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Avian embryonic development does not change the stable isotope composition of the calcite eggshell

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Abstract. The avian embryo resorbs most of the calcium for bone formation from the calcite eggshell but the exact mechanisms of the resorption are unknown. The present study tested whether this process results in variable fractionation of the oxygen and carbon isotopes in shell calcium carbonate, which could provide a detailed insight into the temporal and spatial use of the eggshell by the developing embryo. Despite the uncertainty regarding changes in stable isotope composition of the eggshell across developmental stages or regions of the shell, eggshells are a popular resource for the analysis of historic and extant trophic relationships. To clarify how the stable isotope composition varies with embryonic development, the $\delta^{13}C$ and $\delta^{18}O$ content of the carbonate fraction in shells of black-headed gull (*Larus ridibundus*) eggs were sampled at four different stages of embryonic development and at five eggshell regions. No consistent relationship between the stable isotope composition of the eggshell and embryonic development, shell region or maculation was observed, although shell thickness decreased with development in all shell regions. By contrast, individual eggs differed significantly in isotope composition. These results establish that eggshells can be used to investigate a species' carbon and oxygen sources, regardless of the egg's developmental stage.

Additional keywords: black-headed gull, calcium resorption, carbon isotopes, maculation, oxygen isotopes, shell thickness.

Introduction

The dynamics of the formation and resorption of the calcite layer of the eggshell are an important aspect of avian reproduction with considerable biological and economical significance. In wild birds, a rapid and complete eggshell bio-mineralisation is critical to the individual's reproductive success. Any disruptions to this process, for instance through exposure to environmental toxins (e.g. dichlorodiphenyltrichloroethane (DDT)), can have dramatic consequences on a species' population viability or even its long-term survival (Porter and Wiemeyer 1969; Bitman *et al.* 1970; Schapira 2004; Jagannath *et al.* 2008). Furthermore, avian embryos rely on their eggshell to meet up to 80% of their calcium needs for bone mineralisation, and this dependence on shell calcium can extend even beyond hatching (Crooks and Simkiss 1975; Carey 1983; Deeming 2002*b*). In domestic fowl (*Gallus gallus*) eggs the quality of the calcite shell determines the egg's ability to withstand handling and bacterial contamination and is thus a prominent factor in the production of the hatching egg and the table egg for human consumption alike (Roberts and Brackpool 1993–1994; Poppe *et al.* 1998; Roberts 2004). This critical role of the calcite shell for embryonic development means that the understanding of avian reproduction will benefit greatly from detailed information on how the embryo uses this resource.

Embryonic calcium resorption is best known in the precocial domestic chicken, in which ~5% of the calcite shell is resorbed for bone mineralisation in the course of embryonic development (Crooks and Simkiss 1975; Carey 1983). This process, however, is not continuous but increases during incubation. In chicken embryos mobilisation of eggshell calcium begins only at day 10–12 (i.e. halfway through the incubation period; Carey 1983), and a similar onset of calcium resorption occurs in other precocial species (Vanderst and Richards 1970; Castilla *et al.* 2007). This calcium resorption changes the eggshell structurally (Blom and Lilja 2004; Chien *et al.* 2009) leading to a measureable decline in thickness of the calcite shell (Finnlund *et al.* 1985; Castilla *et al.* 2010; but see Bunck *et al.* 1985) and its breaking strength (Castilla *et al.* 2007). Not all parts of the eggshell are used equally by the embryo during growth and development, resulting in a differential decline in eggshell thickness during incubation. In particular, the blunt pole of the eggshell just above the air sac shows little resorption and maintains its thickness and strength throughout incubation, while the other regions of the shell are more prone to thinning (Beacham and Durand 2007; Castilla *et al.* 2007). Finally, the localised deposition of pigments, in the form of eggshell maculation, has been suggested to affect shell thickness, change the rate of gas transfer across the shell, or even produce a

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localised drop in shell temperature due to reflection of infrared radiation (Gosler *et al.* 2005; Higham and Gosler 2006). Thus, pigmentation may either directly or indirectly influence chemical reactions such as calcium dissolution from the eggshell. The qualitative changes of the eggshell described above suggest that the mobilisation of shell calcium through the avian embryo varies quantitatively with incubation stage and region of the eggshell. As a consequence, the chemical composition of the calcite eggshell may change differentially with embryonic development. This change could provide information about the incubation stage of the egg or the differential use of shell regions.

