Eggs, incubation and hatching asynchrony in gulls

Miran Kim
(miran318@hotmail.com)

Division of Ecology and Evolutionary Biology
Faculty of Biomedical and Life Sciences
University of Glasgow

Thesis submitted for the degree of Doctor of Philosophy
to the University of Glasgow, December, 2008

DECLARATION

I declare that this thesis is my own work and no part of the work has been submitted as a part of any other degree. Supervision by Professor R.W. Furness and Dr. R. G. Nager has helped develop ideas in the thesis.

Miran Kim
December, 2008

To my parents who showed their young daughter birds and nature

SUMMARY

Birds can influence the embryonic development through incubation behaviour because avian embryos develop using parent's body heat. Although previous studies assumed that incubation behaviour influences hatching patterns, few studies have studied the effect of incubation behaviour during egg-laying and early incubation on hatching patterns due to difficulties to determine onset of incubation during egg-laying. I investigated whether incubation behaviour during egg-laying and early incubation affects hatching patterns in gulls using measurements of mean nest attendance and daily change of nest attendance. Hatching patterns were influenced by incubation behaviour during egg-laying and early incubation behaviour. As parents spent more time in their nests, a brood hatched more synchronously and hatching success of the first-laid eggs increased when gulls laid relatively smaller first-laid eggs than other pairs. Within-clutch variation in eggshell colour related to daily change of incubation behaviour. This might relate to hormonal change during egg-laying. Increase of prolactin initiates incubation and accompanies decline of steroid hormones which relate to accumulation of eggshell pigments. Hatching patterns may also be influenced by accelerated development of last-laid eggs. When eggs were swapped to increase interval between eggs, last-laid eggs of herring gulls accelerated their development to catch up. Accelerated development may increase the survival of chicks from last-laid eggs by reducing the disadvantage of small size within a clutch. However, the costs of accelerated development seem to appear during the embryonic period. Hatching success was low in eggs with accelerated development, although there were no differences in growth rate and early nestling survival between accelerated and control last-laid eggs. Eggshell characteristics might be a factor affecting hatching patterns because they are related to embryonic metabolism. Hatching duration was not related to eggshell thickness and total functional area, but chicks which hatched from eggs with higher proportion of mammilllary cone contact area took longer to hatch. Chicks hatched from thicker eggshells showed longer "head plus bill" at hatching and grew faster in skeleton size after hatching. Diet during egg-laying and early incubation affected nest attendance. Females which consumed more marine food during egg formation had lower nest attendance during egg-laying and early incubation. This may relate to longer foraging time required to obtain marine food. In conclusion, this thesis suggests that parents can influence hatching patterns by altering incubation behaviour during egg-laying and early incubation and hatching patterns also may be affected by accelerated development of last-laid eggs, diet during egg-laying and early incubation and eggshell characteristics (proportion of mammillary cone contact area).

ACKNOWLEDGEMENTS

First of all, I would like to thank my supervisors Professor Robert W. Furness and Dr. Ruedi G. Nager for their supervision during my PhD. Bob and Ruedi gave me good ideas and comments. Their criticism and continuous comments improved my scientific writing and knowledge as a researcher.
I am grateful to my family who supported me during my PhD. My parents and brother always send me their love and patience. Without their support, I could not complete my PhD.

During my PhD, I realized that PhD work cannot be done without peoples helps. Natural environmental research council allowed me do stable isotope analysis at the Scottish Universities Research and Reactor Centre (SUERC), East Kilbride. Dr. Rona McGill in the SUERC helped me during laboratory work and gave me comments for Chapter 2. Dr. Stuart Bearhop and Leandro Bugoni gave me brilliant ideas and comments for analyzing diet using stable isotopes. Whoseung Lee introduced me a self-organizing map for analyzing diet (Chapter 2). Dr. Bernie Zonfrillo gave me useful information of diet near the Sanda Island. Kong Tharapoom kindly allowed me to use his unused data and collected eggshells for Chapter 6. For the analysis of eggshell characteristics, I could work independently for preparing eggshell samples and using the scanning electron microscope because Professor Maureen Bain and Nick Cormack in Veterinary school, University of Glasgow, kindly taught me laboratory skills. For the fieldwork In Iceland, Dr. Sveinn Valdimarsson allowed me to use facilities of the Nature Centre and Gunnar Hallgrímsson helped me how to say " Please, do not take eggs this area" in Icelandic - "EKKI TAKA EGG, TAKK" when I had a problem with local people who were collecting eggs in the study site. Anaid Diaz helped me to measure hatching process under the windy weather condition and scary attacks by gulls. I hope that she does not hate gull too much after working in Iceland. Chris Foote, Irene and Liliana D'Alba were good company. They gave me good friendship and advice during my first fieldwork in Iceland. I remember a parcel from Kate Griffith and Irene Leighton when I was doing my second fieldwork in Sanda Island. Their kind present (a bottle of chocolates) made me energetic and happy!

In the department, I also had good advisors. Whenever I needed help, Dr. Jan Lindström, Professor Pat Monaghan and Professor Malcolm Kennedy gave me good advice. I thank them for their help. I am also grateful to Professor Dan Haydon for his help and useful statistics lectures which made me confident using GLM.

I was really lucky to have good friends in Glasgow. My big sister, Adriana Vallarino gave me wise advice and made me strong in a real life. My first flat mates, Adriana and Cesar Gonzalez and I discussed our work, future and life at the dinner table. It helped me realize thousands of interesting things in the world. Douglas Kerlin and Anaid Diaz were good teachers whenever I had a question in statistics. Veronica Neves who has a special view of life and world helped me find delights in Glasgow apart from PhD work. My recent flatmate, Lulu Ruiz fed me on nice Mexican food which always made me happy and forget stress of thesis at the end of my PhD. I also thank my short-term flatmates, Vivi, Valerie and Annabelle. Vivi Carniel introduced me nice Brazilian music and fatty but delicious cream pastas. Valerie and Annabelle gave me some idea of post-doctor and nice cocktail recipes. Maria Bogdanova, David Alvarez, Pierre Bize and John Crane gave me wise advice for my PhD and thesis, I thank them for their kind friendship. My office mates, Davina Hill, Ross McLeod and Juan Hernando were good companies at the hottest office in the department. When I arrived at Glasgow, Yuri Alvores, Sin-Yeon Kim, Jae-bum and In-kyoung Lee helped me settle down in Glasgow and forget homesick. I really thank for their warm friendship and help. Finally, I also thank my examiners Professor David Houston and Jeff Graves. During a viva, they gave me great ideas and comments for the thesis, publication and future work. My viva was the happiest moment during my PhD to me.

I have leant many things from people in the DEEB, not only academic knowledge but also various culture and life. It was a really great time to share experiences with them. Thank you!!

I am grateful to my Korean friends. Yun-kyoung Park, like another big sister has given me a peace and a deep breath whenever I felt depressed under the Scottish winter (dark and wet!) and with stressful PhD work. My friends who are studying in UK often made me feel home. I thank Jinwon Lee, Kiback Nam, Jiyeon Lee and Kanguk Kim and his family for their hospitality and help. Yun-kyoung Lee in Canada also encouraged me to keep a joy of studying nature. Yeona Ko, Inhong Kim, Minkyung Seo, Mina Kim and Jiyoung Ahn always made me laugh and feel happy when I visited Korea. Oak-sik Jeong was taking care of me like a godfather from a long distance. My friend from high school, Kayng-hyeon Lee showed me good friendship. My best friend, Eun-jae Lee often made me really happy through a long international phone call from South Korea to my office. I would like to thank all of them for their friendship.

I am grateful to Professor Suk-pyo Hong in Kyunghee University. He has been my mentor since I started studying biology in undergraduate. Whenever I lost my direction and confidence, he helped me find them by myself. I also thank for my previous supervisor, Professor Jeong-chill Yoo who encouraged me to study abroad.

I should say to thank gulls in the breeding colony of Iceland and Sanda Island (particularly, nest No. 58) for showing (?) me their private life to understand nature. Finally, I am very grateful to Young-soo Kwon for his endless love and support. He believed my passion more than I did and was always with me.

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Chapter I.

General introduction

Animals develop from a single fertilized cell to an organism. Embryos require nutrients, minerals and suitable temperatures during incubation. In viviparous species, females provide nutrients, minerals and heat to embryos within their body during embryonic development. The embryo in oviparous species develops outside of the mother's body and obtains nutrients and minerals from egg contents and eggshell during development. An egg consists of water, organic matter (e.g. proteins, lipids, carbohydrates and pigments) and inorganic matter (e.g. calcium, chlorine, iron, magnesium, sulphur, phosphorus and sodium) (Romanoff & Romanoff, 1949). Yolk and albumen provide the embryo with lipids and proteins as energy resources and the eggshell provides the embryo with minerals which are essential for embryonic development. Embryos obtain calcium for their developing skeleton mainly from eggshell(Romanoff & Romanoff, 1949). Embryos require heat from outside the eggs. In reptiles, embryonic development is usually determined by ambient environmental temperature (Glen *et al.*, 2006; Du & Feng, 2008). Even though reptiles often sit on their eggs, most of them do not exchange body heat with their eggs. Unlike avian embryos obtain heat directly from parents except in the case of megapode species which use external heat sources (such as decaying vegetation) for incubation. In birds, the transfer of heat to eggs is regulated by parents through temperature sensors in the skin of the brood patch which develops before or during incubation to transfer heat to the eggs (Deeming, 2002b). Hence, avian embryos can develop under a stable temperature until they hatch. During incubation, parents regularly turn eggs as well, to exchange respiratory gases through pores on the surface of the eggshell. Hence, parental behaviour plays an important role in controlling embryonic development.

Fully developed embryos are born from the mother's body or hatch from eggs. In viviparous species, embryos in the uterus are born together if more than one embryo is developed together. In many taxa of oviparous species (e.g. insects, reptiles and birds), siblings in a clutch hatch either synchronously or asynchronously. For example, a clutch of burying beetles (*Nicrophorus vespilloides*) hatches within about 30 hours (Smiseth et al., 2006) and White's skinks (*Egernia whitii*) hatched over 2 days (While *et al.*, 2007). Avian species also hatch synchronously (less than 24 hours) (Johnson, 1974, Munro & Bedard, 1977) or asynchronously (a few days to a couple of weeks)(Drent,

1970; Beissinger & Waltman, 1991). For example, Green-rumped parrotlets (*Forpus passerinus*) hatch over 2 ~ 14 days (Beissinger & Waltman, 1991). As avian embryos can develop when parents start incubation, their hatching patterns can be influenced by incubation behaviours such as onset of incubation, nest attendance and incubation temperature. When parents start incubation before completing their clutch, eggs in the clutch hatch asynchronously due to advanced development of earlier laid eggs, while synchronous hatching may occur when incubation starts after completing the clutch. Incubation patterns may change the hatching interval within a clutch due to advanced development of eggs which are laid before starting incubation. Asynchronous hatching results in a size hierarchy within a brood and often induces disadvantage to last-hatched chicks (O'Connor, 1978). In species with hatching asynchrony, more synchronous hatching often results in lower fledging mass or higher mortality of the last-hatched chick in a brood (Sydeman & Emslie, 1992; Mock & Parker, 1997).

Previous studies have made an effort to explain why some birds hatch asynchronously although it induces the waste of parental effort through higher competition, and increased mortality of last-laid eggs. 21 hypotheses have been reviewed by Magrath (1990). Here, I would address 8 hypotheses which focus on nestling stage and incubation period: 1) brood reduction, 2) peak load reduction, 3) sibling rivalry, 4) hurry-up, 5) energy constraints, 6) hormone constraints, 7) egg viability and 8) egg production. As an adaptive behaviour during the nestling period, Lack (1954) proposed that parents make their brood hatch asynchronously to reduce brood size to fit available food resources if food is scarce (The "brood reduction hypothesis"). This hypothesis has been tested in many cases (Hahn, 1981; Fujioka, 1985a; Hebert & Barclay, 1986). However, some studies failed to find the benefits of asynchronous hatching compared with synchronous hatching in terms of breeding success (i.g. Hahn, 1981; Amundsen & Stokland, 1988). The "peak load reduction hypothesis" suggests energetic advantage through asynchronously hatching, even when food is abundant (Hussell, 1972). In avian growth, there is a critical period when chicks grow fast and demand maximum amount of food. When chicks hatch at the same time, parents have to bring food to match peak demands of broods. Hussell (1972) suggested that if food demands of individual chicks have a sharp peak at a particular age, parents may be able to feed broods efficiently by spreading maximum energy demand of chicks through asynchronous hatching. When all chicks demand maximum amount of food at the same time, parents have to work to feed their chicks more than parents with asynchronous broods. Whilst the "sibling rivalry hypothesis" suggests that parents avoid

sibling competition through asynchrony in hatching (Hahn, 1981). Because sibling competition is often high in case of no dominance in a brood, size hierarchy may avoid fights between chicks. In relation to seasonal change of resources during the breeding season, the "hurry-up hypothesis" suggests that when resources decline according to date in the breeding season, earlier hatching may increase reproductive success. Although later-hatched chicks still need food to grow in late breeding season, earlier-hatched chicks in a brood have already grown up enough to fledge. Hence, parent birds may have at least some fledged chicks in late breeding season even though food is scare in late breeding season (Hussell, 1972; Slagsvold, 1986).

Secondly, some studies looked at the reason for hatching asynchrony during incubation period in terms of a non-adaptive and an adaptive view. As a non-adaptive view, hatching asynchrony might occur due to environmental constraints (The "energy constraints hypothesis", Slagsvold, 1986). Females need additional food to produce eggs. But, their foraging time is often limited during egg production and/or incubation. If females have to spend more time to forage, it may reduce the amount of time to laying and incubate eggs and may induce delayed onset of incubation (Slagsvold & Lifjeld, 1989; Eikenaar *et al.*, 2003) and result in a synchronous brood. In species which use their body reserves during laying and incubating eggs, poor body condition constrains the time of nest attendance (Persson & Goransson, 1999). Alternatively, hatching asynchrony might be an epiphenomenon of hormonal change during laying and incubation. Incubation behaviour is controlled by hormones such as prolactin or oestrogen (Mead & Morton, 1985). As prolactin is responsible for both the termination of egg laying and initiation of incubation, females will start incubation with the laying of the penultimate egg. Hence, onset of incubation may be independent of the clutch size. However, it is not clear that prolactin terminates egg-laying. Clack and Willson (1981) showed only 35% of species started incubation on the penultimate eggs. On the other hand, hatching asynchrony has been explained as an adaptive behaviour during egg-laying. In species with a large clutch size, viability of earlier-laid eggs might be reduced because they are exposed for a long time without incubation. Hence, parents might have to start incubation before completing their clutch to avoid hatching failure of earlier-laid eggs (The "egg viability hypothesis", Arnold *et al.*, 1987). Predation is one of the main factors constraining breeding success (Ricklefs, 1969). If predation mainly occurs during the egg-laying and incubation period rather than the nestling or fledging period, parent birds may start incubation earlier to prevent predation and this results in asynchronous brood (The "egg protection hypothesis", Dunlop, 1910).

1.1.1 Aims of the thesis

The aims of the thesis are to investigate factors affecting hatching patterns, mainly incubation behaviour during egg-laying and early incubation. First, I investigate whether incubation behaviour during egg-laying and early incubation affects hatching patterns. Most hypotheses to explain hatching asynchrony assumed that hatching patterns are controlled by parental incubation behaviour (except constraint hypothesis). Because avian embryos can start developing when parents start incubation, onset of incubation or incubation pattern during egg-laying and early incubation period may relate to hatching patterns. Most previous studies considered mean nest attendance as a factor affecting hatching patterns or hatching span. However, incubation patterns might influence hatching patterns. For instance, in Eurasian kestrels (*Falco tinnunculus*), females incubated their eggs with rising, steady and pulsed patterns and these incubation patterns affected hatching patterns (Wiebe *et al.*, 1998). Also, few studies precisely looked at the relationship between hatching patterns and incubation behaviour during laying and early incubation period although it might be a crucial time to determine hatching patterns. There may be few studies during egg-laying and early incubation because onset of incubation is difficult to identify because it is gradually developed rather than turned on at a specific time. Here, I looked at how incubation behaviour during egg-laying and incubation related to hatching patterns.

Second, I investigate the effect of diet on incubation behaviour during egg-laying. Diet not only affects breeding success but also parental behaviour such as nest or territory attendance during the breeding season (Caldow & Furness, 2000; Bearhop *et al.*, 2001; Ojowski *et al.*, 2001; Votier *et al.*, 2004). In Australian reed warblers (*Acrocephalus australis*), higher food availability increases nest attendance during egg-laying and results in asynchronous broods (Eikenaar *et al.*, 2003). In addition to amount of food, prey preferences may affect parental behaviour. For example, in great skuas (*Stercorarius skua*), specialist bird predators spent more time on their territory and less time foraging than specialist fish predators because they may forage near their territories rather than in the sea (Votier et al., 2004).

Third, embryonic developmental rate might affect hatching patterns because embryos can accelerate or delay their hatching to hatch synchronously or to reduce hatching interval with other eggs in a clutch. One mechanism to control development has been shown. At the end of the incubation period, embryos produce clicking sounds and

vibrations. These allow embryos to communicate with other eggs or siblings in a clutch (Vince, 1969; Woolf et al., 1976). Hatching time may be more important for last-laid eggs to survive because of disadvantages from size hierarchy in a brood. It has been well documented that last-laid eggs commonly accelerate their development and have shorter incubation period. Interestingly, last-laid eggs adjust their development when catching-up the development of earlier-laid eggs is necessary (Muck & Nager, 2006) although earlier hatching allows last-laid eggs more benefits. Hence, there may be costs of accelerated development to limit embryos to accelerate. In some species, poorer body condition has been found after embryos hatch earlier than expected. In this case, last-laid eggs can reduce hatching interval with other eggs by hatching before they fully develop (Nilsson & Persson, 2004) or by accelerating developmental rate with fully developed muscular and organs (reviewed in Metcalfe & Monaghan, 2001). Even though they are fully developed at hatching, the cost of accelerated development may be found shortly after hatching or much later in lifespan. For example, wood butterflies which accelerated their development at the larvae stage showed higher mortality as adults under poor food condition than ones which had normal development (Gotthard *et al*. 1994). In the thesis, I investigated whether there are costs of accelerated development and when costs of accelerated development appeared in the embryonic and/or nestling period.

Fourth, I looked at the effect of eggshell characteristics on embryonic development which may affect hatching patterns. Embryos exchange gases, including water vapour, across the shell through diffusion (Paganelli, 1980). Eggs with thinner shell and larger or higher density of functional pores develop faster than eggs with thicker and lower functional pores on the eggshell. Recently, it has been shown that there is within-clutch variation in eggshell characteristics and this affects hatching asynchrony in Snares penguins (*Eudyptes robustus*) (Massaro & Davis, 2005). However, the relationship between characteristics of fresh eggshell and embryonic development is hardly documented. It is very difficult to have both fresh eggshell and hatchlings from the same eggs because eggshell characteristics are dramatically changed during the incubation period. In this thesis, I investigated the effect of eggshell characteristics on hatching duration using two sibling eggs. One of siblings incubated and the other one was collected for investigating eggshell characteristics. I also looked at eggshell characteristics affect prenatal condition and postnatal growth rate of chicks because eggshell characteristics often related to growth rate of offspring (Blom & Lilja, 2004).

Lastly, I investigated within-clutch variation in eggshell colour as a consequence of incubation behaviour during egg-laying and early incubation. Deposition of eggshell pigments is influenced by hormonal change during egg-laying. The eggshell pigments is accumulated in the shell gland after ovulation and deposited on the surface of eggshell. In Japanese quails (*Coturnix japonica*), steroid hormones (progesterone) are involved in the accumulation of eggshell pigments (Soh & Koga, 1994). Initiation of incubation is controlled by hormone. Incubation starts with increase of prolactin in female. I expected there is a relationship between eggshell colour variation and incubation behaviour because there may be the hormonal changes although I have not directly looked at the hormonal changes during egg-laying and early incubation period.

1.1.2 Outline of the thesis

The aim of this thesis is to investigate whether incubation behaviour during egg-laying and early incubation related to hatching patterns. I also looked at the effect of diet, embryonic developmental rate, eggshell characteristics on embryonic development. Within-clutch variation in eggshell colour has been investigated as a consequence of incubation behaviour.

In chapter 2, I investigate diet changes between egg formation and chick rearing period and seasonal changes of diet using stable isotope analysis of chick feathers, and regurgitated adult pellets. Stable isotopes have been recently used for tracing food webs, ecosystem nutrient cycling, habitat use and migration of animals (Alexander *et al.*, 1996; Bearhop & Klaaseen, 2003; Cherel & Hobson, 2005; Hobson & Wassenaar, 2008). Stable isotope values can reflect diet during a certain time period (reviewd in Michener & Lajtha, 2007). I used stable isotope value of hatchling down which may reflect female diet during egg formation. Regurgitated pellets represent diet in a short time scale. They can give detailed information most relevant for analysing diet of breeding birds (Votier *et al.*, 2003; Barrett *et al.* 2007). Diet change during the breeding season was considered as a factor likely to affect incubation behaviour, because higher food availability allows parents to spend more time in the nest and this higher nest attendance induces greater asynchronous hatching (Eikenaar *et al.* 2003). In Chapter 3, I investigated whether incubation behaviour during egg-laying and early incubation relate to hatching patterns. Mean nest attendance and daily change of nest attendance have been used as measures of incubation behaviour. I assumed that daily change of nest attendance might reflect how quickly birds change their incubation behaviour and mean nest attendance might reflect amount of time to spend in the

nests during egg-laying and early incubation. In chapter 4, I examined whether incubation behaviour influenced within-clutch variation in eggshell colour. Incubation behaviour is controlled by hormones such as prolactin (Buntin, 1996). Prolactin increases during egg-laying initiate incubation. Increased prolactin is often accompanied by steroid hormones which involve in breeding behaviour such as nest building. Steroid hormones also accumulate eggshell pigments. Hence, I expected a relationship between onset of incubation and within-clutch variation in eggshell colour. In chapter 5, I investigated whether there is a cost of accelerated development and if there is, when a cost appears either during the embryonic period or chick rearing period because costs can appear much later than hatching (Metcalfe & Monahan, 2001). I investigated this question through the manipulation of laying interval. In chapter 6, I looked at relationship between eggshell characteristics and hatching duration. Eggshells have several structures including mammillary layer, palisade layer, surface crystal layer, pores and shell accessory materials (Solomon & Bain, 1994) and consist mainly of calcium carbonate (Solomon & Bain, 1994; Solomon, 1999). Embryos exchange CO_2 and O_2 by diffusion between inside and outside of eggshell through eggshell pores, and pores also allow diffusion of water vapour so that water is progressively lost from eggs during incubation. I expected that higher porosity would reduce hatching duration due to greater metabolism. I looked at whether eggshell characteristics influence skeleton growth of embryo and chicks after hatching because calcium is one of the essential minerals related to skeleton growth and eggshell is used for main source of calcium.

1.1.3 Study areas in Iceland and UK

I studied lesser black-backed gulls (*Larus fuscus*) and herring gulls (*L. argentatus*) in Iceland and UK. During the first year of my PhD, I studied the cost of accelerated development in lesser black-backed gulls (Chapter 5) at Sandgerði, southwest Iceland (64º03´N, 22º40´W) (Figure 1.1, A), from May to August in 2005. There were approximately 1,000 nests of lesser black-backed gulls in the study area, which was a pasture near the sea coast. This breeding area holds the biggest population of lesser black-backed gulls in Iceland. Breeding of lesser black-backed gulls was recorded in 1920's for the first time in Iceland. After the 1950's, gulls started colonization in southwest Iceland. The breeding population has grown very rapidly (1,000 ~ 2,000 to 24,300 pairs between 1975 and 1992). In 2004 and 2005, the population was estimated at 36,600 pairs and 29,000 pairs, respectively (Hallgrimsson *et al.*, 2007). However, the number of breeding pairs collapsed with 7,395 pairs in 2006. That may due to poor

breeding success in 2005. Although the reason for reduced breeding population is unknown, it may very likely be related to food availability. Egg collecting by local people is very common in the study area and a culling programme has been carried out near Keflavik International Airport near the study area. The poor conditions and particularly the low breeding success and population decline led me to switch my fieldwork away from this study area in following year, to a colony in western Scotland.

In the second year of my PhD, I studied diet change (Chapter 2), incubation behaviour (Chapter 3) and within-clutch variation in eggshell colour (Chapter 3) of herring gulls on Sanda Island, Argyll, Scotland (55°16′N 5°35′W) (Figure 1.1, B) from April to July 2006. Sanda is a small island (82.6 ha) which is located in west Scotland, about 3 km off the south coast of Kintyre. The breeding population of herring gulls was spread over several sub-colonies in Sanda Island and two smaller islands, Sheep Island and Glunimore. The breeding population of herring gulls has dramatically increased in 1970's (though it is unclear whether that relates to the fact that since 1970s egg-collecting by local farmers had stopped). However, the population in Sanda Islands declined from 1,900 to 700 between 1998 and 2002 (- 7.4 % per year) (Mitchell *et al.*, 2004). As causes of decline in Sanda, avian botulism which is a paralytic disease of birds, and mink predation have been considered likely to have played some role (Mitchell et al., 2004; local warden, pers. commun.). In terms of food availability, a fishery for lobster and whitefish is active through the breeding season around Sanda Island, and is thought to provide some discards. There is agriculture land growing crops and stock grazing area in Kintyre where gulls can steal grain put out in spring for cattle. In Sanda, pellets of adult diet during incubation contain large portion of grass and grain and this indicates that herring gulls forage from agriculture land. Herring gulls also feed on crab and marine molluscs and fish but rarely used refuse in Sanda (Armstrong, 1992).

Eggshells of lesser black-backed gulls were used to study the effect of eggshell characteristics on embryonic development. Eggs of lesser black-backed gulls were collected under a license from English Nature during the breeding season of 2004 at the South Walney Nature Reserve, Walney Island, Cumbria, UK (54°08′N 3°16′W) (Figure 1, C). South Walney has one of the largest herring gull colonies in Britain and Ireland. Walney Island is about 130 ha and holds a mixed breeding colony of about 18,000 lesser black-backed gulls and 10,000 herring gulls between 1998 and 2002 (Mitchell *et al.*, 2004). The population of gulls on Walney Island has declined since about 43,852 gulls

were observed in 1978. However, it has been stable since the 1985 ~ 1988 period (Mitchell *et al.*, 2004).

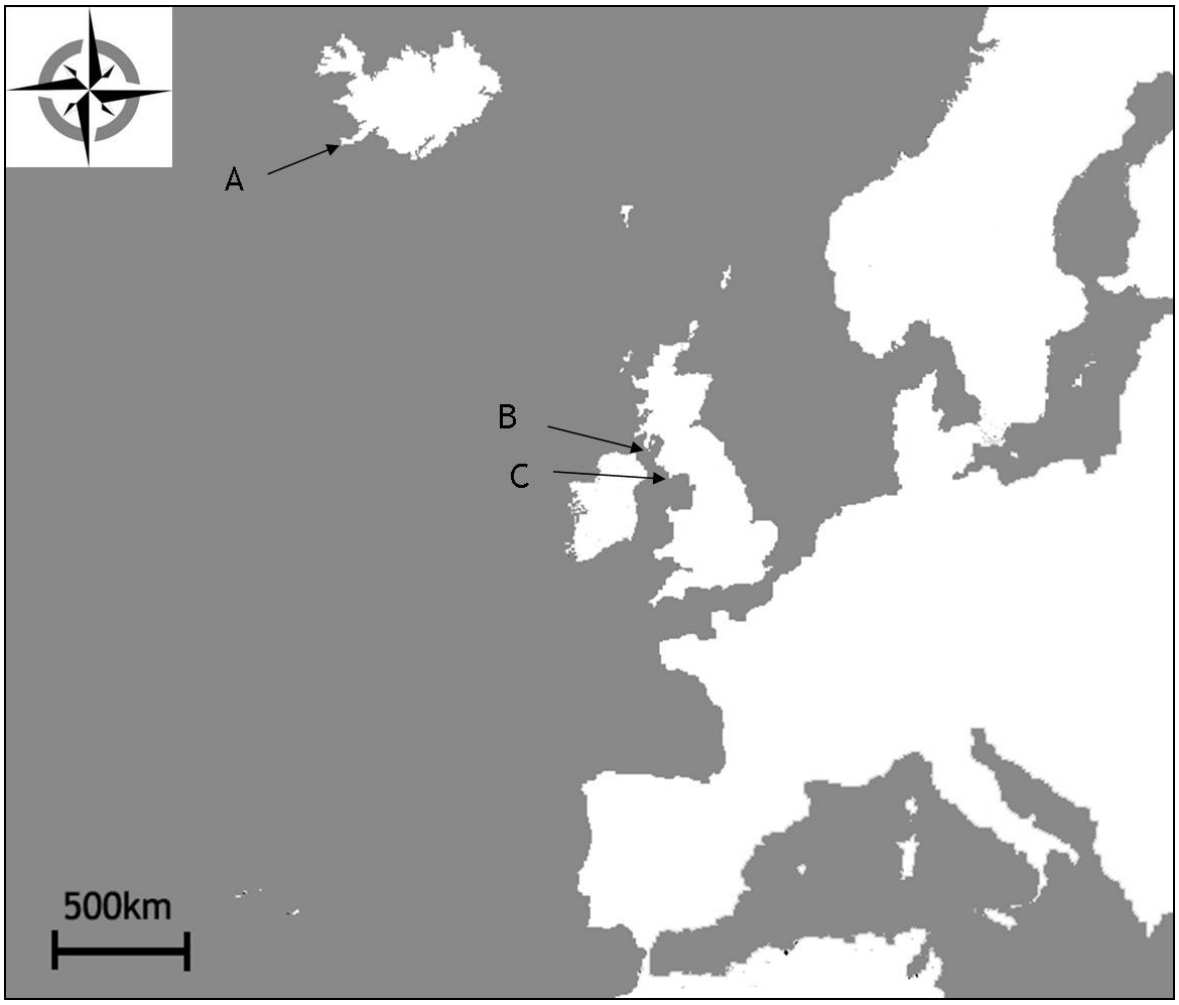

Figure 1.1. Study area in Iceland (A), Sanda Island (B) and Walney Island (C).

