analysis but used as a reference for interpretation of results from principal components analysis (chapter 4).

Digital image analysis is an effective and low cost technique (chapter 4), but does not give measurement of the colour spectrum and cannot measure colour in the UV range. Spectrophotometer technique is a very effective technique (chapter 4). It can give measurement of colour spectrum and can also measure colour in UV range. But the sensor is too big to measure colour in very small areas. This technique is very useful to study coloration at the level of pigments. So far, with ability of indicating the degree of biliverdin in eggshell and the spectrophotometer should be used for measuring shell colour in future works. For measuring amount of eggshell porphyrins in future work, measurement of density of area of spots on the shell may be used as an indicator for amount of porphyrins in the shell.

Normally, calcium content was used as an indicator for Ca-availability to the embryo. But, only mammillary layer which is in contact with the shell membrane functions as a provider of minerals (mainly calcium and magnesium) (Bond *et al.*, 1988; Blom and Lilja, 2004). So, in this thesis I chose to measure Ca-availability from the density of mammilary layer contact area (chapter 3).

For water loss measurement, during a preliminary work, I had a problem with using KOH as humidity absorber in a desiccator. KOH may have a reaction with the egg content of lesser black-backed gull and turned the egg content to be green jelly-liked substance with a bad smell. But I had no problem with using silica gel in a desiccator.

Variations of eggshell characteristics

This study found an effect of laying date on shell thickness, shell porosity, density of mammillary layer contact area, and shell colour (chapter 3 & 4). Laying order did not

have an effect on the density of mammillary layer contact area, and shell colour in late laying birds. In early laying birds, the C-egg had the highest density of mammillary layer contact area, lowest brown pigment and palest shell colour within clutch. The study on relationship between laying bird condition and laying date may give some explanation of the effect of laying date on variation of eggshell quality. At a larger scale, this study found a difference in variation of shell thickness between years. The laying date did not affect the shell thickness on the laying order in egg collection year 2002, but had an effect in egg collection year 2003. So, it would be interesting to investigate variation of shell quality in long-term study. For variation within clutch, C-egg mostly had different shell characteristics (chapter 3, 4 & 6). Ca-supplementation was used to study seabirds for the first time in this thesis (chapter 6). The results of Ca-supplementation suggested that Ca-limitation may influence shell formation within clutch of the lesser black-backed gulls. However, this study did not investigate the effect of Ca-limitation on Ca-availability in shell (density of mammillary layer contact area). For variation within egg, the blunt end had thinnest shell, interestingly even with Ca-supplement, the females seemed to keep producing thinner shell at the blunt end. So, this character may have an advantage for this species. It would be interesting to study the importance of thin shell at the blunt end in future works. There was no variation in shell porosity within the egg. The pointed end had lowest density of mammillary layer contact area. It is still not clear why the lowest Ca-availability would not take place at the blunt end where an air cell blocks Ca-availability.

The third or the last-laid eggs of the lesser black-backed gull hatch later, and these chicks have a higher rate of mortality, than the chick hatching from the first two eggs

in the clutch (Royle and Hamer, 1998). The third-chick disadvantage may be a consequence of being a low quality egg. Earlier studies found that the third egg had the lowest amount of nutrients in the egg contents (Bolton *et al.* 1992; Nager *et al.* 2000; Blount *et al.*, 2002; Verboven *et al.* 2005). The patterns of variation reported in this study on the shell pore density and the density of mammillary layer contact area in relation to laying order does not provide clear supportive evidence of a poorer eggshell quality of the third chick. But birds laying early in the season laid third eggs with greater density of mammillary layer contact area, and hence perhaps with more calcium-availability, than the first two eggs. This may help to increase the rate of the skeletal development of the embryo in the third eggs and then may reduce the effect of hatching asynchrony and sibling size hierarchies in the early laying birds. In Snares penguins, there was a variation of the shell pore density within the clutch, the last-laid eggs having more pore density and hatching before the first-laid eggs (Massaro and Davis, 2005). These are within-clutch differences in eggshell characteristics associated with variation in embryo development time, but it is at present not clear whether these relationships are causal.

This thesis also found variation in the within-clutch pattern of shell thickness between years. Shell thickness did not differ between laying orders in 2002, but shells of last-laid eggs were thinner than first-laid eggs in 2003. Variation of food abundance may also affect the within-clutch pattern of shell characteristics between breeding seasons. It is well known that food abundance can affect the within-clutch variation in egg size (e.g. Horsfall, 1984; Pierotti and Bellrose, 1986; Nilsson and Svensson, 1993; Ramsay and Houston, 1997). Interestingly, in this thesis I found an effect of calcium-supplementation on the within-clutch pattern of shell thickness,

there was no significant difference in shell thickness between the first- and last-laid eggs in calcium-supplementation group. Hence, this suggests that shell thickness of lesser black-backed gull eggs may be constrained by calcium availability.

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