Eggshell formation and resorption occur on very different timescales but use similar mechanisms. The deposition of calcium during eggshell formation is an extremely rapid process, which takes 15–17 h in the domestic fowl, a species that lays in daily intervals like most birds (Roberts 2004). This unusually fast biomineralisation completely depletes the mother's daily calcium intake, forcing her to mobilise calcium for egg production from medullary bone tissue (Simkiss 1961; Dacke *et al.* 1993; Houston *et al.* 1995; Reynolds 1997). During eggshell formation in the shell pouch the egg is bathed in free calcium ions and bicarbonate, which precipitate spontaneously on the surface of the shell membrane to form the calcite shell (Nys *et al.* 1999; Hincke *et al.* 2010). This extra-cellular process depends on the production of bicarbonate through the hydration of CO_2 by carbonic anhydrase (Nys *et al.* 1999) and the transport of calcium ions into the lumen (Bar 2009).

By contrast, calcium resorption from the calcite shell is a greatly extended process that takes 7 days in the quail (*Coturnix coturnix*) and 10 days in the domestic chicken, even though only a fraction of the shell is dissolved (Carey 1983; Castilla *et al.* 2007). Like calcite formation, however, its dissolution occurs in an extracellular milieu, although the exact mechanism of the calcium dissolution is unknown (Packard 1994). For the erosion of the calcite shell, the room between the calcite shell and the chorioallantoic membrane (CAM) may fill with carbonic acid, created by carbonic anhydrase. The acid then dissolves calcium ions from the mammillary cones of the shell, its innermost layer, which can then be absorbed by blood vessels in the CAM (Crooks and Simkiss 1974; Tuan *et al.* 1991; Deeming 2002*b*).

The processes of shell formation and dissolution, like any chemical reaction, lead to isotopic fractionation of the elements involved. As a result, both the oxygen and the carbon used in the creation of the shell occur in a distinct isotope composition that may hold information about the shell calcite use by the embryo. The formation of calcite in an exchange reaction in the lumen of the shell pouch is likely to cause an increase of heavy carbon and oxygen isotopes (enrichment) in the calcite compared with the concentration in the bicarbonate substrate. Similarly, the exchange reaction during the dissolution of calcite in the course of embryonic development should create an increase in the heavier C and O isotopes in the shell calcite (Fry 2006).

Here we use fresh eggshells of a wild bird species to determine the variation in isotope composition caused by embryonic development both in the course of incubation and relative to the sampling location on the egg. We predict first that in our samples the isotope composition ($\delta^{13}C$ and $\delta^{18}O$) of the eggshell will differ with the stage of embryonic development. Specifically, eggs that experienced larger calcium resorption in the course of incubation should show higher $\delta^{13}C$ and $\delta^{18}O$ values than those whose shells lost little calcium. Second, we expect that within a single eggshell, the regions used most by the embryo for calcium requisition should show higher $\delta^{13}C$ and $\delta^{18}O$ values than little used regions. For instance, the calcite shell at the equator of the egg should have higher isotope ratios than the blunt end (Castilla *et al.* 2007). These data will clarify how isotope ratios can be used to track embryonic calcium resorption.

Finally, the dynamics of eggshell stable isotope composition are an important consideration in studies of the trophic relationships in modern and historic environments using eggshell samples (Hobson and Montevecchi 1991). The developmental stage of the shell fragments used in this research or the egg region they originally belonged to are often unclear. It is thus crucial to establish how these factors influence the stable isotope composition of eggshell carbon and oxygen. Our research tests whether eggshells are isotopically homogenous, an implicit assumption of current eggshell isotope research (Emslie and Patterson 2007).