Chapter II.

The use of stable isotopes as indicator of diet change, chick performance, and parental behaviour in herring gulls *Larus argentatus*

2.1 INTRODUCTION

Diet during the breeding season not only affects seabird chick survival and growth rate (Pierotti & Annett, 1991; Golet *et al.*, 2000; Osterblom *et al.*, 2001; Takahashi *et al.*, 2001; Becker *et al.*, 2006; Osterblom *et al.*, 2006; Wanless *et al.*, 2007), but also relates to adult survival after breeding season (Massaro & Davis, 2005). Parent birds can feed chicks according to food availability during the breeding season or can actively choose suitable food items to meet the nutrient demand of chicks. In many gull species, parents switch diet after their chicks hatch. For example, western gulls (*Larus occidentalis*) took more fish when chicks hatched while they consumed mainly garbage in the pre-hatching period (Pierotti & Annett, 1991). This was also observed in herring gulls, which changed their diet from bivalves to marine fish after the first chick hatched (Noorduis & Spaans, 1992) and increased the amount of fish or meat as chicks grew (Nogales *et al.*, 1995). This diet change during the breeding season may occur to meet the energetic and nutrient demands of chicks. It is also important to feed chicks on good quality food to promote rapid chick growth. In 2004, common guillemots (*Uria aalge*) in the North Sea had unprecedented breeding failure although their food provisioning rate was the same as in the previous year (Wanless *et al.*, 2005) This has been explained as the "Junk Food Hypothesis". Guillemots fed chicks on more sprats (*Sprattus sprattus*) that contained fewer lipids instead of the sandeels (*Ammodytes marinus*) as in previous years, and the sandeels fed to chicks in 2004 were much lower in lipid than those fed to chicks in previous years. Although parents may feed chicks on a sufficient amount of food, low quality of food can induce higher mortality of chicks. In the Baltic Seas, common guillemots have also showed a reduced fledging mass of chicks in some years due to poor condition of sprats (Osterblom *et al.*, 2001; Osterblom *et al.*, 2006).

To identify the diet of seabirds during the breeding season, several methods such as regurgitated pellets, prey remains, spontaneous regurgitates, observed feeds and water off-loading have been used (Barrett *et al.*, 2007). Diet items identified from the observation of feeding can provide a biased picture depending on the correct identification of prey items. Water off-loading provides accurate diet items from the latest meal, but it indicates diet over for a short time and it may be stressful. Pellets are commonly used due to the easy access, but it can often overestimate indigestible items because soft prey is more quickly digested (Brown & Ewins, 1996; Votier *et al.*, 2003). It thus underestimates the importance of food items with soft tissue such as worms or fish offal which can be easily digested (Hobson *et al.*, 1994, Votier *et al.*, 2003).

Recently, stable isotope ratios, for example $^{13}C/^{12}C$, $^{18}O/^{16}O$ of deuterium have been used in ecological studies such as migration (Hobson, 1999; Rubenstein & Hobson, 2004; Hobson, 2005), identification of breeding and wintering grounds (Chambelain *et al.*, 1997), pollution (Bearhop *et al.*, 2000), habitat use (Bearhop *et al.*, 2003) and dietary differences between the sexes (Forero *et al.*, 2005) and different ages (Cherel *et al.*, 2002). Isotopes are atoms with the same number of protons and electron and different number of neutrons and various materials have isotopic differences (Michener & Lajtha, 2007). Environmental isotopes such as H, C, N and O are widely distributed in nature. Stable isotope ratios (e.g. $^{13}C/^{12}C$ or $^{15}N/^{14}N$) of diet are reflected in their consumer's tissue. The ratios of carbon isotopes ($^{13}C/^{12}C$: expressed as $\delta^{13}C$) and nitrogen isotopes ($^{15}N/^{14}N$ expressed as $\delta^{15}N$, *see* isotope analysis section in methods) have been used for interpreting the feeding ecology of marine predators (Peterson & Fry 1987). Isotope ratios of marine prey are $\delta^{13}C$ higher compared to values of freshwater or terrestrial prey (Fry *et al.*, 1983). Carbon stable isotope ratios between bird tissue and diet differed only within 1‰ (Peterson & Fry, 1987) and they represent diet sources such as marine and terrestrial diet. Nitrogen isotope ratios reflect trophic level (Hobson *et al.*, 1994; Hebert *et al.*, 1999). For example, nitrogen isotope ratios increased as much as 3-4‰ per trophic level. Diet-tissue differences of isotope-fraction for carbon and nitrogen is consistent among avian species (Mizutani *et al.*, 1992) while turn over rate of stable isotope values varies in different tissues (Tieszen *et al.*, 1983; Hobson & Clark, 1992; Phillips & Eldridge, 2006). For example, turnover rates of ^{13}C in tissues of growing Japanese quail (*Coturnix japonica*) examined by switching the diet from a wheat-based diet (C3 plant) to a corn-based diet (C4 plant) were ranked in order of increasing turn over time: liver > blood > muscle > bone collagen (Hosbson & Clark, 1992). Blood plasma can reflect very short-term dietary resolution while red

blood cells turnover similar to that of muscle tissue (Hobson & Clark, 1993). Feather samples have been used for long-term dietary studies by analysing different portions of feathers corresponding to the feather growing period. Therefore, various samples can be used according to the aim of study: e.g. feather (Bearhop *et al.*, 1999; Bearhop *et al.*, 2000) and blood (Bearhop *et al.*, 2000; Bearhop *et al.*, 2006) for indicating pollution contamination; muscle (Knoff *et al*, 2001), bone collagen (Steele & Hockey, 1991) and egg contents (Hobson, 1995) for identifying diet; eggshell for investigating habitat use (Dutta *et al.*, 1998; Emslie & Patterson, 2007); skin for sexual preference of diet (Tucker *et al.*, 2007) and claw for investigate migration distance (Mazerolle & Hobson, 2005). In birds, feathers have been commonly used to investigate diet because they are mostly pure protein (keratin) and easier to obtain from wild birds compared with other methods. It is also well established that the stable isotope signatures of particular feathers reflect the bird's diet during the period of feather growth (Thompson & Furness, 1995; Hebert *et al.*, 1999; Knoff *et al.*, 2002). Hatchling down can reflect the diet of the adult female during pre-laying and laying, because it is closely related to stable isotope ratios of the yolk (Klaassen *et al.*, 2004).

Using stable isotope ratios has several advantages in diet studies. First, stable isotope ratios allow us to assess diet change during the contentious period (Klaassen *et al.*, 2004). For example, feathers of migrant birds can reflect diet in the wintering ground (Bearhop *et al.*, 2003). Second, they assess diet assimilated without the bias accorded to food items from pellets or stomach contents. Although the stable isotope signature is useful to study a diet, it is difficult to identify a prey to the level of taxon. When diet contains various prey items or similar trophic values, it is difficult to identify from the stable isotope signatures. Stable isotope ratios also allow non-destructive and repeatable sampling.

Herring gulls feed on a variety of marine and terrestrial food such as fish, garbage, small birds, invertebrates and vegetation (Sibly & McCleery, 1983; Ewins *et al.*, 1994). However, previous studies show that individual gulls may specialize on one or a few food types (McCleery & Sibly, 1986) and that diet choice affects breeding performance (Pierotti & Annett, 1991). For example, in one study, intertidal food specialists laid eggs earlier and had a heavier clutch than generalists or terrestrial food specialists (Pierotti & Annett, 1991). In another study, worm specialists tended to spend less time feeding and more time on their territory (McCleery & Sibly, 1986). Pellets and regurgitates are commonly used to identify diet, but it is difficult to use pellet and

regurgitates for identifying the diet of gull chicks, because gull chicks do not produce pellets and regurgitates may be biased depending on prey digestibility.

In this study, I looked at 1) how do pellets indicate diet compare to stable isotope values from chick feathers and 2) how do stable isotope values correlate with hatching success and chick growth rate. 3) Diet change according to breeding season and 4) the relationship between diet and parental behaviour were also investigated using stable isotopes. I expected that gulls on Sanda Island would feed more on the high quality marine food during the chick rearing period, rather than the egg formation period, because of the greater demands of the chicks.

2.2 METHODS

2.2.1 Study area

Herring gulls were studied on Sanda Island (55°16′N 5°35′W), Argyll, Scotland from April to July 2006. Sanda Island (82.6 ha) is close to the mainland (approximately 3 km). A fishery is also active for crabs and lobsters, and trawl fisheries for whitefish and for Norway lobsters (*Nephrops norvegicus*) near Sanda Island. I collected data in 3 herring gull sub-colonies. The distance between sub-colonies was less than 2 km and each sub-colony had total 45 nests, 36 nests and 43 nests of herring gulls, respectively.

2.2.2 Field protocol

48 nests of herring gulls were visited daily to record laying dates and hatching dates in 3 sub-colonies. 3-egg clutches were found at 31 nests and 2-egg clutches were found at 15 nests. 2 nests out of 48 nests had unknown clutch size because they were found when they already had two eggs. 46 nests were found when gulls were building nests or they had one egg in the nest. Fresh eggs were marked with a non-toxic permanent-marker pen to identify laying order and the length and breadth of eggs were measured to the nearest 0.1 mm using vernier calipers. Egg volumes for 41 nests were estimated egg volume was calculated from the equation:

Egg volume (cm^3) = 0.000476 x egg length (cm) x egg $width^2$ (cm) (Harris, 1964).

Hatching success was recorded as two categories: 1) at least one egg did not hatch in the nest; 2) all egg hatched successfully in the nest. Chicks were weighed with an

electronic balance and pesola to the nearest 1 g and tarsus and head plus bill length were measured with vernier calipers to the nearest 0.1 mm approximately every 4 days from hatching to the end of the study in 42 chicks (1 chick per nest).

Chick growth rate was estimated by the linear regression of body mass, head plus bill length and tarsus length. Growth rate was calculated for the period of linear growth for chick ages from 4 to 28 days after hatching.

2.2.3 Chick down and feather sampling

One or two samples of hatchling down per chick were collected from 90 chicks of 48 nests within 24 hours after hatching to investigate the diet of individual females during egg formation. To investigate the difference according to laying order, hatching down of the first and second chicks were collected in 6 nests of 2-egg clutches and hatching down of the first and either second or last chicks in 26 nests. During the chick-rearing period, one or two growing feathers of 29 chicks were taken from the back to investigate their diet during the growing period. Because some chicks ran away when I approached the nests, it was difficult to catch all chicks at the same age. Chick feathers were taken in the wide range of chick ages between 16 to 33 days after hatching. The growth rate of chick feathers was used for estimating chick age because chick feathers grew related to chick ages.

2.2.4 Sample preparation

All samples were washed in distilled water to remove external contaminants potentially affecting the stable isotope signature and dried in an oven for more than 10 hours at 55 - 60 ºC. The base and tips of each chick feather were used to investigate diet change through the chick's growth period. The feather tip was used to indicate the diet during the initiation of feather growth (approximately 1 week after hatching) while the feather base was used to indicate diet of the latter part of the chick-growing period (3-5 weeks after hatching).

Feathers were cut in 2 or 3 pieces depending on the length of feather (or the age of chick) when I collected the samples. To identify the chick age when feathers were synthesized, I measured the length of feather in each age and calculated the feather growth rate following the equation calculated from 44 chick feathers which were taken from chicks of known age:

$Y = 0.243x - 1.138$

where Y is the length of chick back feather (cm) and x is the chick age (days after hatching). Chick feathers were cut in parts synthesized in 1 week (P1), 2-3 weeks (P2) and 3-5 weeks (P3) (Figure 2.1). Down feathers of hatchling (P0) were also used.

2.2.5 Isotope analysis

All samples which had carbon and nitrogen stable isotopic analysis were carried out simultaneously. Analysis of $\delta^{15}N$ and $\delta^{13}C$ isotope was measured by continuous-flow isotope ratio mass spectrometry (CF-IRMS) using a Costech Elemental Analyser (EA) linked to a Thermo Finnigan Delta Plus XP Mass Spectrometer. Approximately 0.7 mg were taken from the tip, middle and base part of chick feather and hatchling down and put inside a tin capsule (4mm by 6mm) for combustion. Samples were alternated with 2 lab reference materials every 10 samples to make a correction for drift. The lab materials are gelatine and alanine and these materials were routinely corrected related to international standard (e.g. gelatine, IAEA-N-2). The lab reference materials were chosen for their $\delta^{15}N$ and $\delta^{13}C$ isotope values, which were similar in carbon and nitrogen contents to the hatchling down and chick feather samples. Isotopic ratios of samples (R_{sam}) were compared to the isotopic ratio of a standard (R_{std}) for that element. R is the ratio of the heavy isotope to the light isotope of the element, and differences in the ratios are expressed in "delta" (δ) notation and are reported in parts per thousand (‰), according to the following equation:

$$\delta X\ (‰) = [(R_{sample} - R_{standard})/R_{standard}] \times 1000$$

where X is ^{13}C or ^{15}N and $R_{standard}$ for ^{13}C is Pee Dee belemnite (PDB) and for ^{15}N is atmospheric nitrogen. Measurements were better than ± 0.3‰ for both $\delta^{13}C$ and $\delta^{15}N$.

2.2.6 Adult pellet samples

Adult's pellets were collected around 43 focal nests (within a radius of 1 m) every 4-day intervals during the pre-laying and laying, incubation and chick rearing period. All pellets near nests were removed to avoid re-counting the pellets at the focal nests. Collected pellets were dried and kept in a plastic container and were identified visually into 10 categories; 1) grain, 2) grass, 3) coleoptera, 4) seaweed, 5) crustacea, 6) gastropoda, 7) fish, 8) refuse, 9) birds and 10) mammals. Chicken bones and various

non-organic matters such as plastic and aluminium foil were included in the refuse category. Herring gulls are omnivorous and their pellets often contained a mixture of several food items. I estimated visually the proportion of each diet item per pellet when pellets contained several items. Proportions of marine prey (including seaweed, crab, mussel and fish) were estimated in each pellet. The mean proportion of each food item per visit was calculated when I collected more than one pellet in the territory. Otoliths contained in adult pellets were identified from reference collections of otoliths dissected from fish caught in the Clyde and the illustration book of otoliths in the Northeast Atlantic and fish size was also estimated from the equations correlation between otolith length and fish length (Härkönen, 1986).

2.2.7 Parental behaviour

During the chick-rearing period, territorial attendance was recorded twice a day (between 09:00 and 12:00 and between 16:00 and 19:00). To avoid disturbance, the absence or presence of each parent was recorded by spot observation from the top of cliffs. Birds were dyed with picric acid during the early incubation period to distinguish individuals. A piece of sponge soaked with picric acid dissolved in a mixture of water and alcohol was tied to the edge of each nest, taking care to avoid contact with eggs. After the bird had returned to incubate, and taken up picric dye onto breast and belly feathers, the location of dyed feathers was recorded for each individual.

Aggression scores of adults were recorded when I visited nests to measure chicks between 0 days and 21 days after hatching. Aggression score was determined in the following 4 categories (Furness, 1984):

Score 0. parents left their territory;
Score 1. parents stayed on their territory or circled above territory;
Score 2. parents swooped regularly but did not hit the observers;
Score 3. parents swooped regularly and hit the observers

The mean aggression score during the chick rearing period was calculated per nests for the analysis.

2.2.8 Statistical analysis

All statistical tests were performed using SPSS 15.0 (SPSS, 2006). For the statistical analysis, 1 egg or 1 chick per nest were used to avoid the variation within nest except when looking at the effect of laying order on stable isotope signatures. Carbon and nitrogen isotope values were normally distributed. A logistic regression was used to investigate the relationship between stable isotope signatures and hatching success. Paired t-test was used for comparing the stable isotope ratios between hatchling down and chick feather. To investigate the relationship between pellet composition and laying date, a self-organizing map (SOM) through the SOM tool box (Alhoniemi *et al.*, 2000) available in Matlab (The Math Works, 1999) was used. SOM is a kind of artificial neural network trained by unsupervised learning. SOM consists of input and output layers. When the input vector was given to the network, the distance between the input vector and weight vector (connection intensity) was estimated through the algorithm. Input vectors were organized depending on the distance and presents the input space of the training samples as a map. In this study, SOM discriminated each food item and calculated the Euclidean distance between food items. The output layer consisted of output neurons in a two-dimensional hexagonal lattice (Kohonen, 1982) and showed which nests had similar diet during the chick-rearing period. SOM placed nests which had similar diet in a hexagonal lattice. 10 types of diet such as grain, grass, insects, seaweed, crustacean, gastropoda, fish, refuse, bird prey, mammal prey and laying date were used as input vectors. Map size of 15 (5x3) neurons (hexagonal lattices) was chosen for obtaining a suitable map.

2.3 RESULTS

2.3.1 Remaining items in adult pellets

Items included in adult herring gull pellets in Sanda Island were mainly grain and grass (Table 2.1). The proportion of grain decreased while the proportion of refuse and fish in pellets increased in the chick-rearing period. The proportion of marine items increased in the chick-rearing period although the proportion of terrestrial item was similar between pre-laying and laying and chick-rearing period. Proportion of insects in pellets was related to the proportion of grass. Mammal, grain, grass and insects tended to be found in the early breeding season while crustacea, fish, bird and refuse appeared mainly in the late breeding season (Figure 2.2). Proportion of marine food significantly increased through the breeding season during the chick-rearing period

(Spearman Correlation analysis: r_s = 0.459, p = 0.032, n = 22) although it was not significantly related to laying date during the laying period (r_s = - 0.015, p = 0.952, n = 18) or incubation period (r_s = 0.240, p = 0.238, n = 26). Five fish species were identified from the otoliths contained in adult pellets during the breeding season: haddock (*Melanogrammus aeglefinus*) (5 otoliths), Norway pout (*Trisopterus esmarkii)* (2 otoliths), whiting (*Merlangius merlangus*) (2 otoliths), blue whiting (*Micromesistius poutassou*) (1 otolith), dragonet (*Callionymus lyra*) (1 otolith). Fish size and fish weight estimated from otolith length of haddock, Norway pout, whiting and dragonet collected from adult pellets ranged from 18 to 26 cm and 43.5 to 142.9 g, while blue whiting was larger than 30 cm and heavier than 250 g.

2.3.2 Stable isotope signature of hatching down and chick feather

Carbon and nitrogen stable isotope values of hatching down and chick feather were between terrestrial and marine potential food items (Figure 2.3). Carbon and nitrogen isotopic values in the feathers increased after chicks hatched (P1, P2 and P3) (Figure 2.4). The stable isotope values from hatchling down represent a terrestrial dominate diet whereas the vales and chick feathers showed a more marine dietary influence. The mean carbon isotope signatures were –23.4 ± 1.88 ‰ and –19.9 ± 1.79 ‰, during the pre-laying and laying period and the chick-rearing period, respectively. The mean nitrogen signatures were 11.51 ± 0.75‰ and 12.61 ± 1.18‰ during the pre-laying and laying period (48 nests) and the chick-rearing period (30 nests), respectively (Figure 2.5). Stable isotope values did not vary significantly according to chick age (One-way ANOVA: $\delta^{13}C$ – $F_{2,68}$ = 1.23, P = 0.297; $\delta^{15}N$ – $F_{2,68}$ = 1.90, p = 0.157)(Figure 2.4).

The proportion of marine food in adult pellets correlated with the nitrogen stable isotope signatures during the laying period and the chick rearing period while it did not relate to carbon stable isotope signatures during the breeding period (Table 2.2).

2.3.3 Stable isotope signatures and clutch size and laying order

Stable isotope signatures of carbon and nitrogen from hatchling down did not differ between 2-egg clutch and 3-egg clutch (independent sample t-test; $\delta^{13}C$: t_{44} = 1.87, p = 0.140; $\delta^{15}N$: t_{44} = 0.59, p = 0.552) (Figure 2.6). They also did not differ according to laying order in 2-egg clutch (paired t-test: t_6 = 0.51, p = 0.627; t_6 = 1.74, p = 0.132) or in 3-egg clutch (mean differences between laying order ± SD - $\delta^{13}C$: 0.08 ± 0.553, n =

26; δ^{15}N: 0.01 ± 0.384, n = 26; 95% confidence interval). Therefore, all clutch size and laying order data were pooled for the analysis of stable isotope variation during the pre-laying period.

2.3.4 Seasonal effect on diet change during the breeding season

Carbon isotope values increased across the breeding season in both the pre-laying period (r = 0.34, p < 0.021, n = 45 nests) and chick-rearing period (r = 0. 57, p < 0.001 n = 29 nests). Nitrogen isotope values were not significantly correlated to laying date in pre-laying and laying period (r = 0.254, p = 0.093, n = 45), but increased with laying date during the chick rearing period (r = 0.512, p = 0.004, n = 29) (Figure 2.7). The differences of isotopic signatures between pre-laying and laying period and chick-rearing period were not related to laying date (δ^{13}C: r = 0.142, p = 0.471, n= 28; δ^{15}N: r = 0.308, p = 0.111, n = 28).

2.3.5 Hatching body mass and size of chicks and hatching success

Egg volume and hatching body condition did not significantly change during the breeding season (egg volume: r = - 0.12, p = 0.455, n = 41; body mass at hatching: r = - 0.01, p = 0.960, n = 42; tarsus length at hatching: r = 0.26, p = 0.089, n = 43; head plus bill length: r = - 0.28, P = 0.063, n = 43). Therefore, there was no detectable effect of egg volume on hatching body condition. None of body mass, tarsus length and head plus bill length and egg volume was related to stable isotope signatures (Table 2.3). I looked at the effect of diet during pre-laying and laying period on partial hatching success. Carbon isotope signature and nitrogen isotope signatures did not relate to the partial hatching failure in the nests (δ^{13}C: B = 0.20 ± 0.234, p = 0.371; δ^{15}N: B = 0.77 ± 0.488, p = 0.113).

2.3.6 Growth rate and stable isotope signatures

Growth rates of chick body mass were positively related to carbon isotope signatures but not related to nitrogen isotope signatures (Table 2.4). Growth rates of chick head plus bill length, and tarsus length, were not related to stable isotope signatures.

2.3.7 Diet and parental behaviour

Parents with higher territorial attendance had more enriched carbon and nitrogen isotope signatures (Figure 2.8) ($\delta^{13}C$: $t_{25} = 2.14$, $p = 0.042$; $\delta^{15}N$: $t_{25} = 2.79$, $p = 0.010$). Territory attendance did not differ according to chick age (repeated measures ANOVA: $F_{2,30} = 5.55$, $P = 0.578$). Aggression score was not related to isotope signature (spearman rank correlation: $\delta^{13}C$: $r_s = -0.10$, $p = 0.585$ $n = 28$; $\delta^{15}N$: $r_s = -0.19$, $p = 0.319$, $n = 28$).

Table 2.1 Proportion (%) of organic items and the mean of terrestrial and marine diet in pellets regurgitated by adult herring gulls in the egg laying, incubation and chick-rearing period in Sanda Island, Scotland, UK in 2006.

	Laying	*Incubation*	*Chick-rearing*
Items			
Grain	88	55	13
Grass	4	35	44
Coleoptera	1	2	2
Refuse (chicken and meat)	0	0.1	10
Bird	0.1	0.1	3
Mammal (rodents)	0	3	<1
Seaweed	0	1	<1
Crustacea	4	2	19
Gastropoda	4	1	<1
Fish and Cephalopoda	0	2	10
Foraging habitat			
Terrestrial	89	95	83
Marine	7	5	17
Total pellet (nests)	26 (19)	79 (29)	115 (37)

Table 2.2 The correlation between the stable isotope of hatchling down and chick feathers (1 week) and adult pellets.

	Laying period (n = 18 nests)		Incubation (n = 28 nests)		Chick rearing (n = 23 nests)	
	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$
% Marine food in pellets	r = 0.37, p = 0.130	r = 0.50, p = 0.032	r = 0.28, p = 0.136	r = 0.11, p = 0.56	r = 0.16, p = 0.456	r = 0.47, p = 0.023

Table 2.3 The relationship between carbon and nitrogen isotope signature and egg volume and body mass and size at hatching. Egg volume and hatching body mass and size did not vary significantly with date. Egg volume was measured from 41 nests (1 chick per nest) because 4 of 45 nests did not measure. 3 chicks from 45 nests did not hatch and excluded from analysis. Tarsus and head plus bill length was missed in 1 nest of 42 nests.

	Egg volume (n = 41 eggs)	body mass and size at hatching		
		Body mass (n = 42 chicks)	Tarsus (n = 41 chicks)	Head plus bill (n = 41 chicks)
$\delta^{13}C$	r = - 0.01, p = 0.983	r = - 0.03, p = 0.877	r = 0.02, p = 0.878	r = 0.04, p = 0.823
$\delta^{15}N$	r = - 0.03, p = 0.849	r = - 0.03, p = 0.865	r = 0.17, p = 0.276	r = - 0.09, p = 0.534

Table 2.4 Growth rate of chick body mass, head plus bill and tarsus and stable isotope signatures of chick feathers. Bold letters indicate significance after Bonferroni correction.

	Growth rate		
	Body mass	Head plus bill	Tarsus
$\delta^{13}C$	$F_{1,14}$ = 8.34, P = **0.012**	$F_{1,15}$ = 0.55, P = 0.469	$F_{1,11}$ = 0.01, p = 0.933
Laying date	$F_{1,13}$ = 2.86, p= 0.114	$F_{1,11}$ = 0.01, p = 0.949	$F_{1,10}$ < 0.01, p = 0.988
Hatching order	$F_{2,11}$ = 1.28, P = 0.314	$F_{2,12}$ = 0.09, P = 0.913	$F_{2,12}$ = 0.28, P = 0.756
Clutch size	$F_{1,10}$ = 0.22, p = 0.649	$F_{1,14}$ = 0.42, p = 0.526	$F_{1,14}$ = 12.20, p = **0.004**
$\delta^{13}C$ * laying date	$F_{1,9}$ = 0.01, P = 0.960	$F_{1,10}$ = 0.68, P = 0.427	$F_{1,9}$ = 0.12, p = 0.735
$\delta^{15}N$	$F_{1,13}$ = 2.29, P = 0.154	$F_{1,15}$ = 1.83, P = 0.195	$F_{1,10}$ = 0.06, P = 0.809
Laying date	$F_{1,14}$ = 7.54, P = **0.016**	$F_{1,11}$ = 0.01, p = 0.976	$F_{1,10}$ = 0.01, p= 0.900
Hatching order	$F_{2,11}$ = 1.76, P = 0.216	$F_{2,12}$ = 0.05, P = 0.948	$F_{2,12}$ = 0.05, P = 0.948
Clutch size	$F_{1,10}$ = 0.31, P = 0.585	$F_{1,14}$ = 0.26, p = 0.618	$F_{1,14}$ = 12.20, p = **0.004**
$\delta^{13}C$ * laying date	$F_{1,9}$ = 0.02, P = 0.888	$F_{1,10}$ = 2.02, P = 0.185	$F_{1,9}$ = 0.10, P = 0.750

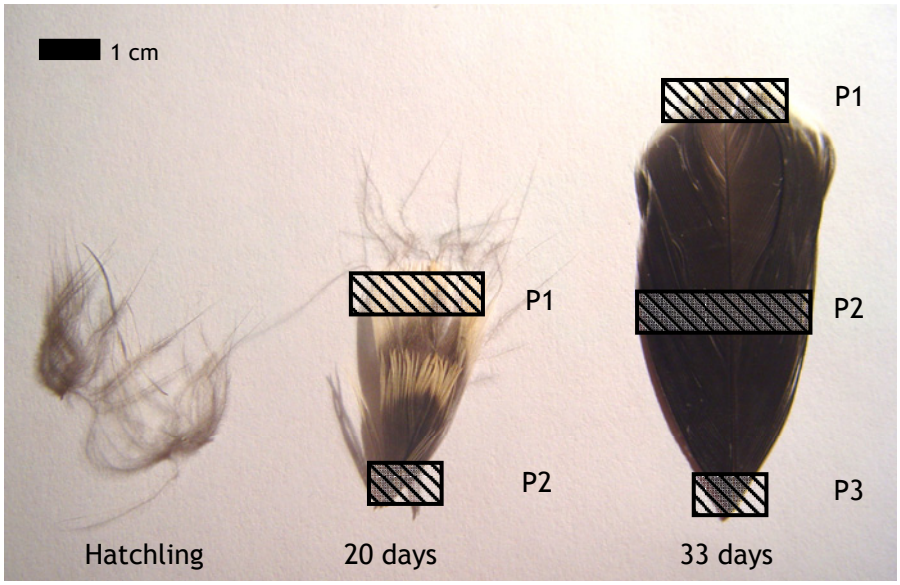

Figure 2.1 Hatchling down and chick feathers used for stable isotope analysis according to chick age (P0: hatchling down; P1: 1 week; P2: 2-3 weeks; P3: 3-5 weeks) after hatching.

