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ORIGINAL ARTICLE

Using eggshell membranes as a non-invasive tool to investigate the source of nutrients in avian eggs

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Abstract Development of minimally invasive techniques to collect nutritional information from free-living birds is desirable for both ethical and conservation reasons. Here, we explore the utility of waterfowl eggshell membranes to determine the nutrient source of egg formation by using stable isotope ratios. We compared $\delta^{13}C$ and $\delta^{15}N$ of membranes from complete king eider (*Somateria spectabilis*) eggs to membranes of hatched or depredated eggs of the same clutch remaining after incubation. Despite large variation among membranes ($\delta^{13}C$: −26 to −14‰) we found a highly predictable relationship between $\delta^{13}C$ of complete egg membranes and remaining (hatched or depredated) membranes from the same clutch. We did not find a consistent change in either $\delta^{13}C$ or $\delta^{15}N$ of eggshell membranes during incubation. We suggest that isotope ratios of membranes can be used to determine the source of exogenous nutrients for egg production in income breeders, and that membranes may offer a clutch-specific reference point for dietary nutrients ('income endpoint') in isotopic mixing models quantifying nutrient allocation in capital or mixed-strategy breeders.

Keywords Eggshell membrane · King eider · Nutrient allocation · *Somateria spectabilis* · Stable isotopes · Waterfowl

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Introduction

Stable isotope ratios of carbon ($^{13}C/^{12}C$), nitrogen ($^{15}N/^{14}N$), and sulfur ($^{34}S/^{32}S$) are increasingly used in ecological studies to reconstruct diets and explore the source of nutrients in animal tissues (Gannes et al. 1998; Fry 2006). If animals rely on a combination of isotopically distinct diets for nutrition, the proportional contribution of each source can be estimated by analyzing the stable isotope ratios of animal tissues and nutrient sources (Phillips 2001; Phillips et al. 2005). The application of stable isotope techniques has advanced several aspects of avian ecology, including quantitative analyses of the origin of nutrients that are incorporated into eggs (Hobson et al. 1997; Gauthier et al. 2003). The general approach in these investigations was to collect whole eggs to analyze their components for ^{13}C, ^{15}N, and/or ^{34}S. Removal of complete eggs may, however, conflict with conservation interests and ethical considerations especially for endangered species (Vucetich and Nelson 2007). Egg removal may also not be feasible if simultaneous studies assessing egg survival rates mandate that clutches are left intact. A potential solution to this problem is to use blood or natal down of hatchlings (Klaassen et al. 2001; Lecomte et al. 2006), or use a syringe to obtain samples of yolk and albumen from eggs left otherwise intact in the nest (Schwabl 1993; Morrison and Hobson 2004). The former approach requires precise

timing of a nest visit to capture precocial young. The second approach requires a fairly delicate operation and may result in contaminated samples and/or affect development of embryos (Finkler et al. 1998; Klaassen et al. 2004). A more robust and less intrusive alternative to sample egg tissues for stable isotope analysis is therefore needed.

Most waterfowl and some shorebirds leave eggshell membranes in the nest bowl after hatching (Milne 1965; Sotherland and Rahn 1987; Mabee 1997). These membranes can be collected for analysis without any detrimental effects on the clutch. Shell membranes are synthesized in the isthmus region of the oviduct at the end of the egg formation process, approximately 20 h before the egg is laid (Taylor 1970; Burley and Vadehra 1989; Alisauskas and Ankney 1992). Nutrients for eggshell and membrane formation are most likely derived from the birds' current diet, which potentially renders membranes a useful tool to determine the diet during egg production (Schaffner and Swart 1991).

The shell membranes facilitate gas exchange during embryonic development. They do not provide nutrients for the developing embryo, but may undergo modification due to incorporation of collagen. This may lead to a difference in the isotopic composition of membranes from hatched or partially incubated eggs (Schaffner and Swart 1991; Hobson 1995). Currently, no information is available on the isotopic change of membranes during incubation.

In this study, we investigate the carbon and nitrogen isotope ratios of king eider (*Somateria spectabilis*) eggshell membranes to examine whether the isotopic composition of membranes changes during incubation. By collecting one complete egg of known incubation stage plus membranes from hatched or depredated eggs of the same clutch, we provide an estimate of changes in isotope ratios in membranes during incubation. Our goal was to evaluate whether membranes can be used as a non-invasive tool to explore the source of nutrients used for egg production.