Materials and methods

Species

Black-headed gulls (*Larus ridibundus*) are a small (200–400 g) colonial gull species that lays 2–3 eggs per clutch (mean length 52 mm × width 37 mm) in shallow ground nests among vegetation (Cramp and Simmons 1983). Incubation can start variably with the first, second or last egg usually resulting in some hatching asynchrony. Black-headed gulls forage in both freshwater and marine habitats and their diet is varied and consists of terrestrial and aquatic invertebrates and occasionally vertebrates (Cramp and Simmons 1983).

Samples

Eggs used in the present study were collected under licence (Natural England #20092237) in early May 2009 from a colony (~100 pairs) in Cambridgeshire, UK, after heavy rains destroyed nests and caused the adult birds to abandon their clutches. As a result of flooding, no reliable nest information was available for the eggs to allow specific sampling of clutches. Instead, we selected the eggshells for our analysis from the total of over 300 eggs in a manner that ensured we sampled across multiple clutches. Based on their contents, the eggs were first classified into four developmental stages: (1) undeveloped, (2) developing yolk, (3) unfeathered embryo and (4) feathered embryo ready to hatch. As black-headed gulls usually commence breeding with the first egg (Cramp and Simmons 1983) eggs of the same stage are likely to belong to different females. Ten eggs of each stage were randomly chosen for further analysis. All eggs were cleaned by removing the blunt end with surgical scissors and washing out their contents under laboratory grade running water. Sampled eggs were soaked in de-ionised water to remove any remnant egg contents or external dirt and dried at room temperature (25°C) for two weeks. Finally, eggs were cut into two equal halves using a Microtorque 2 dental drill

(Milnes Bross, Croydon, UK) with an 817T diamond head (Intensive Swiss Dental, Grancia, Switzerland) to prepare them for measurement of isotope composition and eggshell thickness.

Thickness measurements

The thickness of each of the 40 eggs was measured three times at both maculated and neighbouring plain areas (as judged by a human observer; see Fig. 1) at each of five regions along the long axis of the eggs, resulting in 30 measurements per egg. The five measurement regions were: (1) the blunt end, (2) the shoulder area, halfway between the blunt end and the equator, (3) the equator of the egg, (4) the knee, halfway between the equator and the pointy end and (5) the pointy end (Fig. 1). The exact location of the measurement was chosen according to maculation (speckles) of suitable size. Before measuring thickness, shell membranes were removed by soaking them in a 5% bleach (NaClO) solution overnight. All measurements were taken to an accuracy of 1 μm using a Mitutoyo (Kawasaki, Japan) Series 227–203 constant measurement force micrometer. To enable precise point measurements, both anvils of the micrometer were custom-fitted with an aluminium pin of 1.35 mm diameter and a rounded tip of 0.675 mm radius. Shells were placed in the micrometer so that they were at a 90° angle to the pin and measured on three slightly different locations within a speckle or plain area at a measurement force of 1.5 N.