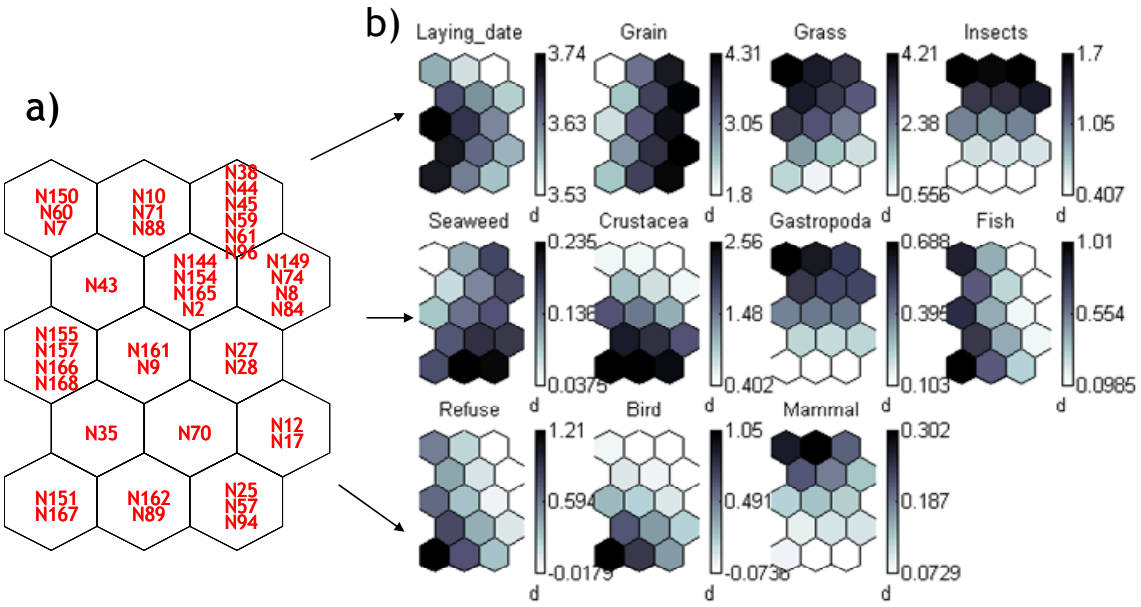

Figure 2.2 Relationship among items in pellets and laying date using the trained self-organizing map (SOM) in grey scale. The left diagram (a) shows the nests in each group and the right diagram (b) shows items in pellets and laying date. In the right diagram, the hexagons are same as hexagons of left diagram (a). Values near the bar in (b) presents the log transformed mean proportion of item in a pellet. Darker shading (larger value) indicates that nests in a hexagon have higher proportion of food item in a pellet or laid eggs later. For example, Nest No. 38 contained mainly grain, grass and insects and laid eggs earlier than others.

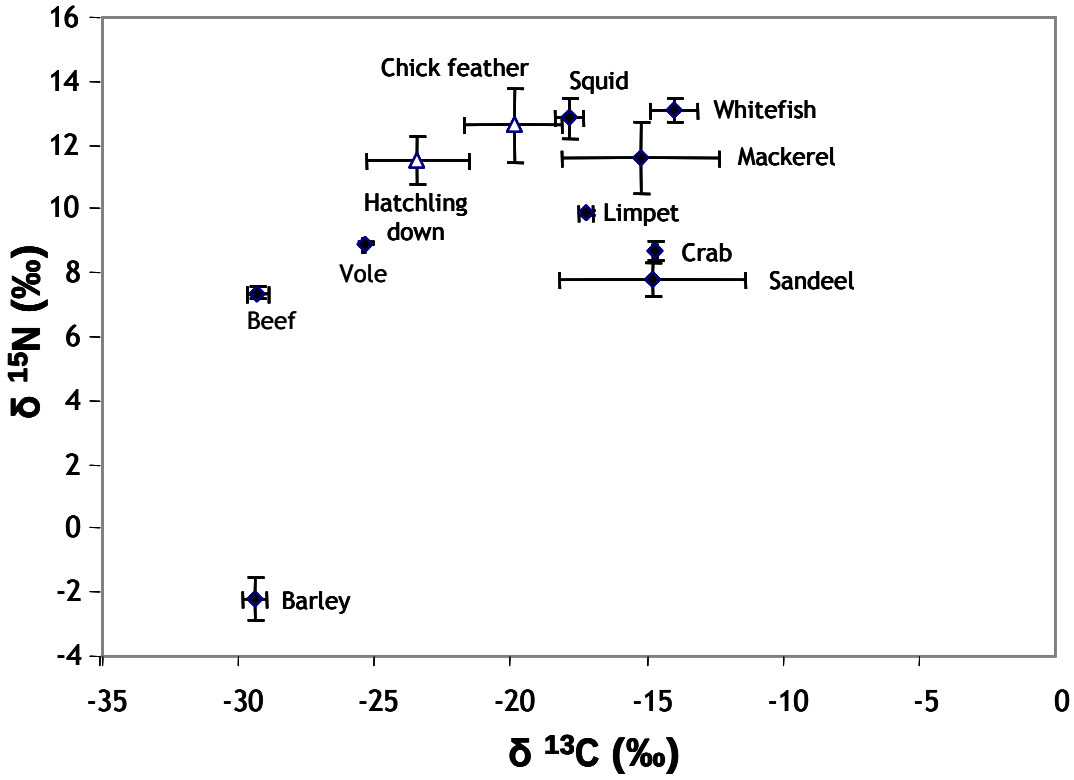

Figure 2.3 Carbon and nitrogen isotopic composition of chick feather and hatchling down (open triangles) and potential and observed diet (closed circles) of herring gulls in Sand Island (bars represent ± SD except limpets and voles with ± SE). Stable isotope signatures of diet items: limpets (*Patella vulgat*) (Andrew, pers comm.); mackerel (*Scomber scombrus);* sandeel, whitefish (Bearhop *et al.*, 1999); Squid (*Lolliguncula brevis*) (Stowasser *et al.*, 2006); blue crabs (*Callinectes sapidus*) (Dittel *et al.*, 2006); qinghai voles (*Lasiopodomys fuscus*) (Li *et al.*, 2004); beef (Bearhop *et al.*, 1999); barley *Hordeum vulgare* (Bort *et al.*, 1998).

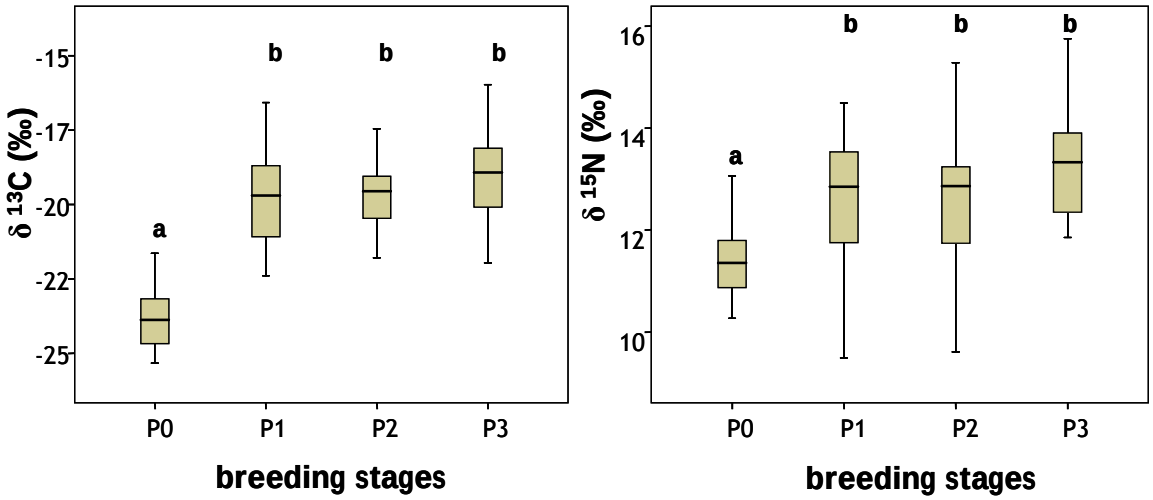

Figure 2.4 Carbon and nitrogen isotope signatures according to breeding stages. Different letters above the bar indicate significant differences. P0: pre-laying and laying period; P1: 1 weeks; P2: 2-3 weeks; P3: 3-5 weeks after hatching. Whiskers indicate ± SD.

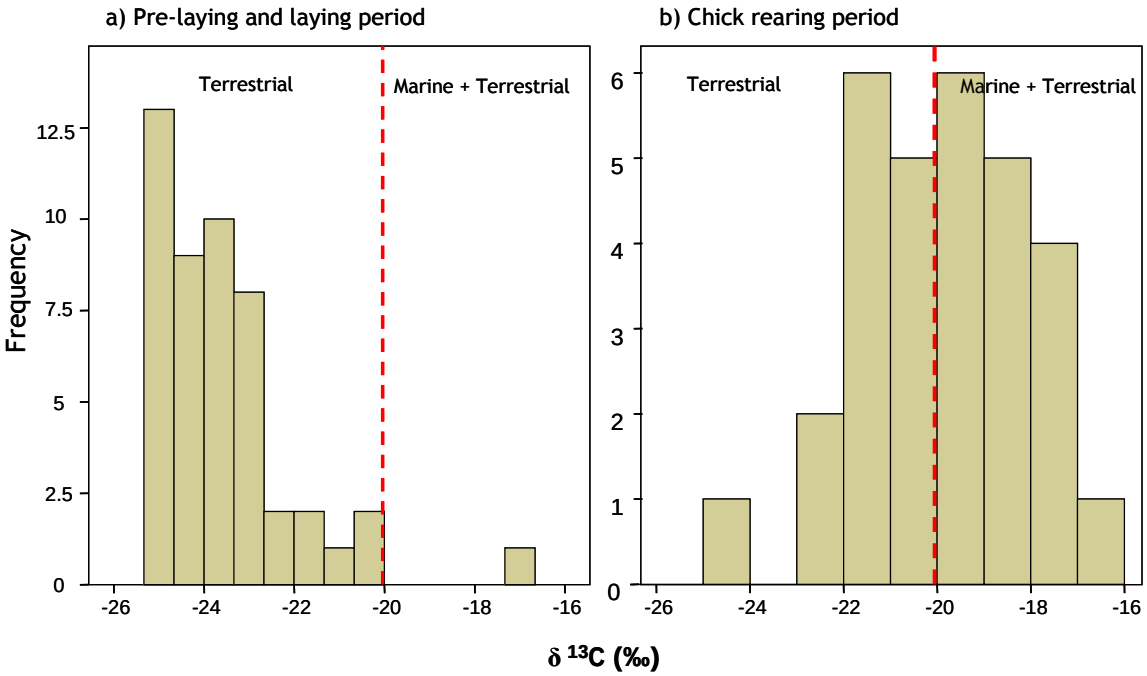

Figure 2.5 Change of carbon isotope signatures between pre-laying and laying period (a) and chick rearing period (b). Stable isotope signatures of terrestrial and marine diet was divided by the previous study in cormorants *Phalacrocorax carbo* (Bearhop *et al.*, 1999)

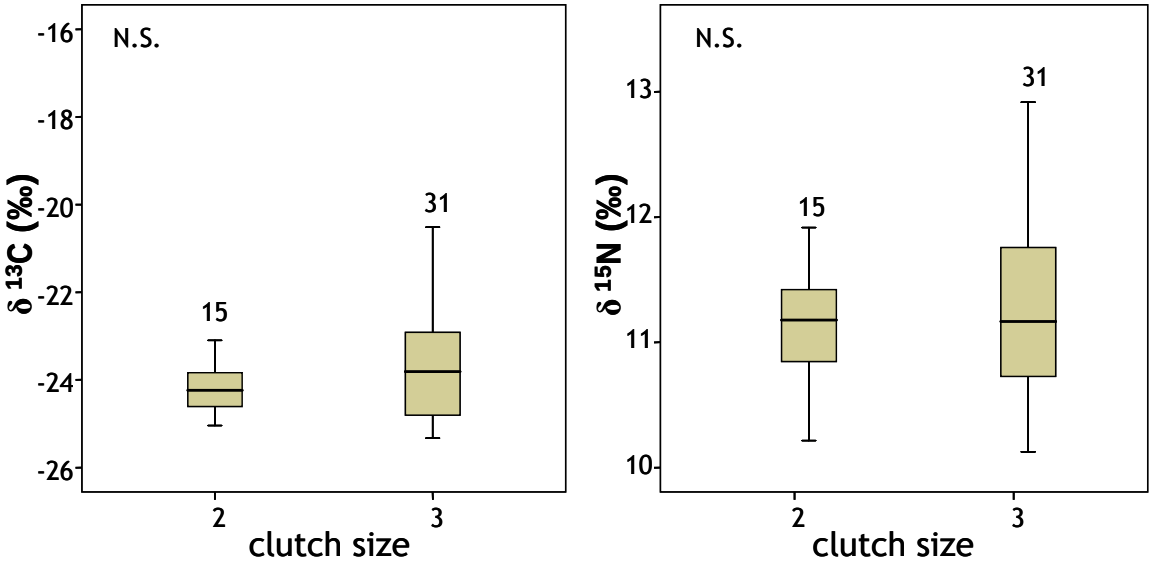

Figure 2.6 Carbon and nitrogen isotope signatures of hatchling down (female diet during pre-laying and laying period) in 2-egg clutch and 3-egg clutch. The number above the bar indicates the number of nests. Whiskers indicate ± SD.

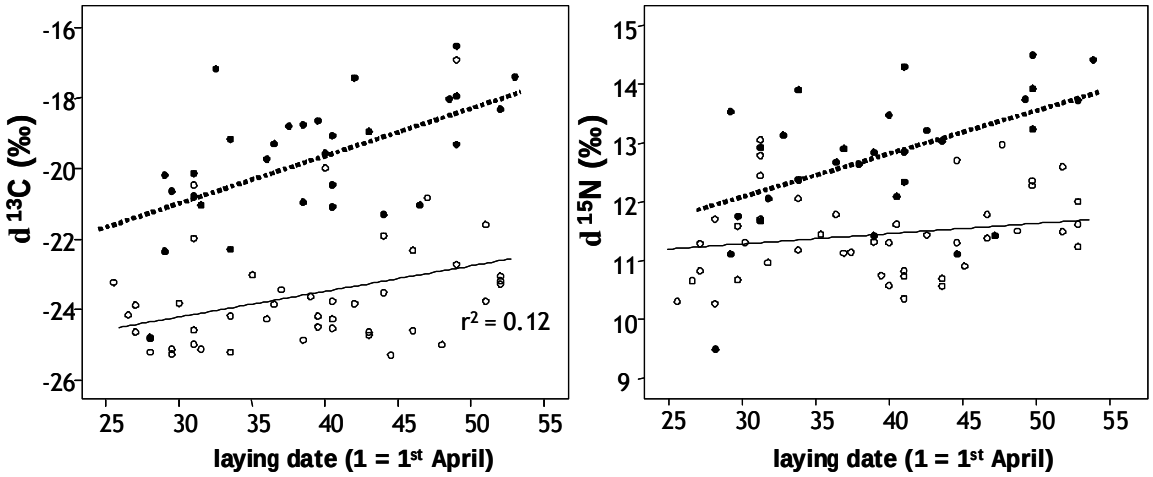

Figure 2.7 Carbon and nitrogen isotope signatures between pre-laying and laying period (45 nests) and chick rearing period (29 nests). Hatching down (solid line, open circle) was used for pre-laying and laying period diet and chick feather (broken line, solid circle) presented chick diet during the rearing period.

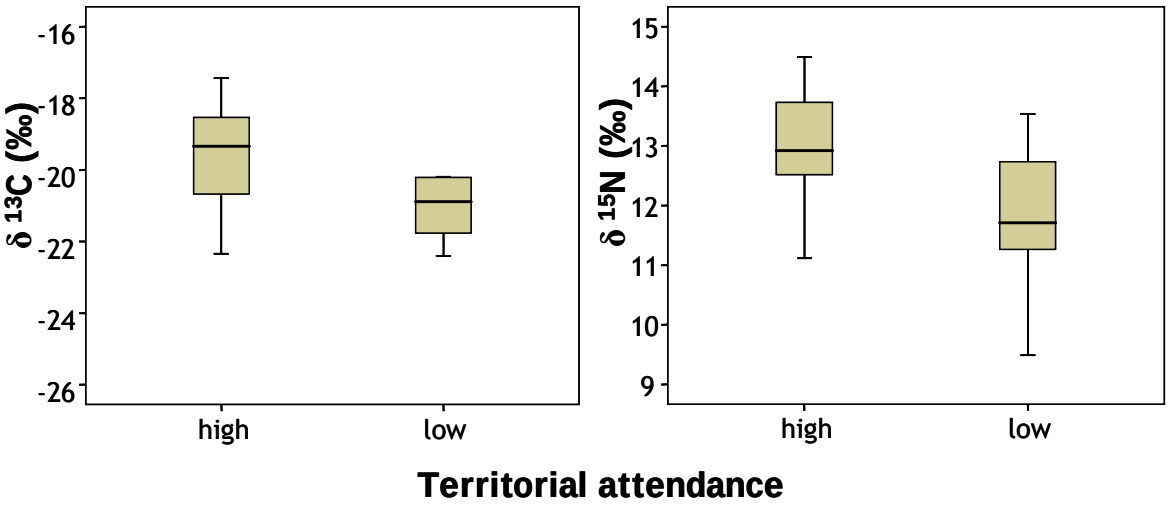

Figure 2.8 Carbon and nitrogen isotopes from chick feather according to territorial attendance of parents. High and low attendance was classified from mean (92%) of total nest attendance in 27 nests (2 nests of 29 nests were excluded due to no data of territorial attendance).

2.4 DISCUSSION

Herring gulls in Sanda Island consumed various kinds of food during the breeding season. Main food items contained in adult pellets were grain and grass. Although many whole grains were found in adult pellets, some pellets contained only husks without grains. Stomach contents of dead chicks included only whole grains. Gulls seemed to eat grains from the farms around the study area. Armstrong (1992) suggested that cereals were an important source of food for adults during the incubation period and during the early chick period. It seems likely the gulls obtain grain from feeding troughs set out in fields for cattle, as they could be seen feeding at these troughs. Farmers probably supplemented cattle diet mainly early in the season when grass had not yet grown much. Adult pellets containing grass were often found with insect remains (beetles). Therefore, grass can be collected coincidently with insects when gulls caught insects in the grass field or ate grass to clean insect remains in their stomach. Gulls which ate insects also showed the tendency to consume mammals (e.g. a small rodent). Pellets from gulls which used crustacean food such as crab contained relatively high portions of fish and seaweed. Herring gulls have been reported as food specialists of intertidal organisms, human refuse or other seabirds during the breeding season (Pierotti & Annett, 1991). Although pairs could not be clearly distinguished in territorial and marine food specialists, herring gulls in Sanda Island may have a tendency to eat either more territorial food or more marine food.

Carbon and nitrogen isotope signatures showed more mixture of terrestrial and marine food compared to items remaining in adult pellets. For example, although pellets contained mainly grains, carbon and nitrogen signatures of hatching down showed higher values than grains. However, carbon and nitrogen isotope signatures of hatching down were in the range of terrestrial food although it is possible that females had a mixture of a large amount of terrestrial food and small amount of marine food. As the proportion of marine food in pellets increased during the chick-rearing period, carbon and nitrogen isotope signatures were more enriched. It may be explained in two ways. One is that parents changed their diet after chick hatched. This diet change after chick hatching was often reported in other studies using pellets (Annett & Pierotti, 1989; Nogales *et al.*, 1995; Bukacinska *et al.*, 1996). Parents may need to change diet to increase chick fitness after the chick hatches. In other herring gull colonies, pairs that did not change their diet at chick hatching suffered higher chick mortality than pairs that did (Bukacinska *et al.*, 1996). However, the other explanation may be possible if there was a diet difference between females and males. Stable isotope signatures from

hatchling down can only reflect the female diet during the egg formation period while isotope values from chick feather included the diet of both female and male. Pons (1994) found that male herring gulls fed more on garbage than females because of higher competition at the garbage tip, which favoured the larger sized males. In Sanda Island, the garbage was seldom used for the diet of herring gulls. However, it is possibility to find different diet between females and males when females and males used the discard fish from the fishing boats. Because obtaining discard fish from the fishing boats is highly competitive (reviewed in Furness *et al.*, 1992), males find it easier to obtain discarded fish than females. Fish size and weight estimated from the otoliths in adult pellets were in the range of discarded fish (Stratoudakis *et al.*, 2001; Palsson, 2003).

There was no significant change in carbon and nitrogen stable isotope values through the chick-rearing period, though there was higher variation of diet between pairs. Carbon and nitrogen isotope signatures of chick feathers did not change during the chick rearing period. It is possible that parents feed larger prey or different prey species within marine or terrestrial foods to older chicks. However, stable isotope signatures of chick feathers did not show this because stable isotope signature cannot tell the diet at the species level.

Carbon and nitrogen isotope values increased through the breeding season. This may be related to seasonal food availability. In Sanda Island, herring gulls often used discards from fishing boats and fishery activity in the area has a peak around July and August (Armstrong 1992). Therefore, increased carbon isotope signatures in late breeding season may be the result of discards use from fishing boats. Adult pellets contained a deep-sea fish (blue whiting), and two demersal fish (haddock and whiting), which it would be difficult for herring gulls to catch alive. In early breeding season, herring gulls may feed mainly on grain or invertebrates from farmland until the discards became available from the fishing boats.

Diet choice affects egg volume and/or clutch size. For example, mussel specialists in herring gulls had larger and heavier clutches than garbage specialists (Pierotti & Annett, 1990). In Florida scrub-jays (*Aphelocoma coerulescens*), females which were supplemented by high fat and high protein diet laid heavier clutches but not larger clutches than females supplemented by high fat and low protein diet or natural diet (Reynolds, 2003). Grains which were found in adult pellets had lower protein and fat than fish or birds. However, in this study, both egg volume and clutch size was not

related to carbon and nitrogen isotope signatures of female diet. Food type or trophic level may not relate to food quality, which can affect egg volume or clutch size.

Hatching body mass, tarsus length and head plus bill length were also not related to carbon and nitrogen signatures. However, growth rate of chicks was positively related to carbon isotope values. Chicks fed on more marine food showed faster growth rate of body mass than chicks fed a lower proportion of marine food. Nitrogen isotope signatures which present the trophic level of diet were not related to body mass growth or skeletal growth. Skeletal growth such as head plus bill and tarsus were not related to carbon and nitrogen stable isotope signatures. Therefore, diets with enriched carbon isotope may affect body mass growth rather than skeletal growth.

To investigate the effect of diet during the pre-laying and laying period on hatching success, it would be better to compare fully unhatched and fully hatched eggs in the nests. However, carbon and nitrogen isotope signatures were compared in partially unhatched nests and fully hatched nests because hatching down could not be collected from unhatched nests. Partial hatching failure did not relate to the type or trophic level of diet.

Territorial attendance has been used to assess the foraging effort in skuas and gulls (Bukacinska *et al.*, 1996; . Bukacinska *et al.* (1996) observed that unsuccessful pairs in herring gulls spent more time foraging and they left their eggs or chicks unguarded. In this study, pairs that fed their chicks on diet with higher carbon and nitrogen isotope values showed higher territory attendance than parents that fed chicks on diet with lower isotope values. This may be related to either foraging efficiency or food availability according to diet, or to intrinsic differences in adult quality correlated with diet choice. In slaty-backed gulls (*Larus schistisagus*), males which fed chicks on seabird chicks, spent more time on their territories than the other males probably because of the easy access to seabird chicks (Watanuki, 1992). Pairs of herring gulls that ate more crab and starfish left their territories unguarded for longer than pairs that ate more fish or bird prey because of longer foraging trips and unguarding chicks can increase the risk of breeding failure (Bukacinska *et al.*, 1996). Great skuas (*Stercorarius skua*) that ate primarily other seabirds “seabird-specialists” also spent less time foraging than the skuas feeding mainly on fish “fish-specialists” or feeding on a wide diversity of foods “generalists” because they can defend nests and feed inside their home ranges (Votier *et al.*, 2004). A higher proportion of marine food could meet the nutritional requirement or be easy to access during the chick-rearing period and it

may let parents stay longer in their territories. Therefore, marine food may be able to increase breeding success in terms of increasing growth rate of chicks and/or nest attendance of parents. In my study, there was evidence that birds feeding their chicks with more marine foods were more successful, and yet also spent less time foraging. The fact that many birds took rather little marine food suggests that there is a constraint, and that perhaps only the best quality birds are able to utilise marine foods successfully. A number of studies have shown that scavenging discards from fishing boats is very competitive (Hudson & Furness, 1988; Garthe & Huppop, 1996), and suggest that only high quality and experienced birds may be able to do this successfully (Garthe & Huppop, 1996).

In order to investigate relationships among diet choice, parental behaviour and breeding success, it is necessary to quantify the diet of study birds with known ecology. This can be done with gulls by collecting pellets of indigestible remains regurgitated by adults. However, pellets do not reflect the whole diet on a one to one basis because some foods such as grains lead to production of more pellets than others such as earthworms (Brown & Ewins, 1996). Furthermore, gull chicks do not produce pellets. Another way to examine diet is to use variations in stable isotope ratios of carbon and nitrogen as indicators of diet type although there is a limitation in identifying diet items through stable isotope values when birds feed on various kinds of diet. In this present study, although items contained in adult pellets during the laying period were mainly grains such as barley, stable isotope values of hatching down were much higher than values of barley. Hence, both analysis of pellets and stable isotope values may be useful to identify diet during the breeding season.

Chapter III.

The effect of nest attendance during early incubation on hatching patterns and egg viability in herring gulls *Larus argentatus*

3.1 INTRODUCTION

In most animals, offspring of the same breeding attempt hatch or are born within a relatively short period of time; hatching or birthing is synchronous. In many birds (Clark & Wilson, 1981), but also in some insects (e.g. Smiseth *et al.*, 2006) and reptiles (While *et al.*, 2007) hatching and birthing can be asynchronous. Asynchronous hatching or birthing establishes age hierarchies among the offspring of a breeding attempt where older siblings have a competitive advantage over their younger siblings (Mock & Parker, 1997). Much research has focused on adaptive hatching patterns in birds during the nestling stage, but why asynchronous hatching has evolved still remains poorly understood (Magrath, 1990, Stoleson & Beissinger, 1995; Stenning, 1996). Causally linked to these adaptive hypotheses on hatching patterns are proximate explanations of incubation patterns, which have received less attention (Stoleson & Beissinger, 1995). Here I focused on the onset of incubation during egg-laying, which is thought to determine the observed hatching patterns.

In avian species, embryonic development is initiated when parents start incubation. Therefore, parents can control hatching patterns behaviourally by adjusting incubation during laying, and incubation pattern over the laying cycle can assume a variety of shapes (Wiebe *et al.*, 1998). If birds start incubation before completing their clutches, eggs which were laid before the onset of incubation start embryonic development earlier than later-laid eggs. In contrast, if pairs start incubation after completing the clutch, chicks have short hatching intervals within a clutch because all eggs start embryonic development almost at the same time (Ricklefs, 1993). Although most emphasis is on the timing of the onset of incubation in determining the hatching pattern (Stoleson & Beissinger, 1995; Mock & Parker, 1997), asynchronous offspring development may also contribute to the hatching pattern (Nicolai *et al.*, 2004; Muck & Nager, 2006).

Since incubation is an energetically expensive activity (Tinbergen & Williams, 2002), resource availability may possibly modify nest attendance during incubation, and this has been termed the nutritional constraint hypothesis. Experiments using supplementary food during laying or incubating showed that pairs given extra food spent more time incubating their eggs (Rauter & Reyer, 1997; Eikenaar *et al.*, 2003). Supplementary-fed marsh tits (*Parus palustris*) initiated incubation earlier and had more asynchronously hatching broods, suggesting that they started incubation early when food condition was good because their onset of incubation was energetically constrained during the laying period (Nilsson, 1993). While chicks of American kestrels (*Falco sparverius*) hatched more synchronously when parents were supplemented (Wiebe & Bortolotti, 1994). Similarly, common eider (*Somateria mollissima*) females in poor body condition initiated incubation earlier, resulting in shorter incubation period and more asynchronous hatching pattern compared to females in good condition (Hanssen *et al.*, 2002). It has been suggested that in species that depend on accumulated body reserves for incubation, unlike small passerines such as marsh tits, individuals in poor condition can maximize their breeding success by having a shorter incubation period through an earlier onset of incubation (Hanssen *et al.*, 2002). Incubation behaviour may not only be affected by food availability altering parental body condition, but also by their diet and the foraging time required for searching and handling it. For example, great skuas (*Stercorarius skua*) which specialized in feeding on more profitable avian prey spent less time foraging than skuas feeding predominantly on fish during the breeding season (Votier *et al.*, 2004). Hence, parents that forage on different diets might be able to spend different amounts of time at their nests and it may affect incubation behaviour.

Onset of incubation may vary with date, and typically earlier breeding pairs start incubation later in relation to clutch completion than late breeders (Sockman *et al.*, 2006). This advance in onset of incubation as the season progresses is thought to shorten the nesting cycle and reduces risk of predation, but also helps late breeders to complete chick rearing before conditions deteriorate at the end of the season (Clark & Wilson, 1981; Slagsvold, 1986). Clutch size may also constrain the onset of incubation. Females with larger clutches started incubation earlier (Magrath, 1992; Potti, 1998) which resulted in increased hatching asynchrony (Magrath, 1992). Larger clutches are laid over a longer period and this may increase hatching asynchrony.

To start incubation at some time before a clutch is completed may be beneficial to the bird in its own right (Stoleson & Beissinger, 1995). Early onset of incubation may reduce the risk of brood parasitism (Kendra *et al.*, 1988) or predation (Clark & Wilson,

1981; Beissinger *et al.*, 1998) and it may be beneficial with respect to territory and nest defence (Beissinger & Waltman, 1991). Arnold *et al.* (1987) suggested that early nest attendance during laying may increase viability of early-laid eggs (egg viability hypothesis). Recently, it has been shown that incubation during the laying period reduces the risk of microbial infection in early-laid eggs (Cook *et al.*, 2003; Cook *et al.*, 2005a; Cook *et al.*, 2005b). Hence, incubation behaviour might be constrained by egg viability.