Materials and methods

Study area

We monitored nests and collected eggs and eggshell membranes (hereafter membranes) of king eiders on the Arctic coastal plain of Alaska in June and July 2006 and 2007 at two study locations near Teshekpuk Lake (70°26′N, 153°08′W) and in the Kuparuk oilfield (70°20′N, 149°45′W). Both areas consist of lowland arctic tundra and support intermediate densities (1–2 nests/km^2) of nesting king eiders.

Study species

King eiders nest in arctic tundra ecosystems around the world and spend about 10 months per year at sea. During the non-breeding season, the birds forage on marine benthic invertebrates by diving to the sea floor (Suydam et al. 2000). On the breeding grounds females forage for aquatic insects and larvae, but also ingest vegetation (Lamothe 1973; Holcroft-Weerstra and Dickson 1997). Female king eiders arrive on their breeding grounds in early June (Phillips et al. 2007) and spend about 2 weeks in a terrestrial/freshwater environment prior to laying a clutch consisting of 4–7 eggs. Eggs are laid at a rate of approximately one per day, incubation starts with the last egg laid, and the female incubates the clutch for 23–26 days before chicks hatch (Kellett and Alisauskas 1997; Suydam 2000).

Field collection

We located nests by systematically searching study areas in June, and collected one complete egg from each nest the first day it was found. Incubation stage of the egg was estimated by egg candling (Weller 1956), and ranged from 0 to 15 days of incubation. At the completion of incubation, we collected all remaining membranes and stored membranes belonging to different eggs from the same clutch separately. If nests were depredated or abandoned, we collected remaining eggs and eggshells with membranes and determined their incubation stage at death by calculating the time difference to the day when the nest was found. For preservation of samples, we boiled complete eggs in the field for 15 min and then kept them frozen until analysis (Gloutney and Hobson 1998). Membranes were air dried at ambient temperature, stored in sealed paper envelopes, and kept in a dry box until analysis.

Laboratory analyses

We separated whole eggs into yolk, albumen, membrane, and shell. We cleaned all membranes with a small brush in de-ionized water (Hobson 1995); oven dried them at 60°C for 24 h, and then broke them into tiny pieces using a mortar and pestle. From each egg, we placed several membrane pieces weighing 0.2–0.4 mg in total into tin cups and analysed them for carbon and nitrogen isotope ratios at the Alaska Stable Isotope Facility using a continuous flow stable isotope-ratio mass spectrometer (ThermoElectron Delta V Plus). We report results of isotopic analyses as ratios in delta notation relative to international standards (Vienna PeeDee Belemnite for C, atmospheric air for N) according to the following equation: $\delta X = ([R_{\text{sample}}/R_{\text{standard}}] - 1) \times 1000$, with X denoting either ^{13}C or ^{15}N, and R representing the ratio of ^{13}C/^{12}C or

$^{15}N/^{14}N$, respectively. Based on the standard deviation of peptone standards run concurrently with samples the precision of measurements was ±0.1 and ±0.1‰ for $\delta^{13}C$ and $\delta^{15}N$, respectively.

Statistical analysis

We first examined whether isotope ratios of membranes differed between our two study years using a non-parametric Mann–Whitney *U* test. We used a reduced major axis regression to examine the relationship between isotope ratios of membranes from complete eggs and from remaining membranes of the same clutch as the isotope ratios on both axes were measured with the same error (Sokal and Rohlf 1995; Bohonak and van der Linde 2004). We tested whether this relationship deviated from a slope of 1.0, and report statistical significance for this hypothesis. We used an ANOVA to test whether within-clutch variation in membrane isotope ratios of all membranes was higher than variation among clutches. To assess temporal change in $\delta^{13}C$ and $\delta^{15}N$ we calculated the difference in $\delta^{13}C$ and $\delta^{15}N$ between the membrane of the complete egg and the remaining membranes of a clutch and the time difference (in days) between collections. We averaged differences in isotope ratios for each clutch from which we had more than one remaining membrane. We then used a linear regression of the average change in $\delta^{13}C$ or $\delta^{15}N$ over the time difference between collections. We hereby assume that a slope different from zero would indicate a systematic change in $\delta^{13}C$ or $\delta^{15}N$ due to incubation of the eggs from which we collected remaining membranes. We present isotope ratios as mean ± standard deviation, and regression coefficients as estimate ± standard error. All tests were two-tailed and used $\alpha = 0.05$.

Results

We collected one complete egg from 28 clutches and 73 membranes of hatched or depredated eggs from those same clutches (range 1–5 remaining membranes per clutch) in 2006, and one complete egg from 18 clutches and 56 remaining membranes (1–5 per clutch) in 2007. Isotope ratios did not differ between years ($\delta^{13}C$: $P = 0.34$, $\delta^{15}N$: $P = 0.35$), and we pooled data from both years in all analyses.