Eggshell sampling for isotope analysis

Eggshell samples for analysis of the carbon and oxygen isotope composition of the calcite shell were collected from maculated and neighbouring plain areas of 10 eggs containing an embryo at developmental stage 3, when considerable calcite resorption could be expected (Carey 1983). Fragments were taken from the five regions along the eggshell as described above (Fig. 1). For 10 eggs each of the remaining three stages, we chose a single sample of plain eggshell in the shoulder region of the egg to assess the variation between eggs of different phases of embryonic development. This region was chosen as it is one of the most active regions for calcium resorption in avian eggs (Booth and Seymour 1987), possibly because most chicks break through the eggshell in this region at hatching (Deeming 2002*b*). All samples for the stable isotope analysis were taken by breaking a small eggshell fragment out of the shell with tweezers. The fragments were then transferred into a 25-mL agate mortar and pestle (Fisher Scientific Ltd, Loughborough, UK) and ground finely. The powder was transferred into a 900-μL glass vial (Chromacol Ltd, Welwyn Garden City, UK) using filter paper (Fisher Scientific). Between each sample, the tweezers and the mortar and pestle were first washed with a 1% solution of hydrochloric acid (HCl) and then rinsed with deionised water to remove any acidic residue and dried thoroughly before the next use. For the stable isotope analysis, 200-μg subsamples of powdered eggshell were weighed out on a Sartorius (Goettingen, Germany) CP2P microbalance and placed into 4-mL glass vials, then sealed with a lid and pierceable septum. The vials were placed in a heated sample rack (90°C) where the vial head space was replaced by pure helium via an automated needle system as part of a GV Instruments (Manchester, UK) Multiflow preparation system. Samples were manually injected with ~100 μL of phosphoric acid and left to react for one hour before the headspace gas was sampled by needle and introduced into a continuous-flow GV Isoprime (Manchester, UK) mass-spectrometer. Samples were calibrated using IAEA standards CO-1 and CO-8. Within-run reproducibility was better than 0.03 per mil for $\delta^{13}C$ and 0.07 per mil for $\delta^{18}O$. The $\delta^{13}C$ and $\delta^{18}O$ values are reported against the Vienna Pee Dee Belemnite standard throughout.

We assessed whether the presence or absence of the membrane influenced the results using five eggshell samples (one of each developmental stage but two samples from stage three eggs) taken from maculated (4 samples) and plain areas in the shoulder (3 samples), equator and knee areas. For each egg we compared the isotope ratios of three samples: (1) untreated whole shell, (2) eggshell soaked overnight in a 5% bleach (NaClO) solution to remove organic components such as the inner shell membrane and (3) untreated inner shell membrane only. Further treatment and isotope analysis was conducted as above.

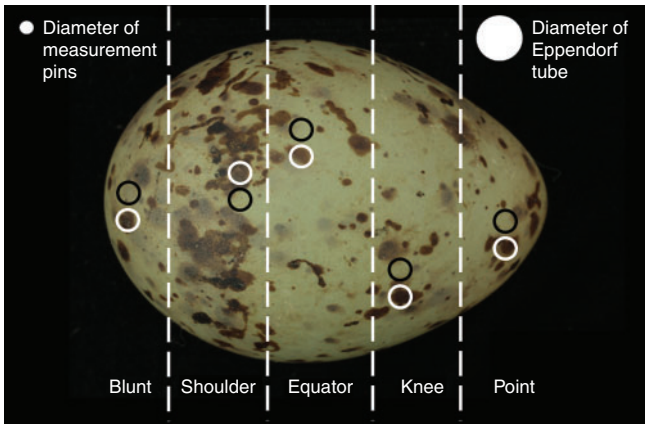

Fig. 1. Regions and plain *v*. maculated sample locations for thickness measurements of black-headed gull (*Larus ridibundus*) eggshells.

Statistical analysis

We used separate generalised linear mixed models (GLMM) assessing the effects of region, maculation and developmental stage on $\delta^{13}C$ and $\delta^{18}O$ of the egg samples respectively. The five regions and maculation (maculated *v*. plain areas) were included in these models as explanatory variables with their interaction, and individual egg code as a random effect. The effect of embryonic stage on eggshell thickness and the isotope ratios of both elements were analysed by general linear model (GLM). We also used GLMs to assess the effect of the presence or absence of the inner shell membrane on the stable isotope ratios and to compare the isotope composition of individual eggs. All analyses were performed in SPSS 16 (SPSS Ltd, Chicago, IL, USA). Results are presented as mean ± s.e. ($\alpha = 0.05$).