Although increasing incubation behaviour during egg-laying has been suggested as the main factor affecting hatching patterns, there are few quantitative data that relate incubation behaviour to hatching patterns. The importance of the timing of onset of incubation in determining hatching patterns relative to other factors that also can modify hatching patterns has not been tested yet. The onset of incubation is difficult to determine directly because incubation gradually increases during egg-laying (Stoleson & Beissinger, 1995). For example onset of incubation was often determined as when parents incubate eggs, or eggs are warm at a nest visit (e.g. Potti, 1998). However, it can be problematic because nest attendance can be altered by the time of day (Palmer *et al.*, 2001) and change gradually rather than being switched on at a specific time (Wiebe *et al.*, 1998). I will therefore use continuous measurements of nest attendance during laying and early incubation in order to characterize the incubation behaviour during egg-laying.

I studied herring gulls (*Larus argentatus*) to investigate factors affecting incubation behaviour during egg-laying and the relationship between behaviour during early incubation and hatching patterns. Nest attendance gradually increases during egg-laying until it reaches a steady level of nest attendance several days after clutch completion (Drent, 1970) and chicks hatch asynchronously (e.g. Hillstrom et al., 2000). I measured nest attendance continuously from the day the first egg was laid until 4 days after clutch completion by recording nest temperature. I expected that early onset of incubation and higher mean nest attendance during egg-laying and early incubation would increase hatching asynchrony but that asynchronous embryonic development, in particular during hatching, would also influence the hatching pattern. I also tested the hypotheses that incubation behaviour is related to laying date, clutch size, diet during egg formation, and egg size, because these parameters also related to female body condition which can be expected to affect incubation behaviour. Pairs in good body condition lay larger eggs and clutches than pairs in poor body condition. Early breeders have been shown to be better quality pairs than late breeders (Bogdanova *et al.*, 2007). I predicted that these factors would be affect incubation

behaviour. Finally I tested whether incubation behaviour during egg-laying was correlated with hatching success.

3.2 METHODS

3.2.1 Study area and field protocol

This study was carried out from late April to July in 2006 on Sanda Island (55°16′N 5°35′W), Argyll, western Scotland. The study population of herring gulls comprised 124 nests spread over 3 sub-colonies. 37 nests which I found before the first egg was laid were visited at least once a day to record laying and hatching progress. The modal clutch size of herring gulls is three eggs (Snow & Perrins 1998) and among my study nests there were 23 nests of 3-egg clutches and 14 nests of 2-egg clutches. Eggs were marked on the day of egg-laying according to the order in which they were laid with a non-toxic permanent marker pen. Length and breadth of eggs were measured to the nearest 0.1 mm using vernier calipers, and egg volume was calculated from egg breadth and length using the following formula (Harris, 1964):

Egg volume (cm^3) = 0.000476 x egg length (cm) x egg $width^2$ (cm).

From the earliest expected hatching date, 20 days after laying the first egg, nests were visited twice a day in approximately half day intervals until hatching had been recorded. During visits to nests, I successfully recorded the stage of the hatching process in a sub-sample of 20 nests (10 nests of 2-egg and 10 nests of 3-egg clutches) failure at other nests mainly being due to depredated and addled eggs. Hatching starts with small cracks, showing fractures of the shell towards the blunt pole (showing a characteristic star-shaped pattern), followed by external "pipping" when the bill breaks through the shell to create a small hole, allowing the embryo to start breathing using its lungs, and then somewhat later the embryo emerges from the eggshell (Schreibler & Burger, 2001). The time from pipping to hatching has been used to investigate hatching duration (see Chapter 5), and I defined hatching duration as the time from the start of external pipping to complete emergence of the chick from the egg.

Chicks were individually marked with a colour dot on their egg tooth through the pipping hole while still in the egg in order to identify which chick emerged from which egg. Hatching date was defined as the day when chicks emerged completely from the

eggshell. Incubation period was defined as the period from the day the first egg was laid until the day the last chick hatched.

3.2.2 Nest attendance during egg laying and early incubation

Herring gulls start incubation before completing their clutch, and nest attendance gradually increases until it reaches a steady level of nest attendance during incubation (Drent, 1970). In this study, rather than recording presence or absence of the adult on the nest, nest temperature was recorded to estimate the incubation constancy during egg laying and the early incubation period. A thermocouple was fixed inside the nest-cup below the egg on the day the first egg was laid. The temperature probe was attached via a long cable (ca. 2 m) to a data logger (Gemini Data Loggers Ltd, Chichester, UK) which was hidden near the nest. Nest temperature was recorded every 2 minutes until 4 days after the last egg was laid. Data recorded during periods when potential disturbance by observers occurred were excluded from analyses. To identify the nest attendance and recess of parents during the laying and early incubation period, I initially used three methods modified from previous studies (Flint & Grand, 1999; Manlove & Hepp, 2000).

- Method 1: the parent's departure from and return to the nest was identified by nest temperature decline and increase more than 0.1°C between two successive records (2 minutes), respectively.

- Method 2: the parent's departure from and return to the nest was identified by nest temperature decline and increase by more than 1°C between three successive records (4 minutes), respectively.

- Method 3: the parent's departure from and return to the nest was identified by nest temperature decline and increase by more than 1°C between three successive records (4 minutes), respectively, or a continuous decline and increase over more than three successive records (4 minutes), respectively.

I compared the incubation attentiveness as estimated by the nest temperature data to direct observations of parental incubation. I observed incubating birds from the top of nearby cliffs to minimise disturbance. Incubating birds were observed approximately 1 hour per nests in 6 nests and 2 hours in 3 nests (total observation time: 734 minutes). Time was recorded when parent bird started and ended incubation. I identified a total

of 18 incubation and recess events where the start and end times of the on or off bout was directly observed. The three nest temperature methods differed in how accurately they predicted the actually observed events of incubation attentiveness and recess (chi-square test: $\chi^2 = 10.87$, df = 2, p = 0.004; Table 1). Duration of nest attendance and recess events estimated from nest temperature data by method 2 and method 3 did not differ from duration estimated from direct observations (paired t-test, method 2: $t_{17} = 1.75$, p = 0.098; method 3: $t_{17} = 1.07$, p = 0.299) while method 1 gave estimates that clearly differed from the observation data ($t_{17} = 3.63$, p = 0.002). Therefore, method 3 was used for estimating the nest attendance. Errors were found in 6 events. These errors might be the effect of nest structure or ambient temperature. In short absence of parents, nest temperature did not change when ambient temperature was high. Sometimes a probe of data logger can be covered by nest materials, because parents still bring nest materials during early incubation period, it might occur errors. Daily nest attendance was estimated for 24-hour intervals starting from the time of finding the first egg.

In many previous studies, onset of incubation was assumed to occur after laying the second-laid egg in herring gulls (Drent, 1970). However, onset of incubation is not easy to characterise as it is not simply a behaviour that is switched on at a particular time, but it gradually develops over a period of time (Wiebe *et al.*, 1998). In the present study, I compared two methods to quantify the onset of incubation: mean nest attendance during laying and early incubation and the change in daily nest attendance over that period. The time was expressed relative to the day the last egg was laid (day 0) in order to be able to compare between 2- and 3-egg clutches. The change in daily nest attendance was estimated by calculating the slope of the mean nest attendance over 24 hours against time over the period from the day the first egg was laid until 4 days after laying the last egg (Figure 3.1). To obtain a linear relationship, I used a natural logarithm transformation of the nest attendance data. I predicted that a higher mean nest attendance and a greater change in daily nest attendance demonstrate an earlier onset of incubation and therefore will result in a larger hatching spread between first- and last-hatched chick. Of the 37 nests, 28 nests yielded attendance data over the entire measurement period, and an additional 6 nests yielded good data only for the first two days and could only be included in the analysis of the hatching success of the first-laid egg with respect to nest attendance between the first- and second-laid eggs. Hatching failure included addled eggs and eggs that disappeared from the nest, presumably taken by predators.

3.2.3 Female diet during egg formation

Diet during egg formation is one of the factors affecting incubation behaviour (Rauter & Rever, 1997). From each chick, one or two hatchling down feathers were collected within 24 hours after hatching as they reflect the female's diet during egg formation since the first down of chicks is synthesised from egg proteins (Klaassen *et al.*, 2004). Higher carbon stable isotope values reflect more marine food in a diet and higher values of nitrogen stable isotope reflect higher trophic level of diet (Fry *et al.*, 1983; Hobson *et al.*, 1994). All samples were washed and oven-dried. Carbon isotope ratio was measured by continuous-flow isotope ratio mass spectrometry (CF-IRMS) using a Costech Elemental Analyser (EA) linked to a Thermo Finnigan Delta Plus XP Mass Spectrometer (see method in Chapter 2).

3.2.4 Statistical analyses

Paired t-test was used for comparing hatching interval and laying interval within clutches in 18 nests where all eggs hatched successfully. The effect of clutch size, relative egg size difference, laying date and stable isotope values on nest attendance was analyzed using GLM. All biologically relevant two-way interactions between explanatory variables were included in the initial full model. Mean nest attendance was transformed using arsine transformation. For the effect of early nest attendance on hatching success of the first-laid egg I used a logistic regression analysis. From the full model I removed stepwise the least-significant term, starting with the interactions, until no more terms could be removed. Statistics are shown for the last step variables included in the model. SPSS (version 15.0 for Windows, SPSS, 2006) was used for all statistical analyses. Mean values are presented with ± 1 S.E. P-values less than 0.05 were considered statistically significant and all tests are 2-tailed. Sample sizes are presented in each model and varied between models because of hatching failure or cases with missing data for some variables.

3.3 RESULTS

3.3.1 Factors affecting incubation behaviour during laying and early incubation

I used two measures to quantify incubation behaviour during egg-laying, mean nest attendance and the daily change in nest attendance over the laying and early

incubation period, and there was a negative relationship between the two measures ($r = -0.808$, $p < 0.001$ $n = 28$). However, if clutch size was controlled, mean nest attendance and daily change in nest attendance were not related. Mean nest attendance during laying and early incubation was related to clutch size, carbon stable isotope values and an interaction between laying date and egg volume (Table 3.2a). Pairs with 3-egg clutches showed higher mean nest attendance during egg laying and early incubation than pairs with 2-egg clutches (Figure 3.2). Mean nest attendance was higher when females ate a diet with a lower carbon stable isotope value during egg formation (Figure 3.3). It meant that females consumed more terrestrial diet during egg formation. However, nitrogen stable isotope value was not related to mean nest attendance. Early in the breeding season, mean nest attendance declined with egg volume of the first-laid egg while in late breeding season, mean nest attendance increased with egg volume of the first-laid egg (Figure 3.4).

Daily changes in nest attendance were significantly related to clutch size and nitrogen isotope values (Table 3.2b). Daily change in nest attendance was greater in 2-egg clutches (0.015 ± 0.003 % per day, 11 nests) than in 3-egg clutches (0.002 ± 0.003 % per day, 17 nests, Figure 3.5) and increased with increasing nitrogen stable isotope values.

3.3.2 Incubation behaviour and hatching pattern

Hatching intervals were shorter than laying intervals (Table 3.3). In 2-egg clutches, hatching intervals between first-laid eggs and second-laid (last) eggs were 1.1 ± 0.35 days shorter than their laying intervals. In 3-egg clutches, the first- and second-laid eggs hatched almost at the same time. Hatching intervals between the second- and the last-laid egg were 0.7 ± 0.37 days shorter than their laying intervals although the difference between the second- and the last-laid egg was not statistically significant. Hatching interval between the two last-laid eggs was similar between 2-egg and 3-egg clutches ($t_{16} = 0.09$, $p = 0.927$).

Hatching intervals between first- and last-laid eggs increased with increasing laying intervals between these eggs. With increasing mean nest attendance during laying and early incubation hatching intervals declined (Table 3.4, model 1) after controlling for the effect of laying interval between first- and last-laid eggs. In addition, the longer it took the last-laid egg to hatch (interval from initiation of pipping until the chick hatched) the larger the hatching intervals when controlling for mean nest attendance during laying and early incubation (Table 3.4, model 1). Clutch size, laying date and

egg volume of last-laid eggs did not affect hatching interval between first- and last-laid eggs (Table 3.4). Daily changes in nest attendance during laying and early incubation, however, were not related to hatching intervals (Table 3.4, model 2). Hatching duration was not correlated to mean nest attendance ($r = 0.05$, $p = 0.877$, $n = 14$) and embryonic age at pipping ($r = 0.22$, $p = 0.446$, $n = 14$). Eggs incubated by pairs with a higher mean nest attendance did not pip earlier ($r = 0.35$, $p = 0.225$, $n = 14$).

Incubation period from the day the first egg was laid until all chicks in a clutch hatched increased with mean nest attendance in 2-egg clutches while it was not related to mean nest attendance in 3-egg clutches (model 1 in Table 3.5, Figure 3.6). Incubation period was not related to daily changes in nest attendance (model 2 in Table 3.5).

3.3.3 Hatching success and nest attendance

To investigate the effect of early nest attendance on hatching success of the first-laid egg, mean nest attendance from laying the first egg to laying the second egg has been used. Mean nest attendance before laying the second-laid egg was related to hatching success of the first-laid egg (Table 3.6), but this depended on the volume of the first-laid egg. There was an interaction between these two variables. To investigate these factors, data were divided in small and large first-laid eggs (Figure 3.6). In small first-laid eggs, hatching success was 14 % higher when pairs incubated their first-laid egg with higher nest attendance than when pairs spent less time incubating their eggs before laying the second-laid egg although this difference was not statistically significant (Fisher's exact test: $p = 0.342$). In contrast, hatching success of large first-laid eggs did not differ between nests with high or low mean nest attendance before laying the second egg. Of the first-laid eggs, 3 eggs were depredated and 6 eggs were addled. Among last-laid eggs, 7 eggs failed to hatch, with 1 egg depredated and 6 eggs being addled.

Table 3.1 Comparisons between 3 methods to estimate nest attendance from nest temperature data and direct behavioural observations of nest attendance (see text for detail). '*Correct*' means that an observed event (bird left the nest or bird returned to an unattended nest) was correctly predicted by the nest temperature data and '*Error*' means that the observed event was not predicted by the nest temperature data. Figures given in the table are the number of correctly and erroneously predicted events.

	Method 1	Method 2	Method 3
Correct	4	13	12
Error	14	5	6

Table 3.2 Mean nest attendance (a) and daily change in nest attendance (b) during laying and early incubation in relation to clutch size, laying date, egg volume of the first-laid egg, carbon and nitrogen stable isotope values (ANCOVA, n = 25 nests, 3 nests had no data on stable isotopes). The other interactions were not significant: carbon isotope * laying date, nitrogen isotope * laying date, carbon isotope * egg volume, nitrogen isotope * egg volume, clutch size * carbon isotope, clutch size * nitrogen isotope, clutch size * laying date, clutch size * egg volume. F and P-values refer to ANCOVA with the clutches. Parameter estimates (B ± 1 S.E.) are shown for significant results.

	F	*P*	*B ± 1 S.E.*
(a) Mean nest attendance			
Constant			6.323 ± 2.084
Clutch size	$F_{1,19}$ = 12.36	0.002	2-egg clutches: - 0.108 ± 0.031
			3-egg clutches: 0
Laying date of first-laid egg	$F_{1,19}$ = 7.80	0.012	- 0.150 ± 0.054
Carbon stable isotope value	$F_{1,19}$ = 6.04	0.024	- 0.031 ± 0.013
Egg volume of the first-laid egg	$F_{1,19}$ = 7.98	0.011	- 0.073 ± 0.026
Laying date * volume of first-laid egg	$F_{1,19}$ = 7.79	0.012	0.002 ± 0.001
Nitrogen stable isotope value	$F_{1,18}$ = 0.02	0.900	-
(b) Daily change in nest attendance			
Constant			-1.01 ± 0.05
Clutch size	$F_{1,22}$ = 10.00	0.005	2-egg clutches: 0.019 ± 0.006
			3-egg clutches: 0
Nitrogen stable isotope value	$F_{1,22}$ = 4.29	0.050	0.009 ± 0.004
Laying date of first-laid egg	$F_{1,21}$ = 2.32	0.143	
Egg volume of the first-laid egg	$F_{1,20}$ = 1.37	0.255	
Carbon stable isotope value	$F_{1,19}$ = 0.80	0.383	

Table 3.3 Laying intervals and hatching intervals in 2-egg clutches (n = 9) and 3-egg clutches (n = 9) where all chicks hatched. t and P-values refer to paired t-test (A: the first-laid egg; B: the last-laid egg in 2-egg clutch, the second-laid egg in 3-egg clutch; C: the last-laid egg).

	2-egg clutch (mean ± 1 S.E.)				3-egg clutch (mean ± 1 S.E.)			
	Laying interval	Hatching interval	t	p	Laying interval	Hatching interval	t	p
A-B	2.5 ± 0.16	1.4 ± 0.36	$t_8 = 3.16$	0.013	2.1 ± 0.11	- 0.1 ± 0.25	$t_8 = 13.15$	< 0.001
B-C		-			2.1 ± 0.15	1.3 ± 0.47	$t_8 = 1.93$	0.086

Table 3.4 Hatching interval between first- and last-hatched egg in relation to the mean nest attendance (model 1) or daily changes in nest attendance during laying and early incubation (model 2) and egg volume of the first-laid egg, clutch size, laying date of the first-laid eggs, hatching duration and laying interval between penultimate and last-laid egg (n = 14 nests where all eggs hatched and information on hatching duration was available). The other interactions were not significant: clutch size* laying date, laying date * egg volume, laying date * mean nest attendance, clutch size * egg volume, clutch size * mean nest attendance, daily change in nest attendance * clutch size, laying date * daily change in nest attendance F and P-values refer to ANCOVA. Parameter estimates are shown in the case of significant results.

	Model 1			Model 2	
	F	p	B ± 1S.E.	F	p
Constant			11.55 ± 3.84		
Mean nest attendance during laying and early incubation	$F_{1,10}$ = 12.74	0.005	-12.66 ± 3.55	-	
Daily changes in nest attendance		-		$F_{1,10}$ = 1.53	0.245
Laying interval between first and last-laid egg	$F_{1,10}$ = 12.89	0.005	0.83 ± 0.23	$F_{1,12}$ = 2.32	0.153
Hatching duration of the last-laid egg	$F_{1,10}$ = 6.86	0.026	1.35 ± 0.52	$F_{1,11}$ = 2.22	0.165
Laying date of the first-laid egg	$F_{1,9}$ = 2.38	0.158		$F_{1,7}$ = 0.08	0.786
Egg volume of the first-laid egg	$F_{1,8}$ = 0.28	0.608		$F_{1,8}$ = 0.08	0.782
Clutch size	$F_{1,7}$ = 0.09	0.769		$F_{1,9}$ = 0.50	0.496

Table 3.5 Incubation period (first laying until last hatching) in relation to mean nest attendance (model 1), daily changes in nest attendance (model 2), clutch size (fixed effect), egg volume of the first-laid egg, interval between laying the first and last egg and laying date of the first-laid egg (all covariates) (n = 15 nests where all chicks hatched). The other interactions were not significant: clutch size * laying date, mean nest attendance * laying date, daily change in nest attendance * laying date, laying date * egg volume, clutch size * mean nest attendance (for model 2). F and P-values refer to ANCOVA with the clutches. Parameter estimates are shown when results were significant (2-egg clutches shown relative to 3-egg clutches).

	Model 1			Model 2		
	F	p	B ± 1S.E.	F	p	B ± 1S.E.
Constant			40.75 ± 5.56			28.29 ± 0.86
Mean nest attendance	$F_{1,11}$ = 1.25	0.288	-	-		-
Daily change in nest attendance	-		-	$F_{1,12}$ = 1.48	0.247	-
Clutch size	$F_{1,11}$ = 11.88	0.005	2-eggs: -27.67 ± 8.02	$F_{1,10}$ = 0.20	0.665	-
Laying interval between first and last egg	$F_{1,10}$ = 0.10	0.756		$F_{1,13}$ = 11.49	0.005	0.84 ± 0.25
Egg volume of the first-laid egg	$F_{1,9}$ = 0.34	0.572	-	$F_{1,9}$ = 0.12	0.732	-
Laying date of the first-laid egg	$F_{1,8}$ = 0.05	0.827	-	$F_{1,11}$ = 1.82	0.205	-
Clutch size * mean nest attendance	$F_{1,11}$ = 10.68	0.007	2-eggs: 23.44 ± 7.17	-		-

Table 3.6 Logistic regression analysis of hatching success of the first-laid eggs in relation to mean nest attendance before laying the second-laid egg, egg volume of the first-laid egg and laying date of the first-laid egg (n = 34 nests; 14 nests of 2-egg clutches and 20 nests of 3-egg clutches).

	x^2_1	*p*
Egg volume of first-laid eggs	6.83	0.009
Nest attendance before laying the second egg	6.28	0.012
Nest attendance before laying the second egg * egg volume of the first-laid eggs	6.14	0.013
Laying date of the first-laid eggs	0.08	0.781
Clutch size	2.01	0.156

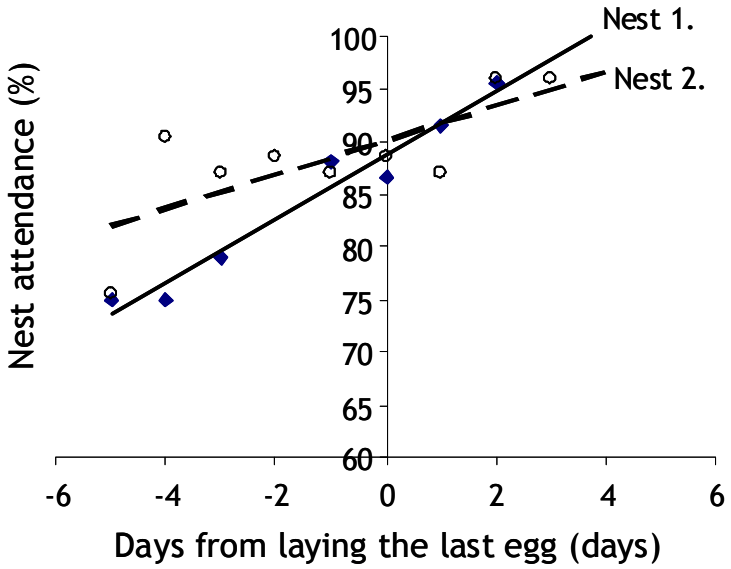

Figure 3.1 The example of calculation of the slope of daily nest attendnace (%) in nest 1 (closed squares with a solid line) and nest 2 (open circles with a broen line). Day 0 presents the day of laying the last egg.

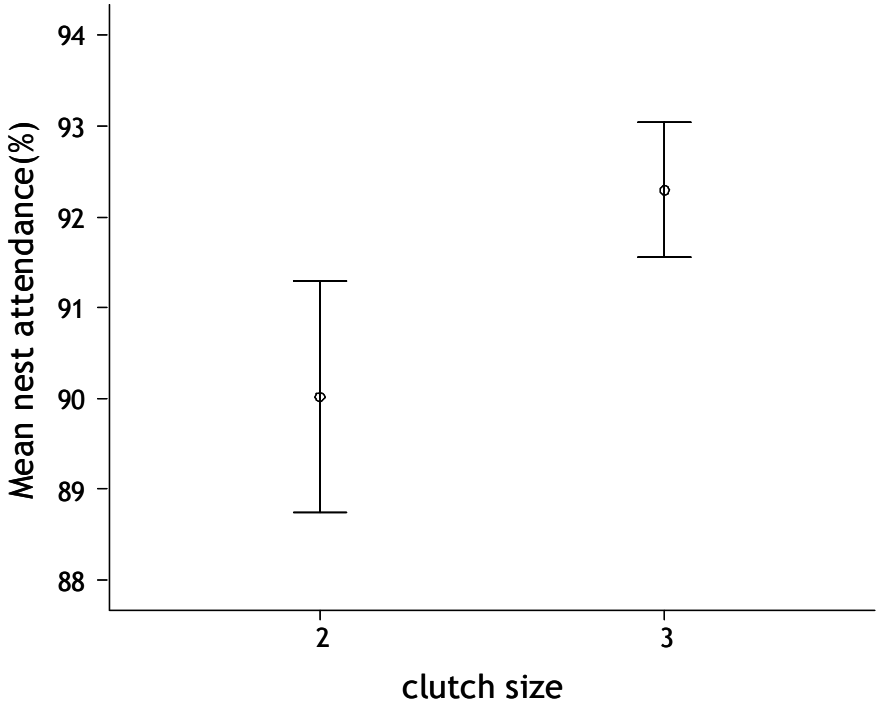

Figure 3.2 Mean (± 1 S.E.) nest attendance for 2-egg (n = 11 nests) and 3-egg clutches (n = 17 nests). Whiskers indicate standard error.

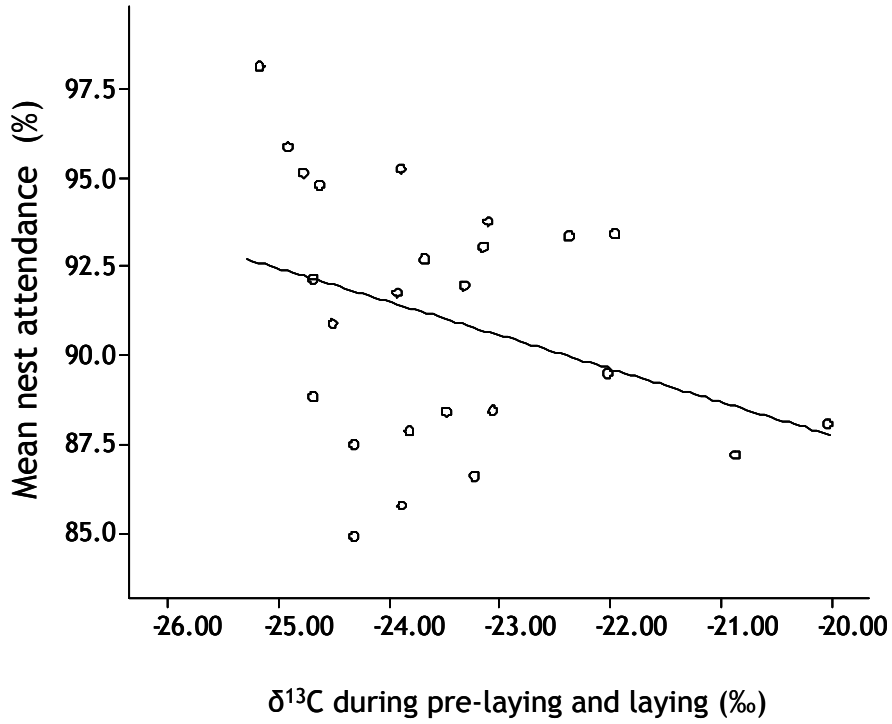

Figure 3.3 The relationship between the carbon isotope value of the diet during egg formation and mean nest attendance during laying and early incubation in 10 nests of 2-egg clutches and 15 nests of 3-egg clutches (3 of 28 nests were excluded due to no data of carbon stable isotope).

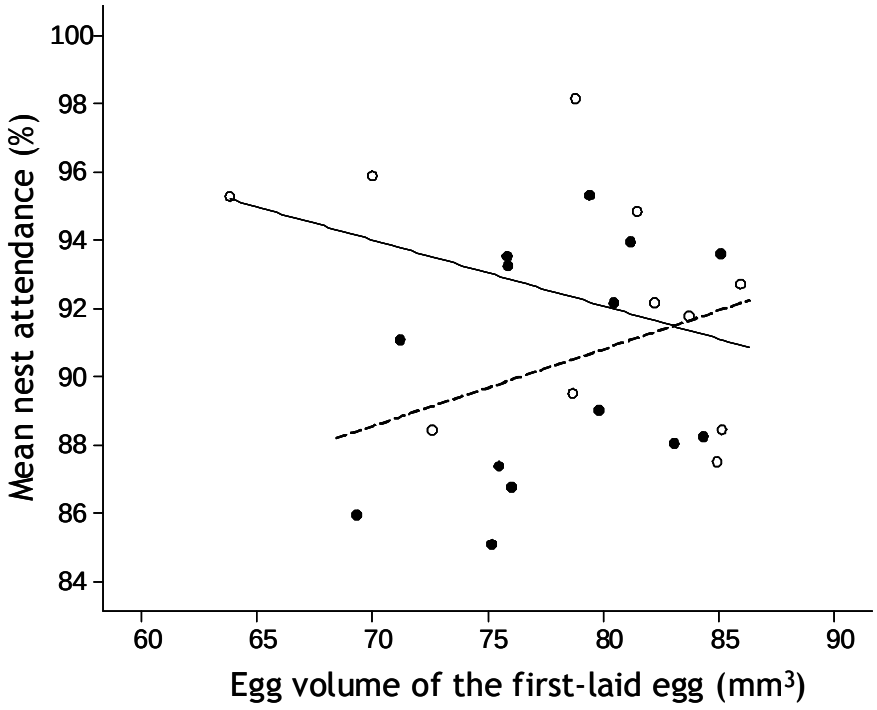

Figure 3.4 Mean nest attendance responded to the volume of the first-laid egg in early ($r = -0.39$, $p = 0.231$, $n = 11$ nests) and late ($r = 0.32$, $n = 14$ nests, $p = 0.253$) breeding season. 3 nests of 28 nests which were measured nest attendance were excluded in these figures due to no data of stable isotopes. Early (open circle with a solid line) and late (closed circle with a broken line) breeding season was categorized by mean laying date. Early breeding season is earlier than mean laying date and late breeding season is later than mean laying date (Day 39.7, Day 1 = 1st April).

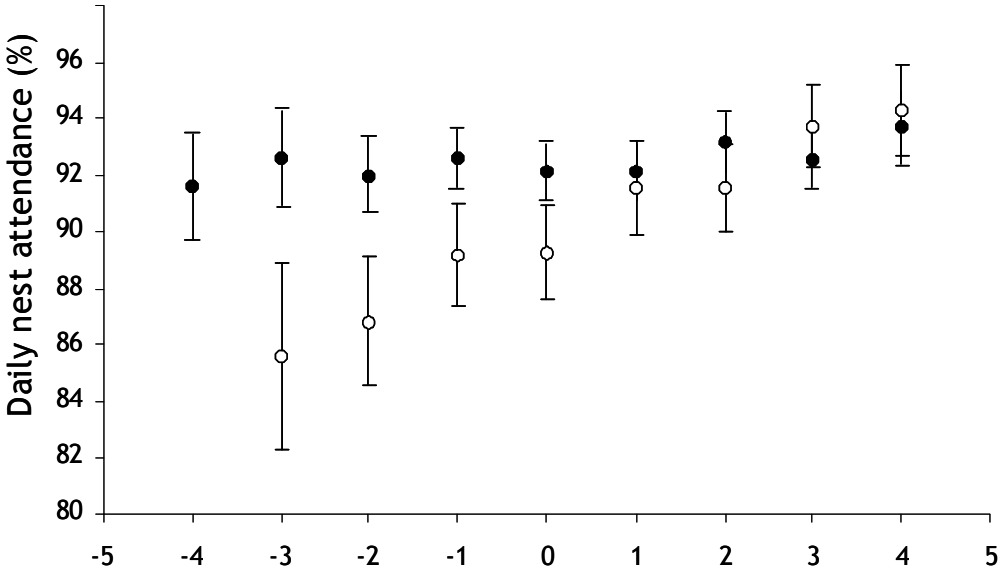

Figure 3.5 Daily nest attendance (mean ± 1 S.E. %) over 24 hrs during egg laying and early incubation period in 2-egg clutches (open symbols, n = 11 nests) and 3-egg clutches (closed symbols, n = 17 nests).

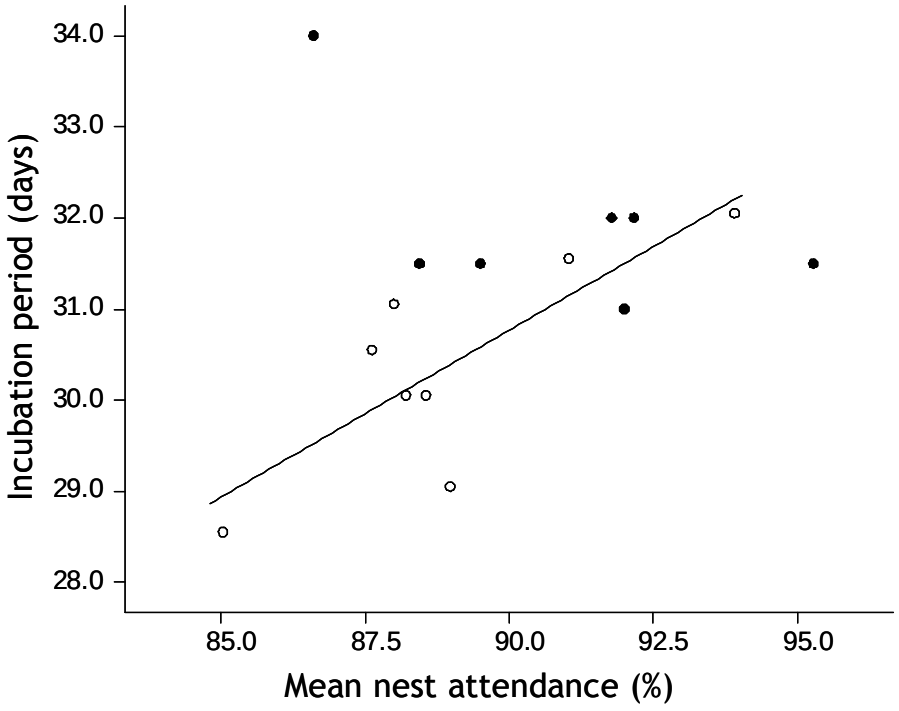

Figure 3.6 The relationship between incubation period and mean nest attendance in 2-egg clutches (closed circle with line, r = 0.79, n = 8, p = 0.018) and in 3-egg clutches (open circle, r = - 0.59, n = 7, p = 0.158).

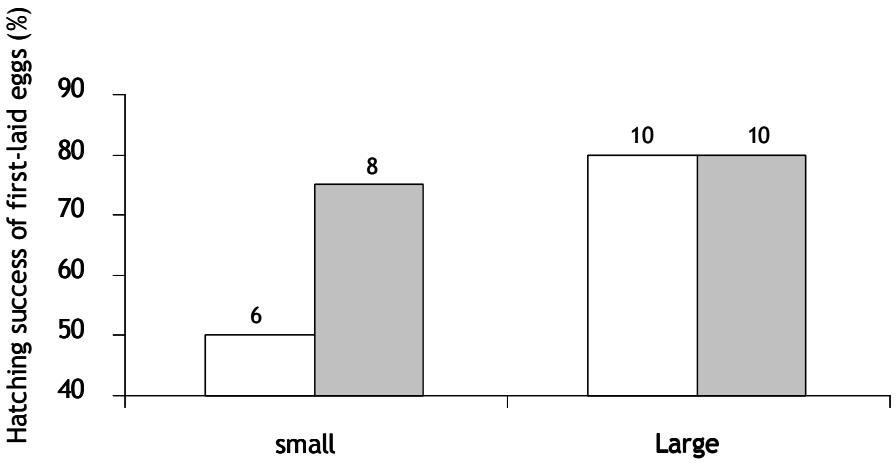

Figure 3.7 Hatching success of first-laid eggs with respect to mean daily nest attendance before laying the second egg and size of the first-laid egg. Small eggs were smaller than the mean egg size (79.04 mm^3) whereas large eggs were larger than the mean egg size. White bars are for nests with low mean nest attendance before laying the second egg below the mean attendance of 87.68% (small), 90.56 % (large) and grey bars are nests with above mean nest attendance. Sample size is presented above the bar.

3.4 DISCUSSION

Incubation behaviour during egg-laying was related to hatching patterns. Higher mean, but not daily changes in nest attendance during egg-laying and early incubation, was correlated with more synchronous hatching, while pairs with lower mean nest attendance had a greater hatching span.

Nest attendance estimated from direct observation of incubation or from temperature of eggs or nests has been commonly used to investigate the relationship between onset of incubation and hatching patterns (Flint & Grand, 1999; Manlove & Hepp, 2000; Hepp, 2004; Ardia *et al*., 2006). As the embryo can develop above the physiological zero (about 27-28 ºC) (Webb, 1987), lower nest attendance might little affect embryonic development in a warm climate. In addition, I also looked at daily change in nest attendance. Wiebe *et al*. (1998) found variation of incubation patterns. In Eurasian kestrels (*Falco tinnunculus*), some females rapidly increased nest attendance while others showed steady or irregular patterns during the egg-laying period, and their incubation patterns affected hatching patterns. In this study, mean nest attendance was negatively related to daily change of nest attendance. However, this relationship may be a result of clutch size, because nest attendance in 2-egg more rapidly changed than in 3-egg clutches. Parent birds having higher mean nest attendance during the laying and early incubation period had a smaller increase in nest attendance over that time, presumably because they already spent more time on the nest from the first-laid egg onwards. After controlling for clutch size, mean nest attendance was not related to daily change in nest attendance.

The timing of incubation onset has been suggested as the primary factor controlling hatching asynchrony (Clark & Wilson, 1981; Mead & Morton, 1985; Magrath, 1990; Wiebe *et al*., 1998). When birds started incubation earlier relative to clutch completion, they had a greater hatching spread, presumably because the earlier laid eggs started embryonic development earlier than later-laid eggs (Parsons, 1972). In European kestrels (*Falco tinnunculus*), onset of incubation determined hatching pattern (Wiebe *et al*., 1998). However, there are also some studies which found no relationship between onset of incubation and hatching patterns. In American kestrels (*Falco sparverius*), hatching day of chicks did not match to the expected day which was estimated from onset of incubation (Bortolotti & Wiebe, 1993). Bortolotti and Wiebe (1993) explained this irregular hatching as an effect of female

body size. Hatching patterns are related to female body size as this can affect the ability to cover all the eggs in a clutch. Higher yolk androgen content can accelerate hatching through developing hatching muscle (Lipar & Ketterson, 2000; Lipar, 2001; Eising *et al.*, 2003). However, the present study showed higher nest attendance during egg-laying and early incubation period induced more synchronous brood. Hence, incubation during egg-laying might not affect embryonic development or other factors might affect embryonic development as well. Embryo developmental rate also affected hatching patterns. Hatching duration of the last-laid egg increased with increasing hatching spans between the penultimate egg and the last-laid egg. It has been shown that last-laid eggs accelerate their development to catch up compared to earlier-laid eggs (Muck & Nager, 2006). Hatching pattern was also related to laying interval. When females laid eggs over a longer period, eggs hatched more asynchronously. Females may therefore affect hatching patterns not only by altering incubation behaviour, but also by adjusting their laying intervals and through differential provisioning of eggs influencing their hatching duration.

Incubation period was related to incubation behaviour only in 2-egg clutches, where pairs with higher mean nest attendance had longer incubation periods. This may have resulted from low nest attendance during egg-laying and early incubation in 2-egg clutches. Higher nest attendance shortened the incubation period (Hanssen *et al.*, 2002). Longer incubation periods may be costly to the parents. For example, extended incubation period may increase the predation risk of eggs as well as of parents (Martin,1992). Incubating parents also have limited opportunities for foraging, other self-maintenance activities and alternative reproductive attempts (e.g. Slagsvold & Lifjeld, 1989). Prolonged incubation also increases embryo energy consumption due to slower embryonic development (Vleck & Vleck, 1987). In 3-egg clutches, incubation period was not related to mean nest attendance, maybe because nest attendance in 3-egg clutches was higher during egg-laying than in 2-egg clutches. Possibly the increase in incubation temperature may also differ between 2-egg and 3-egg clutches, and hence embryo development rate. As higher incubation temperature induces faster embryonic development (Hepp *et al.*, 2006), pairs with 3-egg clutches might create higher incubation temperatures than pairs with 2-egg clutches. However, in my study I can not compare incubation temperature because I measured nest temperature, which is not exactly the same as egg temperature.

Incubation behaviour was affected by several factors. Higher mean nest attendance during laying and early incubation in 3-egg clutches was due to higher nest attendance after laying the first egg than seen in 2-egg clutches. Pairs with 3-egg clutches showed high nest attendance from the first-laid egg onwards and daily nest attendance did not change over the period until 4 days after clutch completion, while in 2-egg clutches nest attendance started with relatively lower nest attendance on the day the first egg was laid and increased during laying and early incubation to a similar levels as in 3-egg clutches. Higher nest attendance of 3-egg clutches during laying may be to protect the first-laid eggs from a potentially longer exposure period until clutch completion compared with 2-egg clutches because un-incubated eggs can have a higher risk of predation (Clark & Wilson, 1981; Beissinger et al., 1998; Persson & Goransson, 1999) or microbial infection (Cook *et al.*, 2003) than incubated eggs.

Parental diet during egg formation was related to nest attendance. The stable isotope values from hatchling down reflect the female's diet during egg formation (Klaassen *et al.*, 2004). Higher carbon stable isotope values indicate a more marine food resources (Michener & Lajtha, 2007), and parents that fed more on a marine diet showed lower mean nest attendance during laying and early incubation compared to parents feeding more on a terrestrial diet. Longer foraging time to obtain marine diet may reduce the time available to stay on the nest during laying and early incubation compared to parents that foraged more on terrestrial food sources. Here I measured only the diet of the female whereas the nest attendance of both females and males was combined. However, in herring gulls, nest attendance of females and males did not differ (Schreiber & Burger, 2001) and males provide courtship feeding to females (Niebuhr, 1981) so that female stable isotope ratios should reflect a combination of their foraging preferences and those of their partner. Nitrogen stable isotopes indicated that pairs which rapidly increased their nest attendance foraged at a higher trophic level than pairs that increased their nest attendance more slowly. In the study area, herring gulls foraged on a mixture of marine and terrestrial food resources from grain to mammal and avian prey (Chapter 2). Lower trophic level diet may be due to the proportion of grain in the diet. Hence, low value of nitrogen stable isotope may indicate that parents foraged more in terrestrial while high value of nitrogen stable isotope may present likely marine food. Hence, pairs which foraged more on terrestrial food showed lower change in nest attendance.

Because there was an interaction between laying date and egg volume of the first-laid egg on mean nest attendance, I analysed early and late breeding pairs separately. Early in the breeding season, mean nest attendance during egg-laying and early incubation was high and declined with increasing egg volume of the first-laid egg although the difference was not statistically significant. High mean nest attendance of early breeders may be related to the quality of parents. Bogdanova *et al*. (2007) showed that mature pairs of herring gulls had higher breeding success and started laying earlier in the breeding season than young pairs. Late in the breeding season, mean nest attendance increased with increasing egg volume of the first-laid egg and only pairs laying the largest eggs maintained a mean nest attendance similar to that of early breeders. Young or poor quality pairs late in the breeding season that laid small eggs may not have been able to afford the costs of higher nest attendance. Alternatively, birds that lay larger eggs may have to maintain a higher nest attendance to avoid the costly re-warming of their eggs, which is more expensive than steady-state incubation (Deeming, 2002a). The rate at which an egg gains heat during incubation correlates positively with its size and so larger eggs take longer to reheat compared with smaller eggs (Tazawa & Whittow, 1994). Hence, if early breeders breed at lower ambient temperatures and lay larger eggs, they may need to keep high nest attendance to minimise their incubation costs.

High nest attendance during laying may be related to egg viability, in particular of the first-laid egg. Egg viability can decline with increasing exposure time when the egg is not incubated (Veiga, 1992; Arnold et al., 1987). This exposure time is usually longest for the first-laid egg and before incubation starts. There may be a number of reasons why egg viability may decline with increasing exposure time. Egg viability may decline when eggs are exposed to cold temperature (Ardia *et al*., 2006). Recently, Cook *et al*. (2003, 2005b) showed that un-incubated egg had a higher possibility to be infected by microbes. They suggested that incubation during laying may be required to prevent infection from bacteria and fungi. Eggshells have many pores on their surface to exchanging gas and water during embryonic development, but theses pores can also be passages for microbes into the egg. Increased egg temperature encourages activation of antimicrobial enzymes in the albumen (Board & Ayres, 1965) and incubating birds transfer antibiotic agents from their epidermal layer and preening gland onto the eggshell during incubation (Menon & Menon, 2000; Shawkey *et al*., 2005). Incubation behaviour may also prevent the build-up of high humidity (which would favour microbial growth in the nest), for example by keeping rainfall out of the nest (Cook *et al*., 2005a). Alternatively, incubation may protect

the egg from predation. Predation risk is usually highest for the first-laid egg (Dunlop, 1910; Drent, 1970). In this study, I cannot distinguish between the different causes of egg mortality. Among failed first-laid eggs there were both depredated and un-hatched eggs. However, the proportion of addled eggs was not different between the first-laid egg and the last-laid egg in the present study. I found a higher predation rate in first-laid eggs compared with last-laid eggs. Hence, predation risk may force the birds to incubate their eggs during laying. Hatching success of first-laid eggs depended on an interaction between nest attendance before laying the second egg and egg volume of the first-laid egg. Smaller first-laid eggs had lower hatching success than larger first-laid eggs when nest attendance was low although the difference was not statistically significant. This non-significant result may be due to small sample size. Brouwer and Spaans (1994) suggested that poor quality pairs laid not only smaller eggs but also guarded their eggs less during incubation and this resulted in a higher risk of egg predation. In this study, hatching success of larger first-laid eggs did not differ between nests with higher nest attendance and lower nest attendance before laying the second egg. As the rate of heat loss of larger eggs is lower than that of smaller eggs (Ar & Sidis, 2002; Deeming, 2002a), this may help to keep larger first-laid eggs warmer when parents were not in their nests compared to smaller eggs.

Few studies have shown the relationship between incubation behaviour during laying and hatching patterns (Magrath, 1990). Daily change in nest attendance did not explain hatching spread and incubation period. Hence, mean nest attendance may be the better method to investigate the effect of incubation behaviour on hatching patterns. In the future, it would be good to assess the effect of daily change in nest attendance on embryonic development or parent body condition to understand why some pairs increase nest attendance more rapidly than others. It would also be good to look at the relationship between embryo temperature and nest attendance.

Overall, this study suggests the hatching pattern is not only influenced by the incubation behaviour, but also the length of the laying period and the hatching duration of the last-laid egg, all of which can be controlled to some extent by the parents. Early onset of incubation increased hatching success of first-laid eggs and induced synchronous brood. Shorter hatching spread may provide the last-laid egg higher survival (Sydeman & Emslie, 1992). However, it might be disadvantageous for parents due to higher levels of competition among chicks (Hahn, 1981). Experimentally synchronous hatched chicks often have lower fledging mass which

can affect survival rate of chicks after fledging (Sydeman & Emslie, 1992). Hussell (1972) suggested that early onset of incubation may encourage early chick hatching in order to avoid reduced food availability late in the season (hurry-up hypothesis). Hence, chicks may have benefit from a reduced incubation period. However, early onset of incubation did not affect incubation period (3-egg clutches), or even increased incubation period (2-egg clutches) in this study. Hence, my data suggest that onset of incubation may more likely to be selected for increasing egg viability of first egg and the survival of last-laid egg.

Chapter IV.

The effect of incubation behaviour and laying interval on within-clutch variation in eggshell colour of herring gulls *Larus argentatus*

4.1 INTRODUCTION

Birds lay eggs that considerably vary in colour and pattern both within and between species. Previous studies have explained eggshell colour variation as an adaptation to increase breeding success. Eggshell colour has been considered to be the result of selection for camouflage to avoid predation (Tinbergen *et al.*, 1962; Leksrisompong *et al.*, 2007; Martin *et al.*, 2007; Westmoreland & Kiltie, 2007) or for avoiding a risk of overheating when eggs of ground nesters are exposed to solar radiation for a long time without incubation (Bakken *et al.* 1978) During incubation, eggshell pigment, protoporphyrin and biliverdin reflects long wavelength of light and plays a role of solar filtering. Eggshell pigments may also be involved in determining the strength of eggshells (Solomon, 1987; Gosler *et al.*, 2000). Recently, the signal hypothesis suggested that eggshell colour may be deposited to withdraw the parental investment from males (Sanpera *et al.*, 2007). Eggshell pigment, biliverdin (the blue-green pigment) is an antioxidant which may reflect antioxidant capacity and porphyrin (brown) is a pro-oxidant which may signal tolerance of oxidative stress. These pigments may be able to indicate the tolerance of oxidative stress in eggs and female's body condition which can affect parental effort. In pied flycatchers (*Ficedula hypoleuca*), deposition of eggshell pigments increased with female immunocompetence (Sanpera *et al.*, 2007) and males with more pigmented eggs invested more chicks than males with paler eggs although experiment is needed to confirm this (Sanpera *et al.*, 2007).

Despite intensive previous studies on egg colouration and patterns between clutches and species, eggshell colour variation within a clutch has received relatively little attention and may require different explanations. Eggshell colour generally changes in the laying sequence (Krist & Grim, 2007; Sanpera *et al.*, 2007) and the last-laid eggs are distinctively paler than the other eggs in the clutch (Kendeigh et al., 1956; Holyoak, 1970; Lowther, 1988; Kilpi & Ost, 1998). The odd-coloured last-laid egg

may actually decrease the camouflage of the clutch and clutches with larger colour variation might be more easily spotted by predators. Hence, within-clutch variation in eggshell colour may be non-adpative and reflect physiological constraints in females during egg laying, Three non-mutually hypotheses have been proposed. First, Holyoak (1970) suggested that the less pigmented last-laid eggs are the result of limited availability of pigments. Accordingly, eggshell colour is determined by the deposition of three pigments, porphyrins generating red and brown eggshell colour, biliverdin, and the zinc chelate of biliverdin which are responsible for blue and green colours and eggshell pigments are deposited from uterus cell glands into the eggshell before oviposition (Kennedy & Vevers, 1976). Females may deposit less pigments in later-laid eggs because they only produce a limited amount of pigments (Holyoak, 1970). An egg removal experiment in house wrens (*Troglodytes aedon*) supported this hypothesis. When females were experimentally induced to lay more eggs than they would usually, the colour of these additional eggs become continuously paler (Kendeigh *et al*., 1956). Secondly, paler last-laid eggs also have been suggested to be the result of a shorter passage time through the shell glad than the earlier-laid eggs of the clutches (Baerends *et al*., 1970). In species with asynchronous hatching survival of last-laid eggs is related to the hatching span (Sydeman & Emslie, 1992) and longer exposure time of eggs without incubation during the laying period could reduce hatchability of earlier-laid eggs (Cook *et al*., 2003). Therefore, females may lay the last eggs in relatively shorter time than earlier-laid eggs and this short laying interval of the last-laid egg may induce paler last-laid eggs.

Finally, Lowther (1988) proposed that hormonal changes in the female after clutch completion may influence the deposition of pigments into the eggshell. Deposition of porphyrin, responsible for the brown eggshell colouration, is related to steroid hormone such as progesterone. In Japanese quail (*Coturnix japonica*), females which were injected with progesterone during egg formation laid more pigmented eggs (Soh & Koga, 1994). Progesterone declines during laying while prolactin which initiates incubation behaviours, increases during laying and early incubation (Vleck, 2002). However, the effect of incubation behaviour on within-clutch variation of eggshell colour has not been studied yet.

In this study, I tested the hypothesis to explain within-clutch variation in eggshell colour that this variation is correlated with laying and incubation behaviour in herring gulls (*Larus argentatus*). Herring gulls lay brownish green eggs with spots or

streaks and their eggs contain all three pigments such as propophyrin, biliverdin and the zinc chelate of biliverdin (Kennedy & Vevers, 1976). The modal clutch size is three (Snow & Perrins, 1998) and chicks hatch asynchronously because parents start incubation before completing their clutches (Parsons, 1972). Within-clutch variation in eggshell colour has been observed in herring gulls (Kilpi & Ost, 1998). Eggshell pigments are deposited from glands just before oviposition. Because pigments are deposited on the eggshell just before oviposition, if eggs stay longer in uterus, they may obtain more pigments. I have not measured the passage time of eggs through the uterus, but it is reasonable to assume that if last-laid eggs show an accelerated passage time the laying interval would become shorter. I therefore predict that clutches with longer laying intervals between penultimate and last-laid eggs show similar eggshell colour between penultimate and the last-laid eggs. Egg size might relate to eggshell colour because females in good quality usually lay larger eggs (i.g. Hanssen *et al.*, 2002) and often have more pigmented eggs than females in poor quality (Sanpera *et al.*, 2007). Hence, I expected that larger eggs may have more pigments. I also predict when parents start incubation earlier relative to clutch completion they have a greater variation in eggshell colour within a clutch than in clutches where parents start to incubate relatively late.

4.2 METHODS

4.2.1 Study area and field protocol

This study was conducted from April to July in 2006 on Sanda Island (55°16′N 5°35′W), Scotland, UK. During the egg laying period (26 April ~ 2 July), I visited daily 49 nests and recorded laying order. Egg size was estimated by measuring the maximum length and width of each egg and egg volume was calculated from the formula (Harris, 1964):

Volume = 0.000476 × Egg length × Egg width^2.

From 20 days after laying the first egg onward, nests were monitored *ca.* every 12 hours to identify the exact hatching date. To identify what chick hatched from what egg, chicks were marked with a colour dot of non-toxic paint on their egg tooth when chicks made a pipping hole into the eggshell. Hatching date was defined as the day when a chick completely emerged from the eggshell. Daily nest attendance was measured during the egg laying and early incubation period (from the day the first egg was laid until 4 days after the last egg was laid) in 26 nests (10 nests of 2-

egg clutches and 16 nests of 3-egg clutches). Daily nest attendance (%) was defined as the proportion of time a nest was attended by the parents and it was estimated using temperature data logger (Gemini Data Loggers Ltd, Chichester, UK). Onset of incubation was compared between nests using the slope of nest attendance (see method section of Chapter 3 for details).

4.2.2 Digital image analysis

Eggshell colour was measured from RGB (red, green and blue) values of digital photographs of the eggs. 129 eggs in 49 nests (16 nests of 2-egg clutches and 33 nests of 3-egg clutches; One first-laid egg and one second-laid egg of 3-egg clutches was predated before photos were taken) were photographed during incubation on average 13.8 ± 1.0 days (n = 48) after clutch completion using a digital camera (Sony Cyber-shot DSC-f707) at 2560 x 1920 pixels. Before photographing, each egg was gently cleaned with a wet tissue to remove dust and placed on white paper and alongside each egg I placed three reference colour chips (orange, green and blue). To control the light, all digital images were taken inside a grey wooden box (30cm x 30cm x 30 cm) with a camera lens-sized hole and only standardized flashlight was used. Focal ratio (F-number) of digital images was fixed at 3.2 and digital image were saved in JEPG format.

I chose three areas excluding spots or streaks at approximately the same places on each egg and measured RGB colour values using Adobe Photoshop software (7.0) (Villafuerte & Negro, 1998). Repeatability (R) of measurements was calculated according to the method of Lessells & Boag (1987). R was 0.71 for red colour ($F_{48,338}$ = 8.54, p < 0.001), 0.60 for green colour ($F_{48,338}$ = 5.54, p < 0.001) and 0.74 for blue colour ($F_{48,338}$ = 9.51, p < 0.001). Although I took a digital image under as standardized light conditions as possible, there was still some variation in RGB colour values of reference colour chips between images. This may result from changes in light sources. To remove this variation, each image was corrected using the measured red, green and blue values of reference colour chips. I compared the red, green and blue (RGB hereafter) of colour chips in each picture with the mean RGB colours of all reference colour chips and used this to correct the egg RGB colour scores. From the corrected RGB scores a single colour variable has been derived by entering RGB colour values into a principal components analysis (PCA) (Figure 4.1). Only one component was extracted and RGB colour scores represented 74.7% of variance.

4.2.3 Statistical analysis

Mean daily nest attendance data were transformed using square root arsine transformation to meet the assumption of parametric analyses. Repeated measures ANOVAs were used to analyse differences in eggshell colour with respect to laying order. Analysis of covariance (ANCOVA) was used for determining whether within-clutch variation in eggshell colour was related to incubation behaviour during laying and early incubation. All possible 2-way interactions between explanatory variables were included in the initial full model and only significant results are presented in the final model. To estimate onset time of incubation, I used mean nest attendance and daily change of the slope of daily nest attendance against time between the first eggs were laid until 4 days after the clutch completion in 26 nests (see the method section of Chapter 3 for the detail). 2 of 28 nests which had data on incubation behaviour were excluded because of missing data on eggshell colour. The absolute difference of eggshell background colour between the first-laid egg and the last-laid egg (within-clutch eggshell colour contrast hereafter) was used to investigate the effect of incubation behaviour on eggshell background colour. To test the passage time hypothesis, I estimated eggshell colour difference between penultimate and last-laid egg and looked at the relationship between eggshell colour difference and laying interval between penultimate and last-laid egg. SPSS (SPSS, 2006) was used. Two-tailed P-values less than 0.05 were considered statistically significant. Mean values are presented with ± 1 standard error (S.E.) and sample size are presented for each analysis. Parameter estimates (B ± 1 S.E.) were presented only in the case of significance.

4.3 RESULTS

4.3.1 Eggshell colour variation

Eggshell colour of first-laid egg did not differ between 2-egg clutches and 3-egg clutches (t_{46} = 0.22, p = 0.825). However, eggshell background colour in 3-egg clutches was paler in the last-laid eggs than in the first- or second-laid eggs (repeated measures ANOVA, $F_{2,94}$ = 4.09, p = 0.020) (Figure 4.2). In 2-egg clutches, eggshell colour did not statistically differ between the first- and the last-laid egg (paired t-test, t_{15} = 1.69, p = 0.112). The PC1-scores of the second-laid eggs (last-laid) in 2-egg clutches were significantly higher than PC1-scores of second-laid eggs in 3-egg clutches (t_{46} = 2.21, p = 0.039) The PC1-scores of eggshell colour in the

second-laid eggs (last-laid) of the 2-egg clutches was similar to the eggshell background colour in the third-laid egg of 3-egg clutches ($t_{47} = 0.15$, $p = 0.877$).

Within-clutch eggshell colour contrast was not related to mean daily nest attendance during laying eggs and early incubation, clutch size, laying date and egg volume of the last-laid egg (model 1 in Table 4.1). Daily changes in nest attendance were related to within-clutch eggshell colour contrast. Pairs with larger increases in nest attendance over the laying and early incubation period showed greater within-clutch eggshell colour contrast than pairs starting incubation later (model 2 in Table 4.1). Eggshell colour differences between penultimate and last-laid egg was not related to laying intervals between penultimate and last-laid egg, clutch size, laying date and egg volume of the last-laid egg (Table 4.2). Laying interval between penultimate and the last-laid egg was 2.2 ± 0.06 days (n = 48; 1 nest was excluded due to a missing laying date).

Hatching interval between the penultimate and the last-laid egg was related to eggshell colour difference between the penultimate and the last-laid egg. Hatching interval increased with eggshell colour differences between the penultimate and the last-laid egg (Table 4.3).

Table 4.1 The relationship between within-clutch eggshell colour contrast and mean nest attendance (model 1) or daily changes in nest attendance (model 2), egg volume, laying date of the last-laid eggs and clutch size in 26 nests (2 of 28 nests did not have a record of eggshell colour). *F* and P-values refer to ANCOVA. Parameter estimates are shown in the case of significant results.

	Model 1		Model 2		
	F	p	*F*	p	B ± 1 S.E.
Mean nest attendance	$F_{1,24} = 0.42$	0.522	-		
Daily change in nest attendance	-		$F_{1,24} = 4.60$	0.042	32.84 ± 15.30
Laying date of the last-laid egg	$F_{1,22} = 0.07$	0.787	$F_{1,22} = 0.22$	0.638	
Clutch size	$F_{1,21} = 0.01$	0.911	$F_{1,23} = 0.63$	0.434	
Egg volume of the last-laid egg	$F_{1,23} = 0.16$	0.688	$F_{1,21} < 0.01$	0.954	

Table 4.2 The relationship of eggshell colour differences between penultimate and last-laid egg with laying interval between penultimate and last-laid eggs, egg volume, laying date of last-laid egg and clutch size in 44 nests (5 of 49 nests were excluded due to missing values for laying date, eggshell colour or egg volume). F and P-values refer to ANCOVA.