Results

Individual eggs differed markedly in their stable isotope composition for both elements: oxygen ($F_{9,90} = 1651.7$, $P < 0.001$)

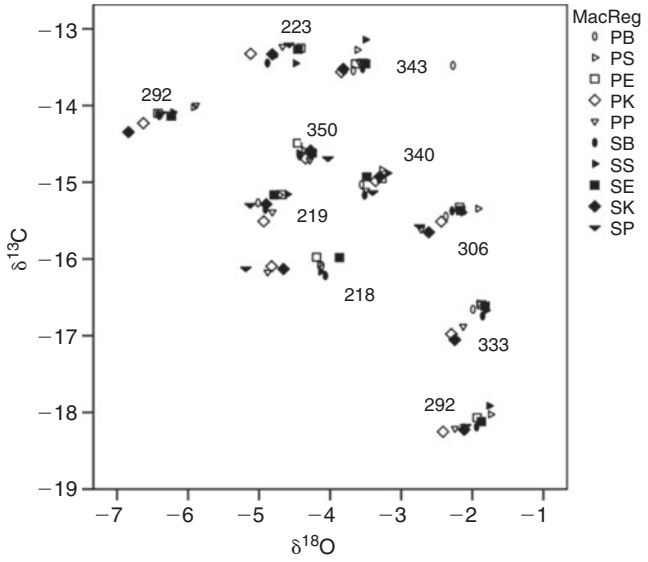

Fig. 2. Individual eggshells of black-headed gull (*Larus ridibundus*) sampled for carbon and oxygen stable isotope ratios at Stage 3 (unfeathered embryo) of incubation ($n = 10$). The numbers above the clusters identify each individual eggshell. Individual shells varied significantly in carbon and oxygen stable isotope ratios. By contrast no consistent variation occurred within individuals, neither between eggshell regions (different symbols) nor between plain (empty symbols) and maculated (filled symbols) areas of the egg shell, as listed in the legend for each symbol as Maculation by Region.

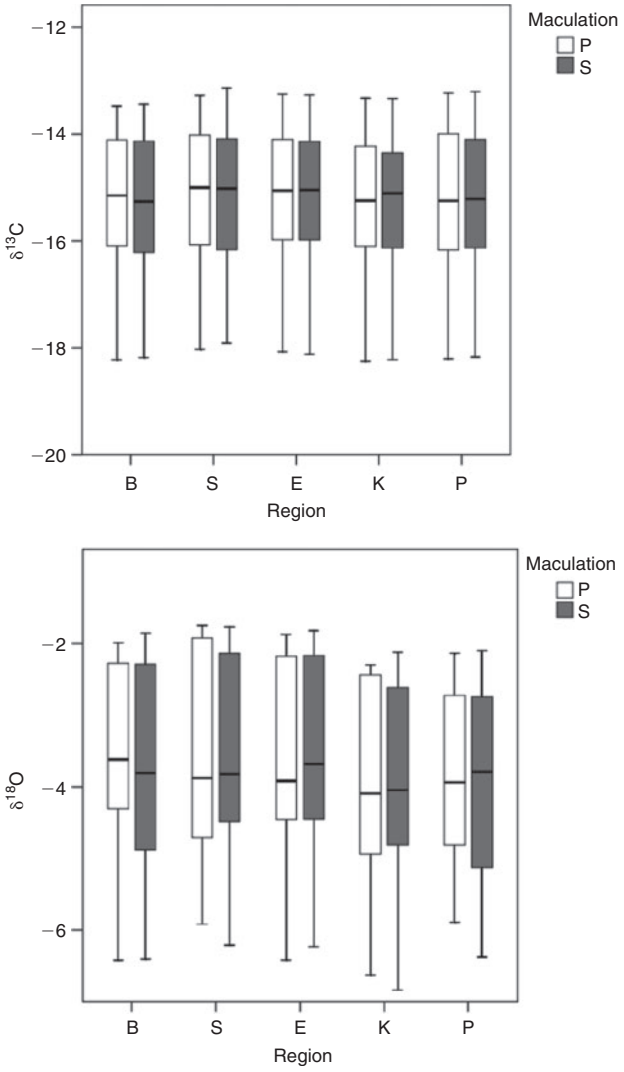

Fig. 3. Carbon (top panel) and oxygen isotope ratios (lower panel) do not differ between plain (P) and speckled (S) areas of black-headed gull (*Larus ridibundus*) eggshells in any region of the shell when measured at Stage 3 of embryonic development (see text for details).

and carbon ($F_{9,90} = 0.57$, $P = 0.86$; Fig. 2). By contrast, isotope ratios did not differ with egg region or maculation for the Stage 3 eggs sampled for either carbon (region $F_{1,93} = 0.003$, $P = 0.96$; maculation $F_{4,93} = 0.060$, $P = 0.99$; region : maculation interaction $F_{4,89} = 0.001$, $P = 0.99$; Figs 2, 3) or oxygen (region $F_{1,93} = 0.009$, $P = 0.93$; maculation $F_{4,93} = 0.271$, $P = 0.90$; region : maculation interaction $F_{4,89} = 0.031$, $P = 0.99$; Figs 2, 3).

Thickness of the calcite shell decreased significantly (average = 6.5%) in eggs containing an unfeathered embryo (Stage 3) compared with unincubated (Stage 1) eggs across all regions of the egg (Fig. 1), regardless of whether the measurements were taken at a speckled or plain location (Table 1). There also was a strong decline in the thickness of the calcite shell in the shoulder region of the egg with progressing embryonic development. This decline was particularly pronounced between the first and second half of embryonic development (Stage 1 = 176.1 ± 1.9; Stage 2 = 167.7 ± 2.0; Stage 3 = 163.9 ± 1.9; Stage 4 = 165.7 ± 1.9; GLM stage $F_{3,113} = 7.7$, $P < 0.01$).

As no effects of maculation or sampling region on isotope ratios were found, the comparison of eggs sampled at different stages of embryonic development was conducted solely on plain samples taken from the shoulder region of the egg. The isotope ratios of both carbon and oxygen as measured in the plain areas of the shoulder region did not change with the progress in embryonic development (carbon $F_{3,36} = 0.77$, $P = 0.52$; oxygen $F_{3,36} = 0.57$, $P = 0.86$; Fig. 4).

Finally, the presence or absence of the inner shell membrane did not lead to significant differences in the measurements of stable isotope ratios of either carbon ($F_{2,12} = 0.59$, $P = 0.81$) or oxygen ($F_{2,12} = 0.29$, $P = 0.76$).

Discussion

Eggshells of black-headed gulls sampled at different stages of incubation showed the dissolution of the calcite shell over the course of embryonic development in the form of significant shell thinning, but not through a change in their $\delta^{13}C$ and $\delta^{18}O$ values. These data indicate that the developing embryo resorbed eggshell calcite for bone formation at least from Stage 3 (unfeathered embryo) onwards. Similarly, across the length of the eggshell the difference in shell thickness (and thus calcite resorption) between unincubated eggs and those containing an unfeathered embryo was much smaller for the blunt end than for

Table 1. Comparison of shell thickness of the calcite shell of black-headed gull (*Larus ridibundus*) eggs from two different stages of embryonic development before and after the presumed onset of shell calcite resorption

Eggs were either unincubated (Stage 1) or contained an unfeathered embryo (Stage 3). $n = 10$ eggs each. Eggs were measured without their inner membrane at maculated and neighbouring plain areas across five regions of the egg (Fig. 1). Results are presented as mean ± s.e.m. or the two stages followed by the test statistics for the GLMs comparing the shell thickness of the two stages at each sampling region

Locations	Stage 1	Stage 3	Stage	Maculation	Stage × maculation
Blunt	162.9 ± 1.1	158.3 ± 1.8	$F_{1,116} = 4.5$, $P = 0.03$	$F_{1,116} = 0.4$, $P = 0.5$	$F_{1,116} = 0.1$, $P = 0.7$
Shoulder	175.7 ± 1.4	162.7 ± 1.5	$F_{1,116} = 38.7$, $P < 0.01$	$F_{1,116} = 0.6$, $P = 0.5$	$F_{1,116} = 0.1$, $P = 0.7$
Equator	178.9 ± 1.4	170.9 ± 1.8	$F_{1,116} = 12.0$, $P < 0.01$	$F_{1,116} = 0.2$, $P = 0.6$	$F_{1,116} = 0.1$, $P = 0.8$
Knee	177.8 ± 2.2	168.2 ± 2.1	$F_{1,116} = 10.0$, $P < 0.01$	$F_{1,116} = 0.1$, $P = 0.8$	$F_{1,116} = 0.0$, $P = 0.9$
Point	187.9 ± 2.2	164.3 ± 1.6	$F_{1,116} = 78.3$, $P < 0.01$	$F_{1,116} = 0.1$, $P = 0.8$	$F_{1,116} = 0.0$, $P = 0.9$