	F	p
Laying interval between penultimate and the last-laid egg	$F_{1,42} = 0.93$	0.340
Clutch size	$F_{1,41} = 0.71$	0.403
Laying date of the last-laid egg	$F_{1,40} = 0.72$	0.398
Egg volume of the last-laid egg	$F_{1,39} = 0.43$	0.516

Table 4.3 The hatching interval between penultimate and last-laid eggs in relation to eggshell colour difference between penultimate and the last-laid egg, clutch size and laying date in 25 nests (2 of 28 nests did not have eggshell colour and 1 of 28 nests was no data of laying date).

	F	p	B ± 1 S.E.
Eggshell colour difference between penultimate and last-laid egg	$F_{1,23} = 5.10$	0.034	0.36 ± 0.16
Laying date of the last-laid egg	$F_{1,22} = 2.33$	0.140	
Clutch size	$F_{1,21} = 0.46$	0.505	
Egg volume of the last-laid egg	$F_{1,20} = 0.10$	0.745	
Laying interval between penultimate and last-laid egg	$F_{1,19} = 0.01$	0.908	

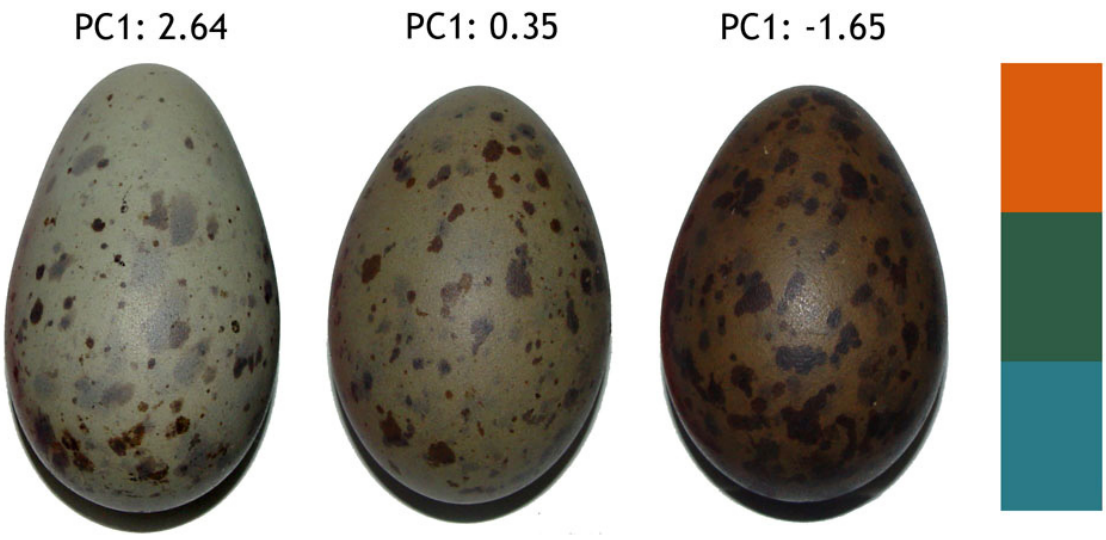

Figure 4.1 Examples of eggshell colour variations in herring gulls. Using principal component analysis a single PC1 score was derived from RGB-values measured from digital images. Orange, green and blue colour chips (right) were photographed with each egg to correct RGB value of the eggshell colour in each image. Digital images were corrected using colour chips. Pictures above are showing uncorrected image but PC1 scores indicate corrected values.

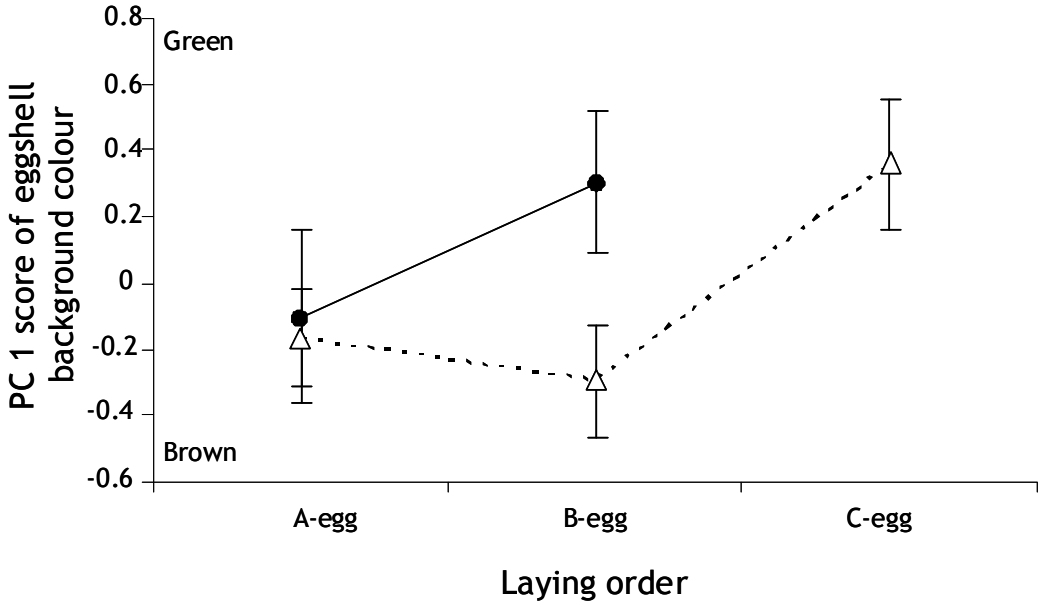

Figure 4.2 Eggshell colour (mean ± 1 S.E.) in relation to laying order for 3-egg clutches (Δ with a broken line, n = 33 nests) and for 2-egg clutches (● with a solid line, n = 16 nests). A-, B- and C-egg refer the first-, second- and third laid egg, respectively.

4.4 DISCUSSION

Eggshell colour varied within a clutch. Eggshell colour of last-laid eggs was related to incubation behaviour during egg-laying and early incubation. As parents increased nest attendance rapidly, variations in eggshell colour were greater.

Moreno and Osorno (2003) proposed that eggshell colour may indicate female body condition because a high deposition of biliverdin (a blue pigment) into the eggshell may reflect a high antioxidant plasma level in the female. In lesser black-backed gulls, RGB-colour of eggshell correlated with orange to red wavelength range and it may reflect porphyrin level (a red or brown pigment). It has been suggested that RGB values of eggshell colour reflect porphyrin (Tharapoom, 2006). In this study, I assumed that RGB-colour present porphyrin. I did not find a difference in eggshell colour of the first-laid egg between females laying two and three eggs, nor did I find relationships between eggshell colour and laying date and egg size, although females laying 2-egg clutches are assumed in poorer body condition than females laying 3-egg clutch. However, the second-laid eggs were paler in 2-egg clutches compared with the second-laid eggs in 3-egg clutches. Egg production is costly (Davis *et al.*, 2005). In great skuas (*Stercorarius skua*), females laying more eggs than their normal clutch size produced lower numbers of new blood cells (Kalmbach *et al.*, 2004), which might relate to deposits of eggshell pigments. When females deposit biliverdin, which is one of the eggshell pigments, this compound is a strong antioxidant and porphyrin is a pro-oxidant, so these pigments may be able to indicate the tolerance of females to oxidative stress. Protoporphyrin induces oxidative stress and increases activation of antioxidant enzymes (Vanore & Batlle, 1999). Hence, females laying only 2 eggs may produce smaller amounts of pigments and run out of eggshell pigments quicker than females laying 3 eggs due to poor body condition.

I investigated whether within-clutch variation in eggshell colour was related to incubation behaviour. Pairs with a larger change in daily nest attendance during laying and early incubation showed greater within-clutch eggshell colour contrasts. Larger daily change of nest attendance during egg-laying and early incubation induced relatively paler colour of last-laid egg. Onset of incubation is hormonally controlled by prolactin and steroid hormones (reviewed in Buntin, 1996). During laying, prolactin levels of parent birds increase and peak in the middle of the incubation period (reviewed in Vleck, 2002) and prolactin level rapidly declines after hatching (Opel & Proudman, 1989). The larger daily changes in nest attendance may be triggered by

steeper increases of prolactin levels. Soh and Koga (1994) found that changes in sex steroids during egg-laying affect the deposition of eggshell pigments in Japanese quails (*Coturnix japonica*). Higher progesterone levels encouraged accumulation of porphyrin in the shell gland. Females which rapidly changed nest attendance may switch earlier from steroid hormone to prolactin than females which started incubation later. This early switch from steroid hormones to prolactin may depress the accumulation of pigments in the shell gland. This may result in pigment depletion on eggs which were laid after onset of incubation. The paler colour of the second-laid egg in 2-egg clutches than of the second-laid egg in 3-egg clutches may be explained by the differences in nest attendance during laying between 2-egg and 3-egg clutches. Because pairs with 2-egg clutches changed nest attendance more rapidly than pairs with 3-egg clutches (see Chapter 3), the second-laid eggs which might be laid after onset of incubation in 2-egg clutches may present paler colour than the second-laid eggs in 3-egg clutches. Within-clutch eggshell colour contrast was not related to clutch size because eggshell colour of first- and last-laid eggs was similar between 2-egg and 3-egg clutches. Laying date and egg volume of last-laid eggs did not affect eggshell colour contrast within a clutch. Although I looked at the effect of mean daily nest attendance on within-clutch variation in eggshell colour, daily nest attendance did not relate to eggshell colour contrast. This result suggests that within-clutch variation in eggshell colour related to the change of nest attendance during egg-laying rather than mean daily nest attendance.

Baerends and Hogan-Warburg (1982) suggested that the odd colour of the last-laid egg could be the result of the shorter time it stays in the uterus. Thus, the last-laid egg may not have enough time to obtain the same amount of pigments than earlier-laid eggs that spend longer in the uterus. In species with asynchronous hatching, short laying or hatching intervals are advantageous to the survival of the youngest offspring due to a less pronounced competitive hierarchy within the brood (Sydeman & Emslie, 1992). Females may lay the last eggs in shorter time to reduce the competitive hierarchy within a clutch. However, differences of eggshell colour did not relate to laying interval between penultimate and the last-laid egg. This result did not support the short passage time hypothesis in the uterus. The results of Kilpi and Byholm (1998) in herring gulls also did not find an effect of laying interval on within-clutch variation in eggshell colour. Furthermore, in this study, laying interval between the penultimate and the last-laid egg did not differ from laying intervals between first- and second-laid eggs in the clutch (see table 3 in Chapter 3), but eggshell colour was still paler in last-laid eggs than in earlier-laid eggs. Pairs with a greater within-clutch difference of

eggshell colour between penultimate and last-laid egg had longer hatching interval between penultimate and the last-laid egg. In other words, within-clutch variation in eggshell colour increased with hatching span.

To conclude, this present study suggests that variation in behaviour during laying and early incubation can explain within-clutch variation in eggshell colour. This may be the result of hormonal changes during the time eggshells were formed but further studies on hormonal changes are required. The benefit of this result is that measures of within-clutch variation in eggshell colour that can be taken at one visit any time during incubation allows inferences on the birds' nest attendance pattern during laying and early incubation.

Chapter V.

Consequence of accelerated embryonic development in lesser black-backed gulls *Larus fuscus*

5.1 INTRODUCTION

As embryonic development starts through incubation in avian species, parents may control hatching through onset of incubation. Females often start incubation during the laying period (Wiebe *et al.*, 1998; Persson & Goransson, 1999; Hebert, 2001; Hanssen *et al.*, 2002, see also Chapter 3) and it may generate developmental asynchrony within a clutch (Stoleson & Beissinger, 1995). But, in many precocial birds, chicks in a clutch hatch within 24 hours although parents start incubation before the clutch completion. Experiments which altered onset of incubation showed that embryos can affect hatching patterns. Vince (1964) found that bobwhite quail (*Colinus virginianus*) eggs accelerated development when placed next to eggs at more advanced development in a clutch. In mallards (*Anas platyrhynchos*) and ring-necked pheasants (*Phasianus colchicus*), embryos also can delay or accelerate their hatching to hatch synchronously (Persson & Goransson, 1999). It has been shown embryonic development can communicate through vocalisation or movement of other nest mates (Vince, 1966). Synchronous hatching may be more important in the survival for last-laid eggs to avoid being left behind in the nest after hatching (Vince, 1964) because parents have to leave the nest with chicks as soon as chicks hatch to feed their hatched chicks (Nilsson & Persson, 2004). Hence I expect stronger selection on embryonic development rate in last-laid eggs than in earlier-laid eggs.

Although many studies on accelerating embryo development have been done in synchronously hatching species, some evidences of accelerated development of embryos in last-laid eggs have also been reported in asynchronously hatching species. For example, last-laid eggs of herring gulls (*Larus argentatus*) and lesser black-backed gulls (*Larus fuscus*) hatched early relative to first-laid eggs (Parsons, 1972). In lesser black-backed gulls, last-laid eggs have been shown to be able to accelerate their hatching when stimulated by older nest mates (Muck & Nager, 2006). Large hatching asynchrony could be disadvantageous to last-hatched chicks in asynchronous species as well as species hatching synchronously (Sydeman & Emslie, 1992). In asynchronously

hatching seabirds, by the time the last-laid egg hatched their chicks are smaller than their older siblings. For example in shags (*Phalacrocorax aristotelis*), on the day the last chick hatched, first-hatched chicks were two times heavier than last-hatched chicks (Stokland & Amundsen, 1988). This physical handicap seems to disadvantage the youngest chick in sibling competition and they obtain less food from the parents (Mock & Parker, 1997) unless parents feed chicks selectively. Sibling competition is one of the most important factors to affect growth rate and survival within a nest (Hahn, 1981; Nilsson & Svensson, 1996). In some species such as egrets and boobies older siblings sometimes directly reduce the survival of last-hatched chicks through siblicide (Fujioka, 1985b). When last-hatched chicks are experimentally hatched synchronously, they have similar survival rates to their older siblings (Hebert & Barclay, 1986). Therefore, shorter hatching spans may be beneficial by reducing size differences between siblings (Sydeman & Emslie, 1992). However, it may be difficult for the last chick to catch up with its older siblings due to the already established hierarchy by the initiation of incubation before clutch completion. The last-laid eggs may be able to reduce hatching interval through accelerating their development during the incubation period and in particular the hatching period (Lipar & Ketterson, 2000; Lipar, 2001). In lesser black-backed gulls, only last-laid eggs can accelerate their development and then only when they hatched last in a brood, but not when hatching first (Muck & Nager, 2006). This result may suggest that fast development might produce not only benefits but also costs to last-laid eggs and therefore last-laid eggs only accelerate when required. Some studies suggest that there is a cost to accelerated embryo development. Acceleration of hatching may affect body condition and survival of the offspring and the development of sensory and neuromuscular systems. Hatchlings of lesser black-backed gulls had poorer body condition when they had shorter hatching duration (Muck & Nager, 2006). In herring gulls, male chicks fledged in poorer condition than female chicks when they developed with other eggs (Bogdanova & Nager, 2008). It may be caused by the different cost during the embryonic development for females and males. In ring-necked pheasants (*Phasianus colchicus*), chicks with shorter incubation periods had poorer locomotion abilities than chicks with longer incubation periods (Persson & Goransson, 1999). The period before hatching is an important period for muscular and organ maturation and in ring-necked pheasants and mallards; chicks with shorter hatching duration had a lower body mass and a shorter tarsus length than control chicks while chicks that delayed hatching had similar or slightly better growth as control chicks (Nilsson & Persson, 2004).

The aim of the present study was to investigate whether there was a cost of accelerated development in last-laid eggs of the lesser black-backed gulls. Lesser black-backed gulls are a suitable species to examine the effects of accelerated development because it has been already shown that last-laid eggs can accelerate their embryonic development depending on their siblings' development (Muck & Nager, 2006). In terms of a cost of accelerated embryonic development, I looked at hatchling body size and condition and hatching success, growth rate and survival of chicks.

5.2 METHODS

5.2.1 Study area and species

This study was carried out in a breeding colony of lesser black-backed gulls at Sandgerði (64º03´N, 22º40´W), SW Iceland, from mid-May to early August in 2005. The study area was a pasture near the sea coast and approximately 1,000 nests of lesser black-backed gulls were found in the study area (see Chapter 1 for details). Lesser black-backed gulls typically lay a clutch of three eggs with 2-day laying intervals between each egg (Cramp, 1983).

5.2.2 Field protocol

During egg-laying, all nests were visited once a day to identify laying sequences and eggs were marked using a waterproof pen. I used only 3-egg clutches for my study. Egg size (the maximum length and width) was measured with vernier calipers to the nearest 0.1 mm and egg volume was estimated using the equation (Harris, 1964):

Egg volume (cm^3) = 0.000476 x egg length (cm) x egg $width^2$ (cm).

To encourage accelerated embryonic development of last-laid eggs, I made 15 experimental nests where I increased the laying interval between the second-laid and last-laid egg. When in an experimental nest the third-laid egg was laid, their first- and second-laid eggs were replaced with other first- and second-laid eggs from two different nests. I aimed to make experimental clutches with a 2-day laying interval between the first- and second-laid egg and a 4-day laying interval between the second- and third-laid egg. However, I was not always able to exactly match the laying dates as planned, and laying intervals were 2.0 ± 0.17 days (mean ± 1 S.E.) between the first- and second-laid eggs and 4.1 ± 0.08 days between the second and third-laid eggs. In 16

control nests I replaced their first- and second-laid eggs with eggs of the same egg order from two different nests that were laid on the same day as the eggs they replaced in order to maintain 2-day laying intervals between the eggs. I replaced first- and second-laid eggs into nests which kept their third-laid egg and the initial eggs of that nest were transferred to unmanipulated nests. Again, I could not always exactly match the laying dates and the laying interval between the first- and second-laid egg was 2.0 ± 0.11 days (mean ± 1 S.E.) and between the second- and third-laid egg was 1.8 ± 0.13 days (mean ± 1 S.E.). In both control and experimental nests, the egg swap was done within 4 days of clutch completion. Parents readily accepted the swapped eggs.

From before the earliest hatching day (22 days after the first egg was laid, incubation period was 24 ~ 30 days) (Muck & Nager, 2006; Bogdanova & Nager, 2008), I visited nests and checked for signs of hatching at least once a day (more often if it was possible). To compare the timing and duration of hatching between eggs, I observed externally visible signs of hatching such as the appearance of cracks and a pipping hole. The first externally visible sign of hatching is a star-shaped crack which has been proceed by the internal pipping. The cracks then grow until the embryo makes a hole into the eggshell and starts breathing using its lungs (pipping stage). It then finally emerges from its eggshell and hatches (Schreiber & Burger, 2001). At each nest visit, I measured the longest length of crack and the diameter of the piping hole using vernier calipers to the nearest 0.1 mm (Figure 5.1). Initiation of cracking and pipping was estimated through regression line from observed length of crack and pipping hole against time. Time of measuring the length of crack and pipping was calculated backward from hatching time. Initiation of cracking and pipping of the third-laid egg was measured from 12 control nests (2 eggs failed to hatch and data were missed in two eggs due to weather condition) and from 6 experimental nests (8 eggs failed to hatch and one egg had missing data).

Hatching time was defined as the time when the embryo completely emerged from its eggshell. It is difficult to observe actual hatching time in the field. Hence, I estimated hatching date using the degree of chick dryness. When I found a hatchling in a nest, I recorded the timing of the visit and the degree of plumage dryness. Plumage dryness was recorded as the percentage of the body surface being dry; from 0 % (wet) to 100 % (dried). The timing of hatching was then determined based on the duration since the last nest visit and plumage dryness. The time it took was based on observations of chicks at the study site during that year and I observed that it took for about half of

the chick to dry was 2 hours and it took about 5 hours to completely dry (pers. obs.). The maximum piping hole diameter that I observed was 3.9 cm,

- Totally wet chick: a chick may hacth just before visiting a nest
- Half dried chick: a chick may hatch 2 hours before visiting a nest
- More than half dried chick: a chick may hatch 5 hours before visiting a nest
- Totally dried chick: a chick may hatch at the mid-point between the last two visits

When there was a sufficiently large pipping hole, I marked the chick by applying a dot of non-toxic paint on their bill through the pipping hole. This allowed me to identify what chick hatched from what egg (Figure 5.1). For each fresh hatchling I measured body mass using an electronic balance to the nearest 0.1 g and head plus bill length, tarsus length and ulna length using vernier calipers to the nearest 0.1 mm. Hatching failure of each egg (%) was monitored as well. For eggs that failed to hatch I recorded whether they died before or after the initiation of cracking. To control the effect of size hierarchy within a clutch, I transferred last-hatched chicks when they hatched more than 2 days later than second-laid eggs. One of last-laid chicks in experimental and two of last-hatched chicks in control nests, which had more than normal hatching interval (2 days), were transferred into other nests within 2 days after hatching to avoid cross-fostering parents recognizing their chicks. Growth rate of body mass and head plus bill was estimated between 0 and 24 days after chicks hatched. To remove an effect of possible differences in hatching condition, I used an instantaneous growth rate of chicks. The instantaneous growth rate is estimated by the slope of weight (W) against age (t):

$\log(W(t + dt) / W(t))$ (reviewed in Moss *et al.*, 1993).

Early chick survival was defined as the survival until 1 week after chick hatching because survival rate until chicks fledged was very low in the study year. When eggs disappeared from the nests, they were assumed to have depredated.

5.2.3 Statistical analyses

I used SPSS (SPSS, 2006) for all statistical analyses. Mean values are presented with ± 1 S.E. p-values < 0.05 are considered to be statistically significant and all probabilities

refer to two-tailed tests. Sample size varies between analyses due to eggs that failed to hatch or had missing data.

5.3 RESULTS

Third-laid eggs of experiment nests were laid on average 7 days later than third-laid eggs in control nests, but egg volume of third-laid eggs did not differ between control and experiment nests (Table 5.1).

5.3.1 Hatching interval in control vs. experiment

I compared the hatching interval and laying interval between the second- and third-laid eggs in control and experiment nests. Hatching intervals between the second- and the third-laid eggs was in both groups shorter than their laying intervals and was 2.0 ± 0.45 days in experimental nests and 1.4 ± 0.23 days in control nests (Figure 5.2). Third-laid eggs in experimental nests reduced the interval to the second-laid egg by 1.9 ± 0.45 days (n = 6) while third-laid eggs in control nests reduced it by 0.6 ± 0.26 days (n =13) (independent t-test: $t_{17} = 2.55$, $p = 0.023$). Laying intervals between second- and third-laid eggs significantly differed between control and experimental nests (Figure 5.2). When third-laid eggs hatched, older nest mates had already hatched in all nests.

5.3.2 Embryonic development rate

Initiation of crack and pipping to hatching intervals in third-laid eggs did not statistically differ between control and experimental (crack to hatching: $t_{15} = 0.75$, $p = 0.461$; pipping to hatching: $t_{15} = 0.40$, $p = 0.692$). However, incubation period of third-laid egg was significantly shorter in experimental nests than in control nests ($t_{18} = 2.49$, $p = 0.023$, Table 5.2). Third-laid eggs started crack earlier in experiment than third-laid egg in control nests ($t_{15} = 2.15$, $p = 0.048$) and started pipping hole earlier ($t_{15} = 2.04$, $p = 0.059$) (Figure 5.3). In second-laid egg, pipping hole started later in experiment than in control ($t_{17} = 2.10$, $p = 0.051$).

5.3.3 Hatching success and body condition at hatching

Hatchling body mass and skeletal size of chicks which hatched from third-laid eggs did not differ between control and experimental nests (Table 5.3). Hatching success of third-laid eggs was lower in experimental nests than in control nests (Fisher's exact

test, p = 0.007; Figure 5.4). Hatching success of the first and second-laid eggs did not differ between control and experimental nests (Fisher's exact test, first-laid eggs: p = 0.467; second-laid eggs: p = 0.675). Of the 15 third-laid eggs in experimental nests 3 embryos died after crack, 4 embryos died before cracking and 2 eggs were predated. Of the 16 third-laid eggs in control nests, 1 embryo died after cracking and 1 egg was predated.

5.3.4 Growth rate and early survival of third-laid chicks

Both growth rate of body mass and head plus bill in third-laid eggs did not differ between control and experimental nests (instantaneous growth rate of mass - experiment: 0.12 ± 0.02 g per day; control: 0.13 ± 0.021 g per day; head plus bill length – experiment: 0.03 ± 0.003 cm per day; control: 0.03 ± 0.004 cm per day Table 5.4). Growth rate of body mass and head plus bill in third-laid eggs was not related to initiation of crack and pipping hole from hatching date, treatment and incubation period. Early survival rate (up to 7 days after hatching) of chicks hatched from third-laid eggs was similar between control (57.1%, n = 14) and experiment (50 %, n = 6). Early survival also has not related to initiation of crack and pipping hole (Table 5.5). Only laying date affected early survival of chicks hatched from the third-laid eggs. In early breeding season, 62% of hatched third-laid eggs survived until 1 week (n = 16) wile 25% of them survived in late breeding season (n = 4).

Table 5.1 Laying date (Day 1 = 1st May) and egg volume of third-laid eggs in control and experimental nest (numbers in brackets refer to sample sizes). Data on egg volume were missing for one control nest and 2 experimental nests.

	The third -laid egg (mean ± 1 S.E.)			
	Control	Experimental	t	p
Laying date	37.9 ± 2.01 (16)	45.1 ± 2.07 (15)	t_{29}= 2.48	0.019
Clutch volume (cm^3)	68.3 ± 1.33 (15)	67.4 ± 6.34 (13)	t_{26} = 0.37	0.708

Table 5.2 Developmental rates with respect to egg order and treatment (control and experimental). Initiation of a crack and pipping hole were estimated from regression of crack and pipping hole size against time in each nest. Sample size varied due to hatching failure and missing data.

	Control (n)			Experiment (n)		
	First	Second	Third	First	Second	Third
Intervals						
Initiation of cracking to hatch (days)	-3.0 ± 0.25 (6)	-3. 8 ± 0.51 (8)	- 3.3 ± 0.38 (11)	-2.8 ± 0.54 (10)	- 4.0 ± 0.25 (10)	- 3.8 ± 0.53 (6)
Initiation of pipping hole to hatch (days)	-1.4 ± 0.11 (9)	- 1.9 ± 0.16 (9)	- 1.4 ± 0.21 (11)	-1.1 ± 0.19 (10)	- 1.5 ± 0.18 (10)	- 1.3 ± 0.16 (6)
Laying to hatching (days)	28.0 ± 0.32 (13)	26.9 ± 0.23 (14)	26.1 ± 0.23 (13)	27.3 ± 0.33(12)	26.8 ± 0.19 (13)	25.0 ± 0.45 (6)

Table 5.3 Mean + 1 S.E. (n) body mass and skeletal size of fresh hatchlings hatched from third-laid eggs in control and experimental nests. Mann-Whitney *U*-test was used to compare between control and experimental nests.

	Control (14)	Experiment (6)	*U*	P
Body mass (g)	54.7 ± 1.20	55.3 ± 0.98	39.0	0.841
Head-bill (cm)	4.6 ± 0.04	4.6 ± 0.054	32.5	0.433
Tarsus (cm)	2.3 ± 0.04	2.3 ± 0.05	37.0	0.680
Ulna (cm)	2.3 ± 0.03	2.3 ± 0.04	38.0	0.741

Table 5.4 The growth rate of third-laid eggs in relation to initiation of cracking and pipping hole, treatments (control and experiment) and laying date. F and P-values refer to ANCOVA. Parameter estimates are shown in the case of significant results. Because the duration from pipping hole to hatching and cracking to hatching were not independent were not independent, but were correlated, I analysed the data in two separate models.

	Growth of body mass			Growth of head plus bill		
	F	P	B ± 1 S.E.	F	P	B ± 1 S.E.
Intercept	$F_{1,7} = 32.69$	0.001	0.19 ± 0.034	$F_{1,7} = 43.45$	< 0.001	0.04 ± 0.007
Initiation of crack from hatching (days)	$F_{1,7} = 4.52$	0.071		$F_{1,7} = 2.65$	0.147	
Treatment	$F_{1,5} = 0.18$	0.687		$F_{1,5} = 0.11$	0.753	
Incubation period (days)	$F_{1,6} = 0.23$	0.645		$F_{1,6} = 2.32$	0.178	
Laying date (1 = 1st May)	$F_{1,4} = 0.06$	0.812		$F_{1,4} = 0.02$	0.887	
Incubation period * initiation of crack from hatching (days)	$F_{1,2} = 0.05$	0.835		$F_{1,3} = 0.06$	0.812	
Laying date * incubation period	$F_{1,3} = 0.08$	0.796		$F_{1,2} = 0.49$	0.556	
Intercept	$F_{1,7} = 2.86$	0.134		$F_{1,7} = 1.58$	0.248	
Initiation of pipping hole from hatching (days)	$F_{1,4} < 0.001$	0.987		$F_{1,4} = 0.008$	0.934	
Treatment	$F_{1,6} = 0.07$	0.795		$F_{1,5} = 0.01$	0.903	
Incubation period (days)	$F_{1,5} = 0.002$	0.969		$F_{1,7} = 0.58$	0.470	
Laying date (1 = 1st May)	$F_{1,7} = 0.41$	0.538		$F_{1,6} = 0.26$	0.629	
Incubation period * initiation of pipping from hatching (days)	$F_{1,2} = 0.46$	0.567		$F_{1,3} = 0.98$	0.394	
Laying date* incubation period	$F_{1,3} = 0.66$	0.475		$F_{1,2} = 0.36$	0.609	

Table 5.5 Logistic regression analysis of chick survival of third-laid eggs until 1 week after chick hatched in relation to laying date, initiation date of crack (model 1) and pipping hole (model 2), treatment and hatching mass in hatched 17 nests (control: 11 nests; experiment: 6 nests). Bold letter indicates significance after Bonferroni correction.

	x^2_1	p
(a) Model 1		
Laying date	6.30	**0.012**
Initiation of crack from hatching (days)	0.73	0.392
Hatching mass	0.67	0.410
Treatment	0.01	0.919
Laying date * hatching mass	0.86	0.354
(b) Model 2		
Laying date	3.68	0.055
Initiation of pipping from laying date (days)	0.001	0.978
Treatment	0.02	0.873
Hatching mass	0.87	0.351
Laying date * hatching mass	0.95	0.329

Figure 5.1 Crack (left) and pipping hole (right) of lesser black-backed gull eggs. Length of a crack and pipping hole was defined as a maximum length of diameter Embryos were marked using non-toxic paint on the bill (green in the right panel) to identify which chick hatched from which egg.

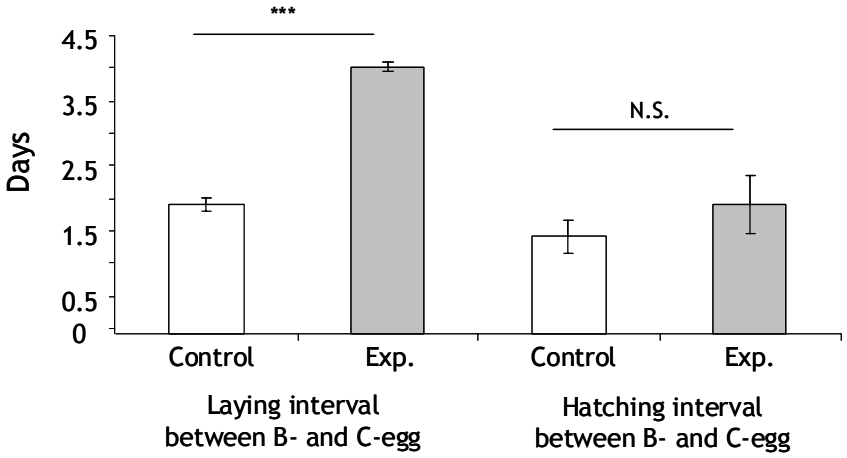

Figure 5.2 Mean (± 1 S.E.) laying interval and hatching interval between second- and third-laid eggs in control (open bars, 13 nests) and experiment nests (filled bars, 6 nests). Statistical differences between control and experimental nests are marked (independent-sample t-tests, ***: $p < 0.001$; N.S.: not significant).

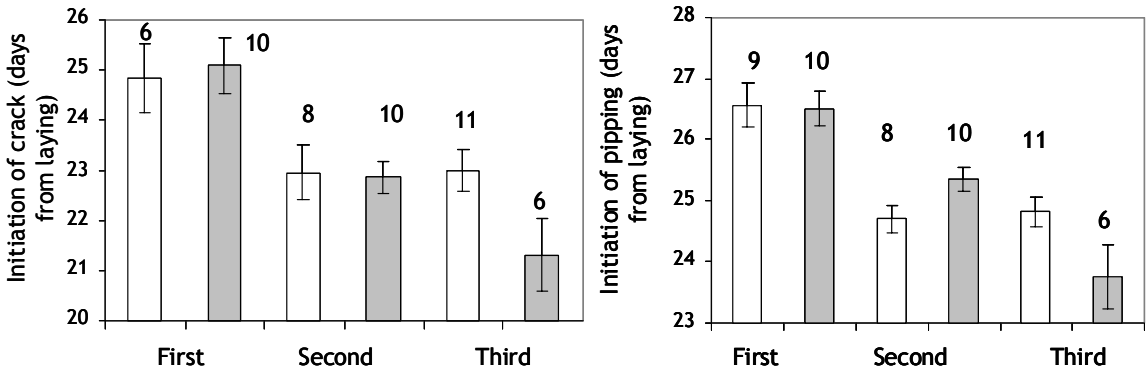

Figure 5.3 Initiation of crack and pipping hole in first-, second- and third-laid eggs between control (open) and experimental (filled) nests. The number above bar indicates sample size and whiskers present ± 1 S.E.

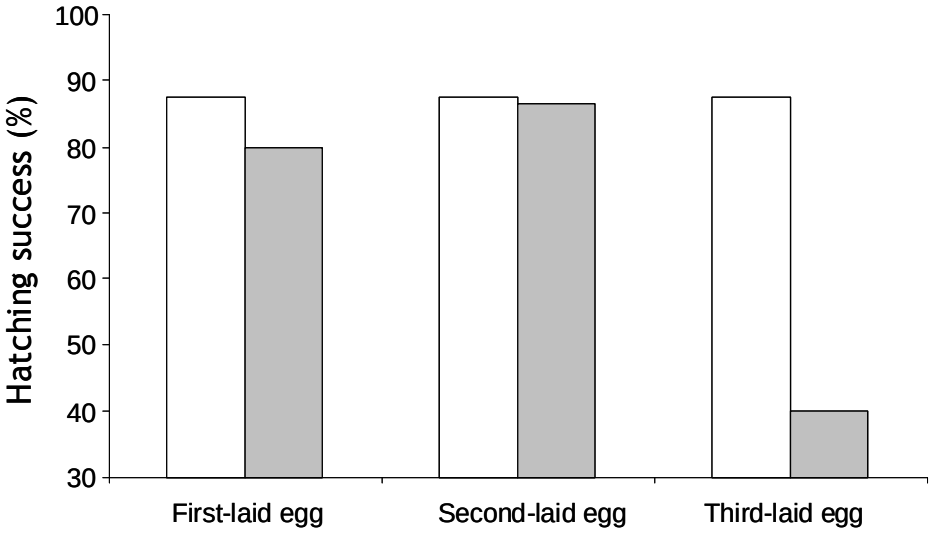

Figure 5.4 Hatching success (%) of eggs between control (open bars) and experimental clutches (filled bars).

5.4 DISCUSSION

The third-laid eggs in experimental nests reduced extended laying interval and hatched with normal hatching interval between penultimate eggs. They may either accelerate development (Metcalfe & Monaghan, 2001) or may hatch before they are fully developed (Nilsson & Persson, 2004) to reduce hatching intervals between the penultimate and last hatchlings. In this present study, the latter was unlikely the case because there was no difference of hatching body condition in experimental compared with control chicks.

Shorter hatching intervals than laying intervals between the penultimate and last-laid eggs are commonly found in birds. In herring gulls, last-laid eggs hatch less than 2 days after the penultimate eggs although gulls lay eggs every other day (Drent, 1970). Results of this present thesis showed the last-laid eggs can even more accelerate their development than they normally do. Reduced hatching interval between older siblings and the last-laid eggs is important for survival of the last-laid eggs due to size hierarchies within a clutch and fledging success of last-hatched chicks decreased with hatching interval (Sydeman & Emslie, 1992). If third-laid eggs do not accelerate development to hatch at the same time as their siblings though they can, there may be constraints controlling embryonic development.

I looked at whether there is a cost of accelerated development during pre-natal and post-natal period. Hatching success of third-laid eggs was significantly more than two times lower in experimental than in control nests. In experimental nests, addled eggs, which parents incubated but which did not hatch in the nest, were the main cause of nest failure. In experimental nests, embryos died at the early stage of development or after crack. There are two possibilities to explain the cause of hatching failure. Firstly, it might be the result of egg neglect during hatching. When the third-laid egg hatched, earlier-laid eggs were already hatched in a clutch, and parents may not be able to fully incubate third-laid eggs because they need to feed earlier hatched chicks (Beer, 1962; Drent, 1970; Beissinger & Waltman, 1991). Lower incubation temperatures which may occur during neglect retards embryonic development (Webb, 1987; Evans, 1990; Astheimer, 1991) or increases mortality of the last-laid eggs (Cannon *et al.*, 1986; Nilsson & Persson, 2004). In experimental nests, neglect of the last-laid egg might be greater than in control nests because of a longer laying interval. However, hatched third-laid eggs in experimental nests had similar hatching interval as third-laid eggs in control nests because they reduce laying interval through accelerated development.

Furthermore, Lee *et al*. (1993) suggested that in herring gulls, pipping temperature of third-laid eggs is still higher than physiological zero (27ºC) and did not affect hatching although egg temperature of third-laid eggs is lower than earlier-laid eggs at pipping. Hatching failure before a shell crack occurred more in experimental nests than in control nests. It still remains to be tested whether lower hatching success is the result of egg neglect. Secondly, lower hatching success in experimental nests may be the cost of accelerated development during the embryonic period. Costs of accelerated development may appear through the life span (reviewed in Metcalfe & Monaghan, 2001). To investigate the cost of accelerated development during nestling period, I compared hatching body condition and growth rate between experimental and control nests. Unlike data for the hatching success, in the present study, there were no differences in hatching condition and growth rate between control and experimental eggs. Although previous studies suggested the costs of accelerated development have been shown. For example, mallards (*Anas platyrhynchos*) which accelerated embryonic development had a lower body mass, a shorter tarsus length, a poorer balance and mobility than chicks in control (Nilsson & Persson, 2004). Accelerated hatchling in lesser black-backed gulls had lower body mass than hatchlings in control (Muck & Nager, 2006). There are sex-specific costs of hatching last in other studies. In herring gulls, although a single chick grew in a nest, last-hatched males showed lower fledging body mass than last-hatched females although hatching time did not differ between female and male chicks (Bogdanova & Nager, 2008). There is the possibility that all hatched chicks were females in the present study. Because the costs of accelerated development differ between females and males, their body condition may be less affected by accelerating development. Unfortunately, chicks were not sexed in this study. I also compared early chick survival between experimental and control nests and did not find any difference. It might be that the cost of accelerated development is already paid chicks through the successful hatching. No difference of growth rate and early survival of third-laid chicks might be explained as third-laid eggs compensated the cost of accelerated development with reducing size hierarchy in a clutch. Alternatively, chicks hatched from third-laid eggs in experimental nests might be better quality chicks than dead embryos. However, it needs larger sample size to confirm this.

It is not clear when and how third-laid eggs in experiment accelerated embryonic development. The pipping to hatching interval has been used to measure hatching duration (e.g. Muck & Nager, 2006; Bogdanova & Nager, 2008) because embryos communicate with sibling eggs by producing clicking sounds and vibration during the

last few days before hatching when the auditory system of the embryos is developed (Driver, 1965; Vince, 1969). This acts as a signal between siblings to control the speed of the last stage of embryonic development (Vince, 1969). Woolf *et al*. (1976) suggested that embryos of Japanese quail (*Coturnix japonica*) can accelerated only if they were stimulated during the last 3 days before hatching. In the present study, the cracking to hatching and pipping to hatching intervals did not statistically differ between control and experimental eggs while the incubation period of third-laid egg was much shorter in experimental compared with control clutches. It might be due to the low power of a small sample size. I also looked at the initiation time of cracking and pipping relative to when the egg was laid. Third-laid experimental eggs started cracking and pipping at an earlier stage of embryonic development than in third-laid control eggs. Third-laid eggs might accelerate their development just before they started to crack, but took the same amount of time between cracking and hatching and between pipping and hatching. Herring gulls might develop sensory organs early or might be sensitive to light because parents might be away nests to feed hatched chicks. There is hardly any documented evidence about when embryos of herring gulls develop sensory organs. It needs further study to investigate which factors influence the third-laid eggs to accelerate their development.

In conclusion, third-laid eggs in experimental nests might reduce the experimentally imposed age disadvantage by accelerating their development. This could possibly provide a benefit to the offspring after hatching. However, the cost of accelerated development seems to appear during the embryonic period. When considering the accelerated development of last-laid eggs to catch up with earlier-laid eggs, we not only need to consider the potential benefits from reduced social hierarchies but also the costs of accelerating development. Although there were costs of accelerated development during the embryonic period in the present study, it still needs experiments with larger sample sizes to confirm the causes of these costs of accelerated development.

Chapter VI.

Effects of eggshell characteristics on offspring development in lesser black-backed gulls *Larus fuscus*

6.1 INTRODUCTION

Parents invest their efforts to increase offspring fitness and their inclusive fitness. In birds, females can allocate their resources during egg formation through egg size (Christians, 2002) and egg components such as yolk lipids (Royle *et al.*, 1999) or hormone levels (Verboven *et al.*, 2003; Verboven *et al.*, 2005) depending on their body condition and environmental condition. However, the effect of eggshell characteristics on offspring development has not been studied very well. Eggshell not only protects the embryo from physical damage and against microorganisms until the chick hatches (Mine *et al.*, 2003) but also contributes by exchanging gas and water between the external environment before the initiation of lung ventilation (Tazawa & Whittow, 1994). Oxygen and carbon dioxide are exchanged by diffusion through the pores of the eggshell. Eggshell also provides calcium to the embryo during embryonic development. Although embryo uses calcium from yolk in the early embryonic development, eggshell becomes important to provide calcium during the rest of embryonic development (Romanoff & Romanoff, 1949). For example, a domestic hen obtains about 80 % of calcium requirement from the mammillary layer of eggshell and only 20 % of calcium requirement from yolk (Simkiss, 1961).

Variation in embryonic developmental rate has been explained by differences in eggshell characteristics. Higher porosity of eggshell with thinner eggshell thickness and/or larger pore area allows the embryo more respiratory gas exchange during development than lower porosity (Ar *et al.*, 1974; Tullett & Deeming, 1982) and increases embryonic developmental rate (Zimmermann & Hipfner, 2007). In seven species of Alcidae, species with higher porosity have shorter incubation period than ones with lower porosity due to fast development (Zimmermann & Hipfner, 2007). The effect of porosity on the embryonic development is also found within species. In snare penguins (*Eudyptes robustus*), inverse-hatching patterns between first- and second-laid eggs are the result of the differences of eggshell characteristics. The second laid-eggs

with higher pore density hatched earlier than the first-laid eggs (Massaro & Davis, 2005). In addition, the mammillary layer, the inner part of the eggshell, is also related to developmental rate (Blom & Lija, 2004). Precocious species such as Japanese quails (*Coturnix japonica*) had a higher density of mammillary cones and more extensive calcium removal compared to altricial species such as fieldfares (*Turdus pilaris*). This is because slower growing species take more calcium from the mammillary layer and had higher levels of ossification of their skeleton during embryonic development than the faster growing species.

Although previous studies suggested that eggshell characteristics affect embryonic developmental rate, many of the studies compared variations of eggshell characteristics of fresh eggshells between species (Zimmermann & Hipfner, 2007) or used hatched eggs from different individuals to investigate the effect of eggshell characteristics on the development (Massaro & Davis, 2005) because it is difficult to have both fresh eggshell and chick from the same egg. Furthermore, eggshell characteristics change during incubation (Bond *et al.*, 1988). For example, mammillary cones are eroded due to calcium uptake of embryo (Blom & Lilja, 2004; this study Figure 6.1) and eggshells get thinner and pore size and shape also change during incubation (Balkan *et al.*, 2006). To investigate the effect of eggshell characteristics on offspring development, I used the similarity of eggshell characteristics between first- and second-laid eggs of lesser black-backed gulls (*Larus fuscus*). They normally lay three eggs in a clutch and the first two eggs are very similar in egg mass, eggshell characteristics (Tharapoom, 2006) and egg compositions (Royle *et al.*, 1999; Nager *et al.*, 2000). Therefore, I used fresh eggshell of either first-laid egg or second-laid eggs as a proxy for eggshell characteristics of its sibling egg that was incubated to hatch.

In this study, I investigated the effect of eggshell characteristics on embryonic developmental rate and skeletal growth development. I tested whether chicks from eggs with poorer gas exchange capacity and/or thicker shells took longer to hatch duration and whether eggshell characteristics are related to hatchling skeletal size and post-hatching skeletal growth.

6.2 METHODS

6.2.1 Study area

This study was conducted on Walney Island (Cumbria, NW England, 54°08´N, 03°16´ W) from April to June in 2004. Walney Island has a mixed breeding colony of lesser black-backed gulls and herring gulls (see Chapter 1).

6.2.2 Field protocol

Eggshells from 48 three-egg clutches of lesser black-backed gulls were collected from an area near the centre of the breeding colony under a licence from English Nature. Laying date interval was 6 days between first nest and last nest. Nests were visited daily and fresh eggs marked with a non-toxic maker pen to identify laying order. Fresh egg mass was weighed on the day the egg was laid using an electronic balance to the nearest 0.1g. The first-laid or second-laid egg was randomly selected to be collected on the day of laying. Collected eggs were replaced with dummy eggs to avoid disturbing normal laying and incubation behaviour. Eggshells from collected eggs were air dried after removing egg contents in the field.

The other one of the first two eggs in a clutch that was not collected was fostered to a nest laying on the same day. It was incubated together with the hosts' first or second egg and third egg until it hatched. From the expected hatching date onwards from 20 days after laying first eggs, nests were visited twice a day in regular intervals. Hatchlings were marked with a small colour dot on their egg tooth while still in the eggs so that each chick hatched from a known egg. Chicks from 39 of the 48 fostered eggs hatched. Hatching time in which is defined as the time when chicks completely emerged from the eggshell was recorded in 39 nests. However, the first observation of external pipping (the puncturing of the eggshell before hatching) was recorded in 32 eggs of hatched 39 eggs due to weather condition. Hatchlings were weighed with an electronic balance to the nearest 0.1 g and tarsus, wing and head plus bill length were measured with vernier calipers to the nearest 0.1 mm within 24 hours of hatching. Hatching body mass and sizes were estimated in 27 nests because 12 chicks were not measured on the day of hatching due to the weather condition.

When the chick hatched, shell membranes containing the embryo's blood vessels were peeled off and stored in ethanol for subsequent molecular sexing (Jensen *et al.*,

2003). This was used to determine the sex of chicks using the molecular methods (Griffiths *et al*., 1998). After hatching, each nest was visited every 4 days until 35 days when chicks were close to fledging. Chick survival was recorded and if chicks were not found on the visiting day and on 3 following days, they were assumed dead, except more than 30 days old chicks that were likely to have fledged. Chicks were weighted with a pesola spring balance to the nearest 1 g and tarsus and head plus bill length of chicks were measured to the nearest 0.1 mm at each visit. Growth rate of chicks was calculated using the slope of chick growth between 4 days and 24 days (linearly growing period) after hatching in body mass, head plus bill length, tarsus length and wing length. To avoid the effect of hatching condition on growth rate, I used instantaneous growth rate that was transformed by logarithmic transformation.

6.2.3 Measurements of eggshell characteristics

● Eggshell thickness - I measured eggshell thickness including eggshell membrane to the nearest 0.005 mm using a micrometer (Draper PM 025) with a modified tip to fit the curvature of the eggshell. Measurements were done in three different areas of the equatorial zone of the shell and a mean of the three measurements was used for the analysis. The repeatability of the eggshell thickness measures was high ($r > 0.99$) ($F_{2, 93} = 0.003$, $p = 0.997$).

● Mammillary layer contact area - I cut pieces of eggshell using an electric hand drill approximately 1cm^2 and soaked them in distilled water for 2-3 days to facilitate the manual removal of the shell membrane. To remove the remaining membrane from the inner surface, I put eggshell fragments into bleach containing sodium hypochlorite (NaClO) for approximately 30 minutes, and rinsed them with distilled water after removing all eggshell membrane by bleaching, eggshell fragments were oven dried at 37° for 10 minutes. Eggshell fragments were mounted on aluminium stubs and coated with gold-palladium under vacuum and examined under a scanning electron microscope (SEM) (x 250 magnifications). To estimate percentage of mammillary cone contact area, I traced mammillary cone contact area on a known eggshell fragment area (500 x 500 pixels) of SEM image using the software Image J (Rasband, 2006).

● Pore density - The membrane of eggshell fragments was removed by flooding with Decalcifier II for 2 minutes and put into water for few seconds to stop the reaction. To make pores visible, the eggshell was flooded with Decalcifier II for 15 minutes again and put into water for a few seconds to stop the reaction. After drying, the eggshell

fragments were placed under a dissecting microscope (x 25 magnification) and the pores were counted from a known surface area of shell (9 mm^2). Pore density (the number of pore per cm^2) was estimated from a mean of four counts. The repeatability of 4 pore counts was low ($r = -0.004$; $F_{3,124} = 0.679$, $p = 0.567$). This may be because pores are randomly distributed on the eggshell, or pores were counted from only a small area.

● The total functional pore area (*Ap*) - Eggshell fragments were placed into bleach for 45 minutes to remove the eggshell membrane and organic materials. After bleaching, eggshell fragments were rinsed with distilled water and oven dried at 37°C. They were mounted on the aluminium stub and coated with gold-palladium mixture before viewing pores from outer surface. I randomly selected 5 pores from each fragment and took a digital image of each pore under SEM (x 1000 magnification). The width of the pore was measured from the SEM image using the software Image J (Rasband, 2006, Figure 6.2). The total functional pore area was calculated using the equation (Ar *et al.*, 1974):

Ap = total number of pores per egg x mean area of individual pores on the egg

Mean area (*S*) of individual pores on the egg was calculated according to the following equation (Hoyt, 1976):

$S\ (cm^2) = [4.393 + 0.394 \times \text{egg length} / \text{egg breadth}] \times \text{egg volume}^{0.667}$

6.2.4 Statistical analysis

All variables were checked for normal distribution. The total functional pore area (*Ap*) required a logarithmic transformation to make normal distribution. Percentage of mammillary cone contact area was transformed using arcsine. The residual of hatching duration was used after controlling for the effect of laying order and laying-to-pipping interval. All means are reported with standard deviation. SPSS (version 15.0 for Window, 2006) was used for statistical analysis. Repeated measured ANOVA was used to test whether there was a difference in eggshell characteristics and egg mass between collected and hatched eggshells and between laying orders. For the analysis of the effect to eggshell characteristics on hatching duration, body size and growth rate, I used general linear models. The full models included the following effects: laying date, laying order, egg mass, laying-to-pipping interval (laying-to-hatching

interval for skeletal size and growth), offspring sex, eggshell thickness, total functional pore area and percentage of mammillary cone contact area. Interactions were reported only when they were significant ($p < 0.05$). Parameter estimates (B ± 1 S.E.) were presented only in the case of significance.

6.3 RESULTS

6.3.1 Eggshell characteristics and hatching duration

Collected (fresh) and hatched eggs were similar in egg mass, laying date and laying order and there were no differences of eggshell characteristics between the first- and second-laid eggs in collected eggshells (Table 6.1 and 6.2). Therefore, it was assumed that the eggshell of collected eggs was similar to the eggshell of the other egg that was kept in the nest to hatch.

Hatching duration significantly related to mammillary cone contact area (Table 6.3, Figure 6.3). Chicks from eggshells with higher mammillary cone contact area took longer from pipping to hatching than chicks from eggshells with lower mammillary cone contact area. Hatching duration also related to an interaction between laying order and laying to pipping interval although it was not significant. In the first-laid eggs, hatching duration decreased with laying to pipping interval. However, in the second-laid eggs, hatching duration increased with laying-to-hatching interval. However, offspring sex, laying date, eggshell thickness, total functional pore area and egg mass did not affect hatching duration.

6.3.2 Eggshell characteristics and hatching body condition and growth rate

The skeletal size at hatching was affected by eggshell characteristics (Table 6.4). Hatchlings from eggs with thicker eggshells had shorter tarsus at hatching (parameter estimates: B = - 11.6 ± 5.76 mm) although it was not significant. Tarsus and head plus bill length were related to egg mass while wing length was independent on egg mass. Head plus bill length was longer when chicks hatched from thicker eggshell although it was not significant after Bonferroni correction. Eggshell characteristics did not affect wing length at hatching.

Skeletal growth rate has been driven by entering instantaneous growth rate of tarsus, head plus bill and wing into a principal components analysis (PCA). Skeletal growth rate increased with eggshell thickness (Table 6.5, Figure 6.4). Mammillary cone contact area, total functional pore area, offspring sex, egg mass and laying data were not related to skeletal growth rate.

6.3.3 The effect from foster parents

Eggshell characteristics and egg mass did not differ between collected eggshell and eggshell characteristics from collected eggs in fostered nests (independent t-test, eggshell thickness: $t_{80} = 0.53$, $p = 0.59$; mammillary cone contact area: $t_{75} = 0.008$, $p = 0.99$; total functional area: $t_{74} = 0.566$; egg mass: $t_{80} = 0.03$, $p = 0.974$; sample size varied due to missing data of eggshell characteristics in fostered nests). Skeletal growth rate of collected eggs was not related to eggshell characteristics of collected eggshells from fostered parents (Table 6.6).

Table 6.1 Comparisons of egg mass in hatched and collected fresh eggs on the day of laying. Type indicated hatched and collected eggshells. *F* and p-values refer to repeated measured ANOVA.

	Collected and hatched eggs	
	F	p
Types	$F_{1,38} = 1.41$	0.243
Laying date	$F_{1,38} = 1.44$	0.237
Laying order	$F_{1,38} = 0.85$	0.363
Types * laying date	$F_{1,38} = 1.50$	0.229
Types * laying order	$F_{1,38} = 2.40$	0.130

Table 6.2 Comparisons of eggshell characteristics between the first-laid eggs and the second-laid eggs in 41 fresh eggs. t and p-values refer to independent t-test.

	Collected eggshells	
	First-laid eggs	Second-laid eggs
	t	p
Eggshell thickness	$t_{39} = 1.11$	0.276
Mammillary cone contact area	$t_{39} = 1.01$	0.319
Total functional pore area	$t_{39} = 1.45$	0.156

Table 6.3 The relationship between hatching duration and eggshell characteristics, offspring sex, laying order, egg mass, laying date and laying-to-pipping interval in 32 nests. 1 of 33 nests with data of hatching duration was excluded due to no data of offspring sex. *F* and p-values refer to ANCOVA.

	Hatching duration		
	F	p	B ± 1 S.E.
Eggshell thickness	$F_{1,24} = 0.29$	0.594	
Mammillary cone contact area	$F_{1,27} = 6.07$	0.020	1.46 ± 0.594
Total functional pore area	$F_{1,22} < 0.01$	0.960	-
Offspring sex	$F_{1,26} = 2.29$	0.142	-
Laying order	$F_{1,27} = 4.52$	0.043	11.95 ± 5.617
Egg mass	$F_{1,23} = 0.02$	0.880	-
Laying date	$F_{1,25} = 0.58$	0.453	-
Laying-to-pipping interval	$F_{1,27} = 3.87$	0.059	-
Laying order * laying-to-pipping interval	$F_{1,27} = 4.11$	0.052	-

Table 6.4 The effect of eggshell characteristics on skeletal size at hatching. Hatching body condition was estimated in 27 eggs with chick measurements within 24 hours after hatching. *F* and p-values refer to ANCOVA. Bold letters indicate significance after Bonferroni correction.

	Tarsus			Head pus bill			Wing	
	F	p	B ± 1 S.E.	*F*	p	B ± 1 S.E.	*F*	p
Eggshell thickness	$F_{1,23}$ = 4.13	0.054	-	$F_{1,24}$ = 5.88	0.023	18.97 ± 7.82	$F_{1,20}$ = 0.01	0.898
Mammillary cone contact area	$F_{1,22}$ = 2.26	0.146	-	$F_{1,22}$ = 1.66	0.210	-	$F_{1,19}$ = 0.04	0.843
The total functional area (*Ap*)	$F_{1,20}$ = 0.36	0.551	-	$F_{1,19}$ = 0.20	0.658	-	$F_{1,21}$ = 0.06	0.807
Laying order	$F_{1,24}$ = 4.48	0.045	- 0.51 ± 0.24	$F_{1,23}$ = 4.17	0.053	-	$F_{1,22}$ = 2.89	0.103
Egg mass	$F_{1,24}$ = 72.65	**< 0.001**	0.14 ± 0.01	$F_{1,24}$ = 11.64	**0.002**	0.07 ± 0.02	$F_{1,25}$ = 2.33	0.139
Offspring sex	$F_{1,19}$ = 0.07	0.783	-	$F_{1,20}$ = 0.76	0.392	-	$F_{1,23}$ = 2.38	0.137
Laying date	$F_{1,21}$ = 0.33	0.571	-	$F_{1,21}$ = 1.07	0.313	-	$F_{1,24}$ = 2.52	0.125

Table 6.5 The relationship between skeletal growth rate and eggshell characteristics in 35 chicks. 1 of 36 chicks with growth rate was excluded due to no data of offspring sex. Skeletal growth rate was estimated from instantaneous growth rate of tarsus, head plus bill and wing. *F* and p-value refer to ANCOVA.

	Skeletal growth rate		
	F	p	B ± 1 S.E.
Eggshell thickness	$F_{1,33} = 9.35$	0.004	23.76 ± 7.770
Mammillary cone contact area	$F_{1,27} < 0.01$	0.998	-
Total functional pore area	$F_{1,28} = 0.05$	0.810	-
Offspring sex	$F_{1,31} = 2.17$	0.151	-
Laying order	$F_{1,29} = 0.26$	0.613	-
Egg mass	$F_{1,32} = 2.45$	0.127	-
Laying date	$F_{1,30} = 2.54$	0.121	-

Table 6.6 Chick growth rate and foster parents' eggshell characteristics in 29 nests. 1 egg of 36 nests with growth rate was excluded due to no data of offspring sex and 6 eggs were excluded due to no data of foster parents' eggshell characteristics. Skeletal growth rate was estimated from instantaneous growth rate of tarsus, head plus bill and wing. *F* and p-value refer to ANCOVA.