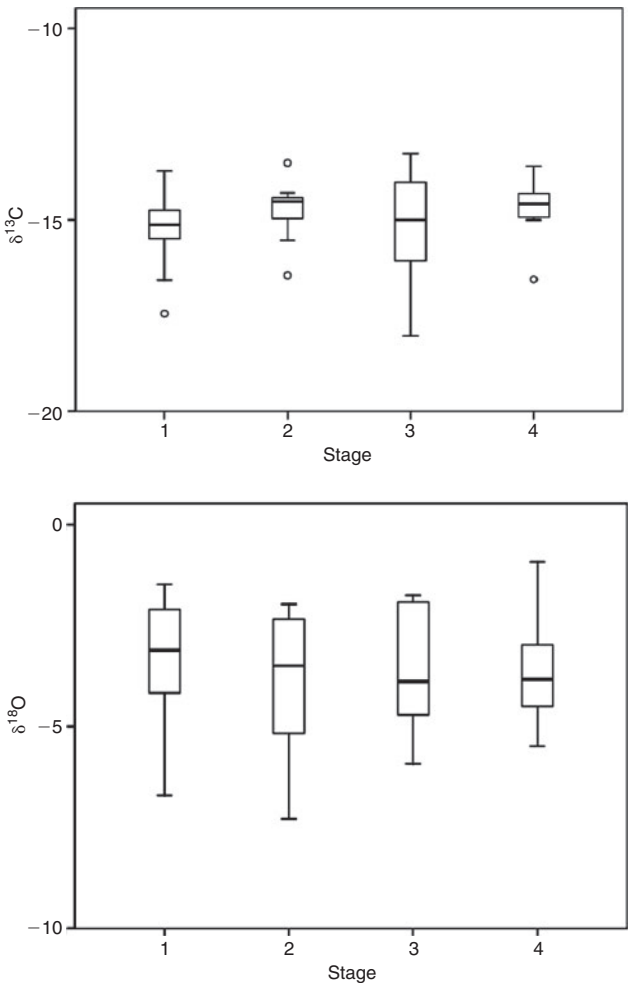

Fig. 4. Carbon (top panel) and oxygen isotope ratios (lower panel) do not differ between developmental stages of black-headed gull (*Larus ridibundus*) eggshells sampled at unspeckled locations within the shoulder region of the shell.

the remainder of the shell, yet no difference was found in the stable isotope composition of either element between any of the eggshell regions sampled at Stage 3. Finally, speckled and neighbouring plain shell areas of eggs containing unfeathered embryos did not differ in their $\delta^{13}C$ and $\delta^{18}O$ values. Our results thus suggest that the formation and dissolution of shell calcite in extracellular systems leads to a homogenous composition of both carbon and oxygen isotopes across the eggshell and at different stages of embryonic development.

The lack of differentiation in isotope ratios despite variation in shell thickness with both embryonic development and eggshell region is remarkable, especially given that individual eggs could be distinguished clearly by the isotope ratios identified in our analysis. This individual variation, which is inevitable due to the destructive nature of isotope sampling, may have masked the difference in stable isotope composition for eggs of different stages. Regional differences in isotope composition, on the other hand, could have been 'diluted' by our sampling of the entire depth of the shell, rather than the inner layer of the calcite shell only, which faces the chorioallantoic membrane and appears to be most affected by dissolution (Karlsson and Lilja 2008). Our findings thus suggest that stable isotope ratios of both carbon and oxygen from eggshell calcite are unsuitable to assess the incubation stages of wild bird eggs. However, their potential to track eggshell resorption in a more controlled experimental set-up remains to be explored. Stable isotope ratios may be useful to follow calcium resorption in eggs produced by the same mothers kept on a constant (or even isotopically labelled) diet and environmental temperature (Grossman and Ku 1986; Fry 2006) or in an *in vitro* system (Packard 1994).