	Growth rate		
	F	p	B ± 1 S.E.
Eggshell thickness of foster parents' eggshell	$F_{1,24} = 0.49$	0.488	-
Mammillary cone contact area of foster parents' eggshell	$F_{1,23} = 0.70$	0.410	-
Total functional pore area of foster parents' eggshell	$F_{1,21} = 0.03$	0.850	-
Offspring sex	$F_{1,27} = 2.00$	0.169	
Laying order	$F_{1,22} = 0.86$	0.773	-
Egg mass	$F_{1,25} = 2.05$	0.164	-
Laying date	$F_{1,26} = 2.05$	0.164	-

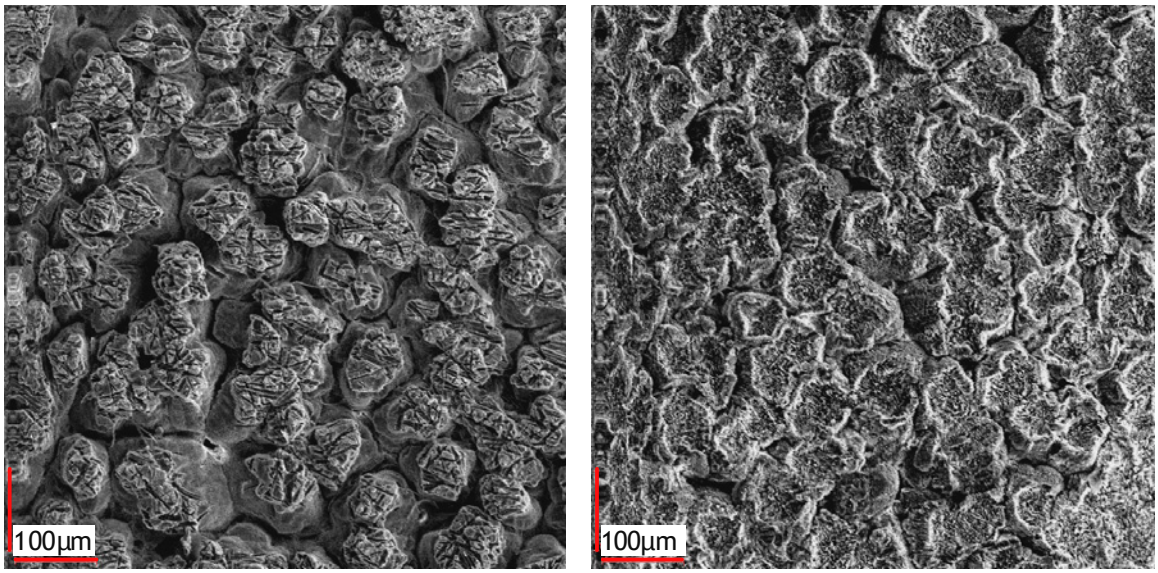

Figure 6.1 SEM of mammillary layer in collected fresh eggs (left) and incubated eggs (right) of lesser black-backed gulls (x 250).

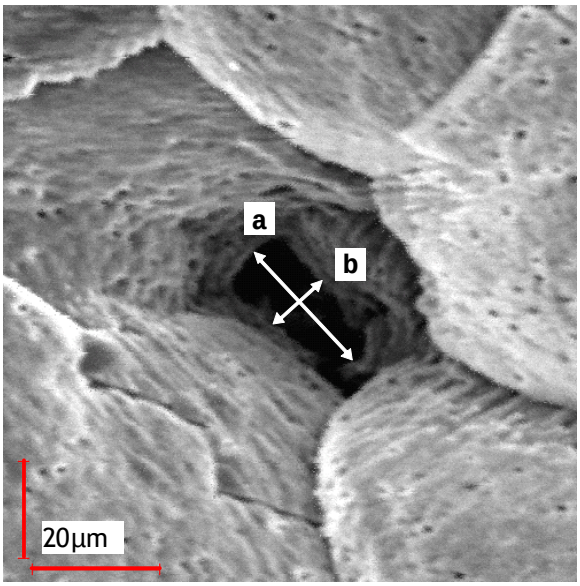

Figure 6.2 Measurement of eggshell pore length (a) and width (b) in lesser black-backed gulls using SEM (x 1000).

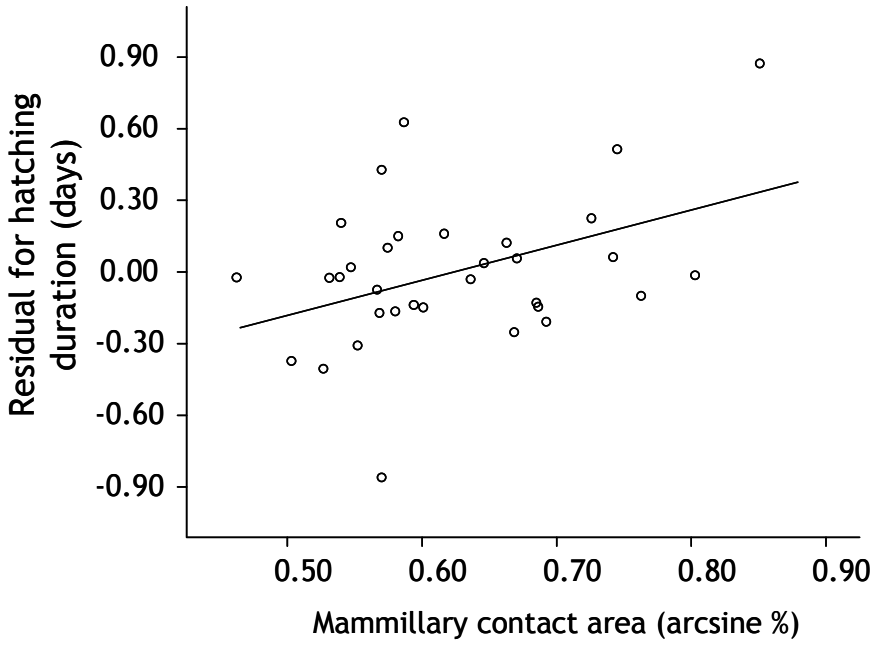

Figure 6.3 The relationship between mammillary contract area (arcsine %) and hatching duration of lesser black-backed gulls in 32 eggs (7 of 39 eggs were excluded due to no data of pipping date).

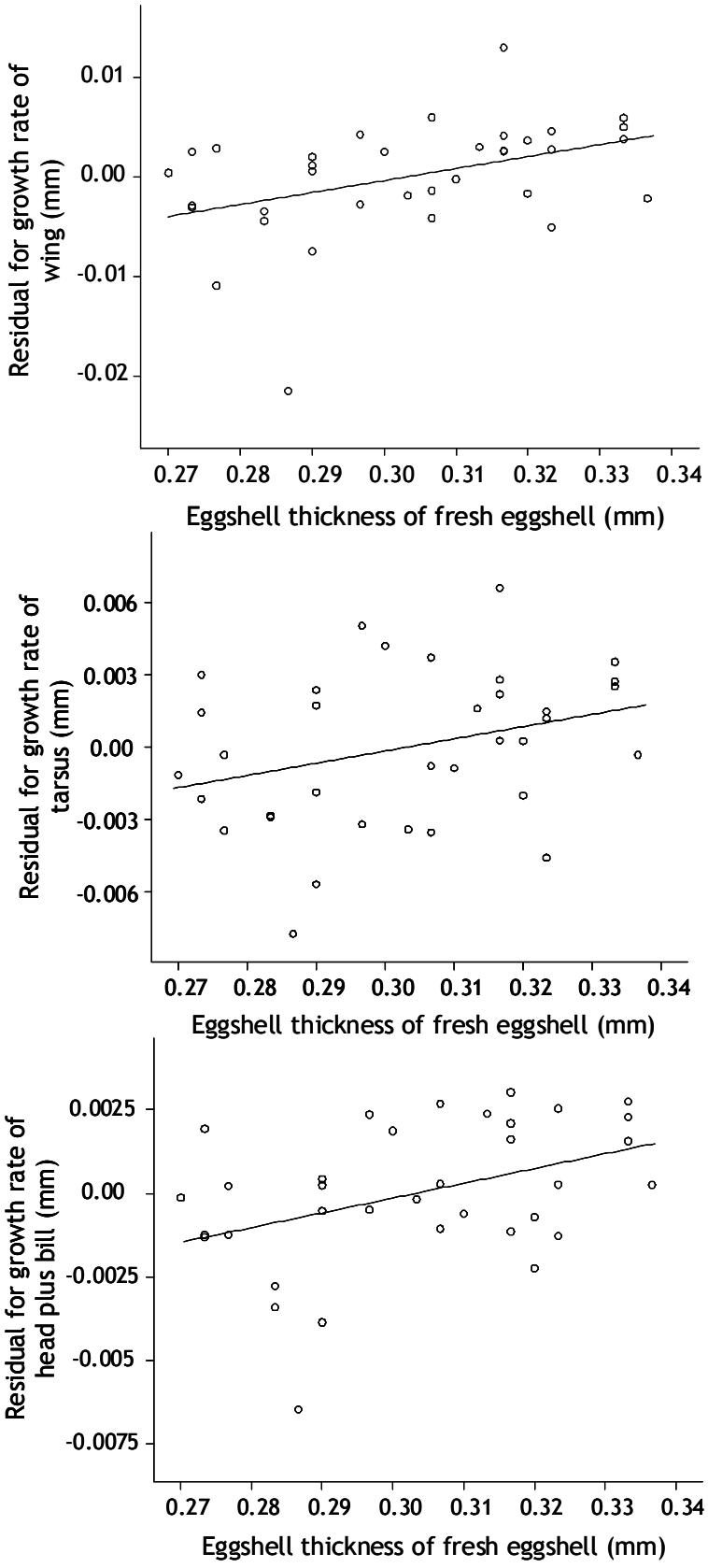

Figure 6.4 Eggshell thickness and growth rate of wing, tarsus and head plus bill in 34 nests of lesser black-backed gulls (5 of 39 nests were excluded due to chicks died before 24 days old).

6.4 DISCUSSION

Mammillary cone area of eggshell characteristics affected hatching duration. An embryo hatched from an eggshell with higher mammillary cone contact area took more time from pipping to hatching than one hatched from an eggshell with a lower mammillary cone area. Longer hatching duration in the eggshell with higher density of mammillary cones may be related to how much calcium the embryo takes from mammillary cone contact area. Blom and Lilja (2004) found that slowly growing species such as precocial species had a higher density of mammillary cone contact area. During the embryonic growth period, species with slow growth rates took calcium more extensively from mammillay cones and had higher degree of ossification of their skeleton than faster growing species such as altricial species. The degree of ossification was inversely correlated with growth rate (Williams *et al.*, 2000; Blom & Lilja, 2004). For example, Japanese quails (*Coturnix japonica*) with high growth rate had less ossification compared to quails with low growth rate (Blom & Lilja, 2004). As in previous studies, embryos of lesser black-backed gulls might spend more time to obtain calcium from the mammillay cones when it is available.

I expected that total functional pore area would be related to hatching duration because it is related to gas exchange rate which affect embryonic metabolism. For example, lower porosity limits oxygen exchange (Tullett & Deeming, 1982) and slows embryonic metabolism (Burton & Tullett, 1983). However, total functional pore area did not affect hatching duration. Total functional pore area may not affect hatching duration because the embryo could breathe through its lung after pipping. It is also possible that functional pore area which was estimated from eggshell thickness and function pore size is not an accurate measure of actual gas exchange. Pores of lesser black-backed gulls had various shape and size (range: 0.001 ~ 0.022 cm^2). The channel of the pore was narrow and sometimes straight through the eggshell. It might bias the measure of actual total functional area.

It is possible that chicks hatched from thicker eggs may have a difficulty in breaking thicker eggshells when they it hatches and have longer hatching duration. However, eggshell thickness did not relate to hatching duration in this study. Although thicker eggshell is difficult to break at hatching, chicks hatched from thicker eggshells might have a stronger skeleton because chicks have been able to intake more calcium from the thicker eggshell.

Hatching duration also related to offspring sex. For example, male embryos hatched one day faster than female embryos in sexually monographic black guillemot (*Cepphus grylle*) (Cook & Monaghan, 2004). Although lesser black-backed gulls are a sexually dimorphic species, hatching duration was similar in female and male embryos in the present study.

In the first-laid eggs, hatching duration decreased with laying-to-pipping interval while in the second-laid egg, hatching duration increased with laying-to-pipping interval. Although it is difficult to explain, it might be related to different onset of incubation between the first- and second-laid eggs. As the first-laid eggs were not incubated immediately after they were laid, they may have longer laying-to-pipping interval than the second-laid eggs.

Eggshell is the main source of calcium requirements during embryonic development (Romanoff & Romanoff, 1949). Chicks hatched from thicker eggshells, had longer head plus bill length at hatching. Thicker eggshell may provide the embryo with more calcium to develop a longer skeleton than thinner eggshell. However, tarsus at hatching was negatively correlated to eggshell thickness and wing at hatching did not relate to any eggshell characteristics. It may relate to different growth rates of each skeleton. Developed head plus bill might be more important to break eggshell during the hatching process than tarsus or wing. If it is, the embryo may develop head plus bill first when it can take more calcium from thicker eggshell.

Mammillary contact area and total functional pore area did not affect the skeletal size at hatching. The level of ossification at hatching can differ among chicks although they have same skeletal size (Dobado-Berrios & Ferrer, 1997; Tilgar *et al.*, 2004). To investigate, it would be required to know the degree of ossification at hatching rather than comparing skeletal size of chicks hatched from shells with high and low mammillary density. Calcium in the yolk can also affect skeletal size at hatching because embryos mainly use calcium in yolk at an early stage of embryonic development. Therefore, further experiments are needed to investigate the relationship between eggshell thickness and calcium in yolk and skeletal growth of the chick.

Skeletal growth rate controlled for hatching skeletal size and was also positively related to eggshell thickness. Although previous study showed that the effect of calcium from egg on chick growth did not persist through the post-hatching period due

to parental feeding (Tilgar *et al*., 2005), this result showed that chicks hatched from thicker eggshells showed faster growth rate until at least 24 days after hatching. Parental quality is more important for chick survival than egg quality (Bolton, 1991). So, I looked at the relationship between fostered parents' eggshell characteristics and growth rate of chicks. Good quality parents which encouraged chicks grow faster may lay eggs with better eggshell characteristics. However, there was no relationship between chick growth and fostered parents' eggshell characteristics. Also, cross-fostering design removed the effect of parental quality. Therefore, this result suggested that faster skeletal growth of chicks hatched from thicker eggshells resulted from eggshell characteristics rather than parental quality.

In conclusion, eggshell characteristics can affect not only embryonic developmental rate but also chick growth after hatching. Although the effect of egg composition or egg mass on chick development has been studied, the effect of eggshell has not been studied very well. In the future, it needs to consider the effect of eggshell characteristics on embryo or chick development.

Chapter VII.

General discussion

Hatching patterns in birds' clutches have been studied many times, as they are believed to be an important factor affecting parental fitness and reproductive success. Synchronous hatching may lead to costs to parents by increasing parental effort during the nestling period (Hussell, 1972), or may reduce fledging mass of chicks (Potti, 1998) which can affect their survival after fledging. Synchronous hatching may reduce fitness due to increased competition within a clutch (Hahn, 1981). However, synchronous hatching often increases the survival of last-laid eggs and reduces the period of parental care. With asynchronous hatching, parents may get benefits through brood reduction, spread peak work load time or reduce competition within the brood, while asynchronous hatching induces higher mortality of last-laid eggs and requires a longer period of parental care. In nature, hatching patterns vary not only between species but also between individuals. Birds may alter hatching patterns depending on their body condition and environmental conditions. Hence, there might be trade-offs to decide optimal hatching patterns under any given conditions.

The findings from this study confirmed that egg laying and early incubation is a critical period to determine hatching patterns. Parents can influence the hatching pattern of their clutch through incubation behaviour during these periods. Herring gulls (*Larus argentatus*) which frequently attended their nests during egg-laying and early incubation had a brood with more synchronous hatching. Higher nest attendance during egg-laying and early incubation gave benefits of high hatching success of first-laid eggs to parents when they laid smaller eggs. This might be due to accelerated development of the last-laid eggs. The last laid egg developed faster than the last-laid egg in a control clutch when it needed to catch-up earlier-laid eggs. Hence, hatching patterns might be selected for by egg viability and the survival of last-laid eggs. Although higher nest attendance during egg-laying and early incubation may increase asynchronous hatching in a brood, this may in part be compensated by acceleration of embryonic development in last-laid eggs which may reduce hatching asynchrony.

However, accelerated development usually brings not only benefits but also costs even including costs that may not be evident until adult life. For example, costs at a physiological level can affect later life time but the ecological costs may occur

immediately (reviewed in Metcalfe & Monaghan, 2001). In this thesis, accelerated eggs had higher hatching failure although it is difficult to entirely confirm that these results were due to accelerated development because of small sample size and a possibility of egg neglect in last-laid eggs. If it is true, the cost of accelerated embryonic development may be paid during the embryonic period rather than during the post-hatching period in lesser black-backed gulls (*Larus fuscus*), because growth rate and survival of last-laid eggs were similar between treatments. Strategy of catch-up growth may vary in species and individual. If birds can overcome the costs of accelerated development through greater benefits, it may be that this strategy is selected. Alternatively, the time scales of costs and benefits of accelerated development may give birds overall higher fitness. For example, if the costs of accelerated development appear after reproductive success, it will be still benefit them although accelerated development might shorten life span (Metcalfe & Monaghan, 2001). For a future study, it would be helpful to understand the strategy of the last-laid eggs to investigate whether there are costs in later life. If hatching synchrony in this thesis is adaptive behaviour to increase breeding success, parents may need to account for the cost of accelerated development of last-laid eggs as well.

As other factors affecting embryonic development exist, I looked at the relationship between eggshell characteristics and hatching duration. Gas exchanges which affect embryonic metabolism occur through the eggshell pores (Ar & Rahn, 1985). Eggshell thickness and total functional pore area are involved in the diffusion rate of respiratory gases. Although I expected total functional pore area to relate to hatching duration, the relationship was not present. It might be that this was due to inaccuracy in the estimation of total function pore area. The shape varied considerably between pores and the channel of the pores is often narrower than the opening of the pore on the outer shell surface. The variable pore shape and the uncertainty of the narrowest diameter yielded a total functional pore area that probably does not accurately reflect actual porosity. In the near future, I plan to measure directly water vapour conductance. It would be worthwhile to compare the total functional area estimated from pore size and measured from actual water vapour conductance. Unexpectedly, I found that only mammillary cone contact area is related to hatching duration. Larger mammillary cone contact area on eggshell induced slower embryonic development. Chicks might spend more embryonic time to absorb calcium when calcium is available. Although mammillary cone contact area did not relate to skeleton size at hatching, it might be possible to relate it to the degree of ossification. Eggshell thickness positively related to skeleton size at hatching because embryos obtain calcium from eggshell

(Simkiss, 1961). The effect of eggshell thickness has been detected later during the nestling period. Chicks which hatched from eggs with thicker eggshell had faster growth of skeleton after hatching. This result may indicate that calcium is limited in the study area and chicks can not compensate through the nestling period. A previous study of lesser black-backed gulls found that supplemented calcium to adults during egg formation increased eggshell thickness (Tharapoom, 2006). A limitation in calcium availability in adults may also be present. Hence, eggshell thickness in gulls might be an indicator of the calcium availability in the area. While fish contain large amounts of calcium, many terrestrial foods taken by gulls, such as grain, have rather low levels of calcium.

Diet during egg-laying and early incubation may affect hatching patterns through altering incubation behaviour of parents (Eikenaar *et al.*, 2003). Findings in the thesis showed that when females consumed marine food and food with higher trophic levels, nest attendance during egg-laying and early incubation declined. Marine food and food with higher trophic levels may require more time to forage and this may limit incubation time. Herring gulls often specialized on a certain type of food during the pre-laying and incubation period (Pierotti & Annett, 1991). For a specialist of marine diet, low availability of marine food might result in lower nest attendance during egg-laying and early incubation, and this might induce an asynchronously hatched brood since lower nest attendance increased hatching asynchrony in this thesis (Chapter 3). By comparison, a specialist of terrestrial food might have higher nest attendance and a synchronous brood. Hence, hatching patterns might relate to diet.

Lowther (1988) suggested that hormonal change during egg-laying and early incubation may influence the function of the pigment glands. In the present study, I looked at the effect of incubation behaviour on within-clutch variation in eggshell colour. As expected, findings from the present thesis showed that females that rapidly increased daily nest attendance showed paler last-laid eggs when females changed incubation behaviour. A possible mechanism of the relationship between incubation behaviour and within-clutch variation in eggshell colour is that an increase of plasma prolactin level in females initiates incubation behaviour and accompanies a decline of steroid hormones. Depression of steroid hormone may affect deposition of eggshell pigments. There were few studies using captive birds to show the relationship between eggshell colour variation and hormonal change during egg-laying and early incubation period (Soh & Koga, 1994). For wild birds, it might be difficult to catch birds just before oviposition. Hence, manipulation of steroid hormone or prolactin may be helpful to

understand the mechanism of within-clutch variation in eggshell colour. To understand the relationship between hormonal change and eggshell colour variation, it may be worth investigating whether there is a difference in hormonal change and incubation behaviour between species with and without eggshell colour variation within a clutch. In this thesis, differences in eggshell colour between penultimate and last-laid eggs related to hatching interval between them. It might reflect the change of incubation behaviour during egg-laying. If it has been confirmed in other species, within-clutch variation in eggshell colour might be useful as a predictor of hatching interval.

Results in the thesis showed that hatching patterns were determined not only by parents but also by embryos (in the last-laid eggs) (Figure 7.1). In addition, other factors such as diet and eggshell characteristics (mammillary cone contact area) also affected hatching patterns. Hence, these complex factors should be considered in the study of hatching patterns. Parents may decide incubation behaviour through benefits and costs of hatching patterns. Herring gulls may have benefits of higher hatching success but also it may accompany with costs such as synchronous hatching, which may increase competition and parental efforts. However, benefits and costs of hatching patterns may differ depending on environmental condition and physiological condition of parents.

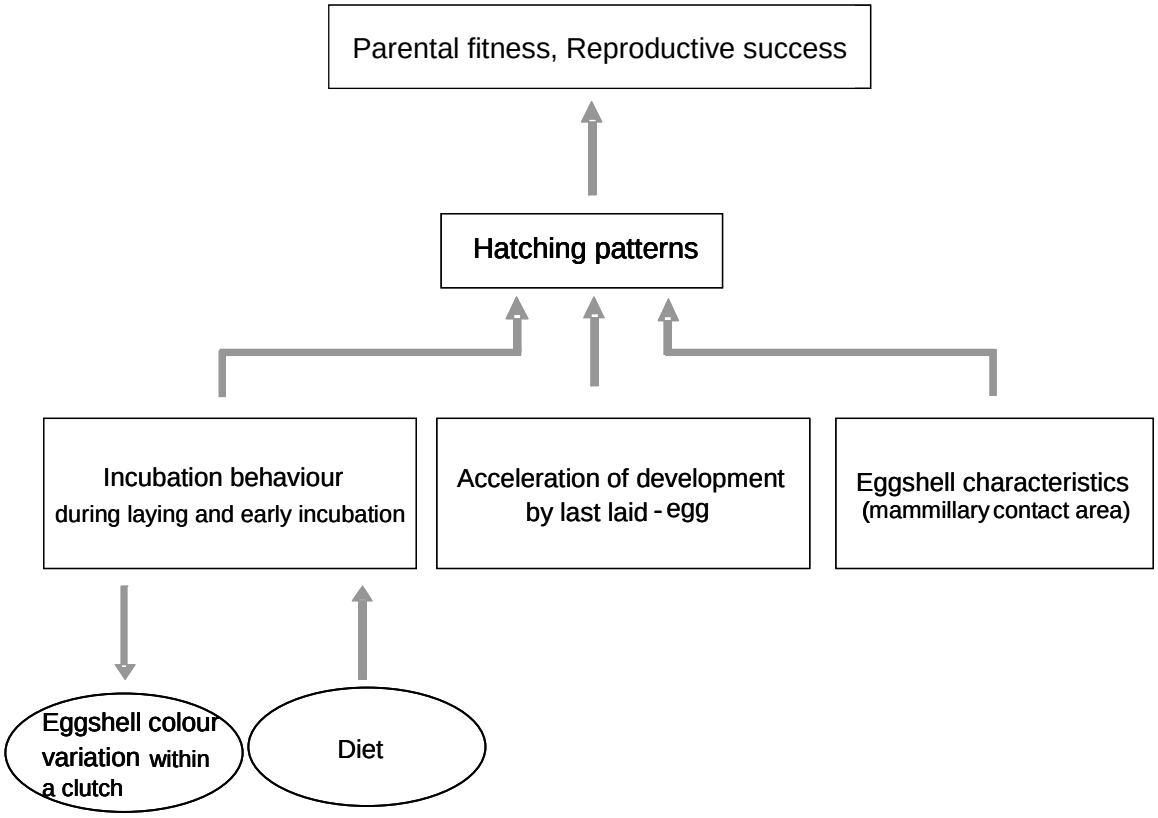

Figure 7.1 Factors affecting hatching patterns and eggshell colour variation in relation to incubation behaviour. Hatching patterns were affected by not only incubation behaviour during egg-laying and early incubation but also embryonic developmental rate and eggshell characteristics. Diets during egg-laying and early incubation may affect hatching patterns through altering incubation behaviour. As a consequence of incubation behaviour, within-clutch variation in eggshell colour may be explained.

APPENDIX

Here, I discuss some of the ideas and objectives that I considered, or was unable to carry though during my PhD study. During the first year of my PhD, I had planned two experiments to investigate costs and benefits of accelerated development and retarded development in last-laid eggs, although I managed to do only one experiment (accelerated development). At the beginning of fieldwork, I manipulated clutches with expanded laying intervals and with no laying intervals between the penultimate and last-laid eggs. These experiments needed large population numbers because three eggs in each experimental nest had to come from biologically unrelated nests and laying dates need to be matched between fostered and experimental nests. After hatching, last-laid chicks should be transferred into host nests which are not manipulated and have normal hatching interval to control hatching span which can affect size hierarchy within a clutch. During the fieldwork in Iceland, I found 487 nests in the study area. However, egg collecting was common in Iceland and some of my experimental nests were collected by people. Another difficulty in Iceland occurred after chicks hatched. Although the gull population was large in 2004 (36,600 pairs) and 2005 (29,000 pairs) (Hallgrimsson *et al.*, 2007), breeding success was very low in 2005. Breeding Arctic terns (*Sterna paradisaea*) near the study area also had very low breeding success. It may be that the low success was related to food availability. I found some chicks dead far from their nests (more than 20 m) and big chicks more than 20 days old died without a sign of injury. It may be that these observations indicate starvation of chicks. Chicks in the study area might be also vulnerable to predation because of short vegetation cover. I often found some chicks dug in the ground to hide themselves. In 2006, the number of breeding pairs in Iceland collapsed with 7,395 pairs (Hallgrimsson *et al.*, 2007). That may due to low breeding success in 2005. It would be worth investigating how quickly a gull breeding population in Iceland changes and recovers depending on environmental change such as food availability. As a consequence of the low breeding success, I dropped one experimental group for retarding development and I could not manage to foster hatched last chicks after hatching. Thereafter, almost all hatched chicks grew in their incubated nests except three nests with longer hatching interval than normal. Small sample size reduces the power in statistical analysis (Chapter 5).

The relative proportion of marine food in a pellet correlated with nitrogen stable isotope during egg-laying period and chick rearing period although the proportion of marine food items was low in pellets. Klaassen *et al*. (2004) suggested that pre-laying female diets can be evaluated from hatchling down. The carbon stable isotope ratio of yolk was related to the ratio in the hatchling down although on average values in down were 3.1 ‰ higher than in yolk. It was interesting to see how stable isotope values changed after the chicks hatched. Diet change after hatching is consistent with other studies using regurgitates (Annett & Pierotti, 1989; Bukacinska *et al*., 1996). To analyze preference of food items in each nest, I used a self-organizing map (SOM). SOM estimates the distance between factors and organizes a group. In Chapter 2, I input the proportion of each diet item per nest and nests with similar diet composition are organized according to the SOM learning rule (Kohonen, 1982). Results of SOM visually showed the relationship between diet items and the preference of diet in each pair. For example, pairs which had insects and mammal (rats) in their pellets also had grass in their pellets. It may be that gulls found insects or rats in grass land. Although SOM has not been commonly used for analyzing ecological data before, it may be a useful tool to identify individual diet preference in study area because it visually shows the group which consumes similar diet.

Eggshell colour was analyzed using digital images. It has been commonly used for colour analysis in various studies, for example eggshell colour variation of museum specimens (Surmacki *et al*., 2006) and iris colour variation of live birds (Bortolotti *et al*., 2003). Although digitalized photography is an easy tool to quantify eggshell colour in the field, it may need reference of actual colour spectrum and UV ranges to use RGB-colour values as an indicator of spectrum or eggshell pigments. In lesser black-backed gulls, RGB-colour value did not indicate blue-green chroma (Tharapoom, 2006) which were correlated with biliverdin (Sanpera *et al*., 2007) whilst RGB-colour correlated with the wavelength of brown colour which may indicate porphyrin in eggshell colour. In the thesis, I did not investigate pigments in eggshell of herring gulls. In herring gulls, eggshell contains all three pigments, porphyrin, biliverdin and zinc chelate of biliverdin (Kennedy & Vevers, 1976). In the future, it would be good to know how the amount of pigments in the eggshell relates to RGB-colour values to use RGB-colour as an indicator for the amount of pigments in the eggshell.

For studying eggshell characteristics, plasma etching has been usually used for removing organic materials from the eggshell surface (Hunton, 1995; Macleod *et al*., 2006). Although this method precisely removes organic materials from the eggshell without damage of surface, it takes more than 4 hours. In the lab, I used bleach to remove all organic materials and visually compared the SEM images between etched and bleached eggshell surface. Mammillary layer and outer surface of eggshell were visually identical. Use of bleach may reduce the amount of time to remove organic materials eggshell surface although it still needs more precise comparison in eggshell structure.

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