The function of eggshell maculation is still a topic of intense debate and both signalling and structural explanations have been put forward (Underwood and Sealy 2002; Gosler *et al.* 2005; Higham and Gosler 2006; Kilner 2006). In particular, a structural function of eggshell speckling could imply small-scale variation in metabolic processes as the flow of substrate for chemical reactions, the gas exchange or the temperature could be different between maculated and plain areas. For instance, an increase in calcite dissolution in maculated areas could explain the occurrence of a 'corona' of eggshell speckles in the shoulder area (Gosler *et al.* 2005). Since the chicks of many bird species hatch by breaking through the shell in the shoulder region (Bond *et al.* 1988; Deeming 2002*a*), increased calcite resorption and thus shell thinning in this area could benefit the hatchling. We showed, however, that calcite dissolution does not differ between speckled and plain areas of the same eggshell region and thus provide evidence against a function of eggshell speckling in regulating the calcium metabolism on a small scale.

Eggshells and their isotope composition have now taken an important role in modern and historic studies of trophic relationships (Hobson and Montevecchi 1991; Schaffner and Swart 1991; Johnson *et al.* 1998), long-term environmental changes (Emslie and Patterson 2007), archaeology (Beacham and Durand 2007) and even quality control of organic and conventional chicken eggs for consumption (Rogers 2009) because they preserve well, sometimes over centuries, and even then can be identified to species level (Beacham and Durand 2007; Emslie and Patterson 2007; Igic *et al.* 2010). Furthermore, substantial data-rich collections of eggshells are available for environmental research (Green and Scharlemann 2003) and fresh eggshells can be sampled with little disturbance to the bird (Schaffner and Swart 1991; Oppel *et al.* 2009). For isotope studies of trophic interactions in particular, eggshells are attractive because they are assembled over a short period of time (Reynolds 1997) and very accurately reflect the food sources of the female bird on the breeding grounds. However, these uses of eggshells require a thorough understanding of how the incubation stage and sampling location of the egg influences the eggshell isotope composition, so as to separate resorption effects on eggshell stable isotope ratios from the effects of food sources (Hobson 1999).

An important result of our research is to confirm the implicit assumption – that eggshells are isotopically homogenous – which is at the basis of previous studies using the isotope composition of modern and historic eggshells to infer trophic relationships or environmental conditions (von Schirnding *et al.* 1982; Schaffner and Swart 1991; Stern *et al.* 1994; Hobson 1995; Emslie and Patterson 2007). In fact, our data show that modern and historic eggshell fragments can be used for trophic studies even without knowledge of the incubation stage of the egg, the origin of the fragment on the egg or its pigmentation. Variation with developmental stage, eggshell region and pattern are all negligible. In addition, 'posthumous' bacterial decay of the eggshell could influence isotope ratios but should only affect its organic component (Smith and Hayward 2010). However, we found, in accordance with earlier studies (von Schirnding *et al.* 1982; Schaffner and Swart 1991), that the organic component of the eggshell, inner shell membrane or protein matrix (Hincke Nys *et al.* 2010) does not influence the analysis of the isotope composition of the calcite shell. These findings establish the isotopes in the calcite shell of bird eggs as a reliable and convenient resource for environmental research, similar to the use of shell membrane (Oppel *et al.* 2009).

In summary, our results show that the carbon and oxygen isotope ratios of eggshells cannot be used to track the calcium resorption by the embryo over the course of incubation, at least for the eggs of wild birds. Future developmental research should explore the potential of isotope analysis to track embryonic calcium resorption for eggshells produced under more controlled conditions. By contrast, the convenient and increasingly popular use of eggshells in studies of modern and historic trophic relationships receives further support through our work; our data confirm the long-held but untested assumption behind such studies, i.e. that variation of isotope ratios within eggshells and between eggs of different developmental stages is negligible.

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