Membrane $\delta^{13}C$ ranged from −26.1 to −14.6‰, and membrane $\delta^{15}N$ ranged from 5.5 to 11.9‰. The mean standard deviation for membranes of different eggs from the same clutch was 0.6‰ (range 0.5–1.7‰, $n = 46$) for $\delta^{13}C$ and 0.7‰ (range 0.5–1.7‰, $n = 46$) for $\delta^{15}N$. The variation among membranes from different clutches was higher than among membranes of the same clutch ($\delta^{13}C$: ±2.1‰, $F_{45,127} = 30.44$, $P < 0.001$; $\delta^{15}N$: ±1.0‰, $F_{45,129} = 5.40$, $P < 0.001$).

We found a highly predictable relationship between $\delta^{13}C$ of complete egg membranes and remaining membranes ($b = 1.024 \pm 0.046$, $r^2 = 0.75$, $P = 0.60$, $n = 127$, Fig. 1a). The relationship for $\delta^{15}N$ was less predictable, but the slope of the relationship did not indicate a deviation from 1.0 ($b = 0.995 \pm 0.077$, $r^2 = 0.17$, $P = 0.95$, $n = 129$, Fig. 1b).

We found no evidence for systematic isotopic change in membranes during incubation. The difference in both $\delta^{13}C$ and $\delta^{15}N$ between complete egg and remaining membranes did not deviate from zero (paired *t* test, $\delta^{13}C$: $t_{126} = 0.69$, $P = 0.49$; $\delta^{15}N$: $t_{128} = 0.51$, $P = 0.61$). There was no change in either $\delta^{13}C$ or $\delta^{15}N$ over the time interval between collections of membranes from the same clutch ($\delta^{13}C$: $b = -0.02$, $P = 0.26$, $\delta^{15}N$: $b = -0.03$, $P = 0.17$, Fig. 2).

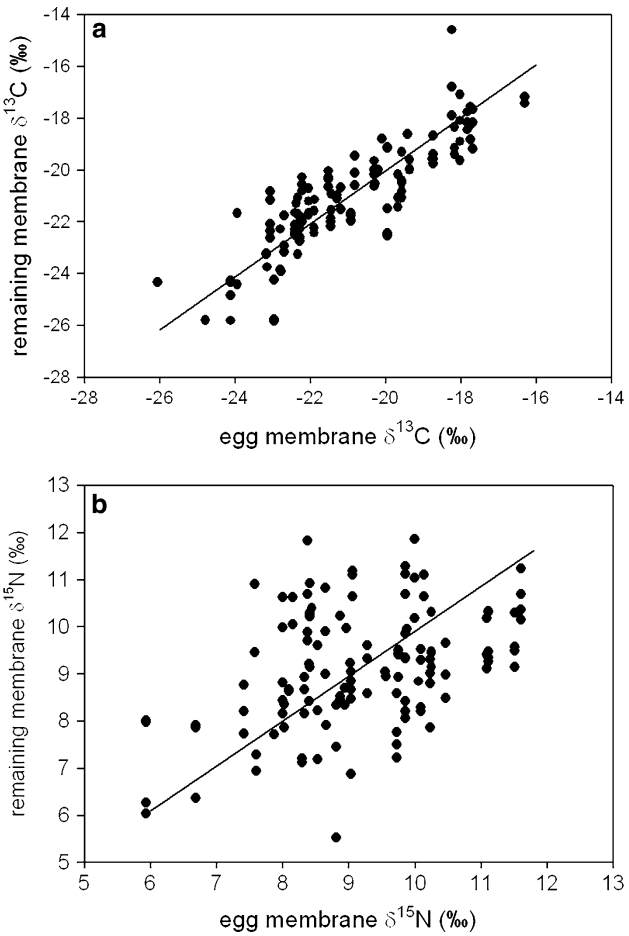

Fig. 1 Relationship of $\delta^{13}C$ (**a**) and $\delta^{15}N$ (**b**) of king eider eggshell membranes between complete eggs and remaining membranes of the same clutch collected in summer 2006 and 2007 on breeding grounds in North Alaska. *Solid line* represents reduced major axis regression (Bohonak and van der Linde 2004). $\delta^{13}C$: $r^2 = 0.75$, $n = 127$; $\delta^{15}N$: $r^2 = 0.17$, $n = 129$

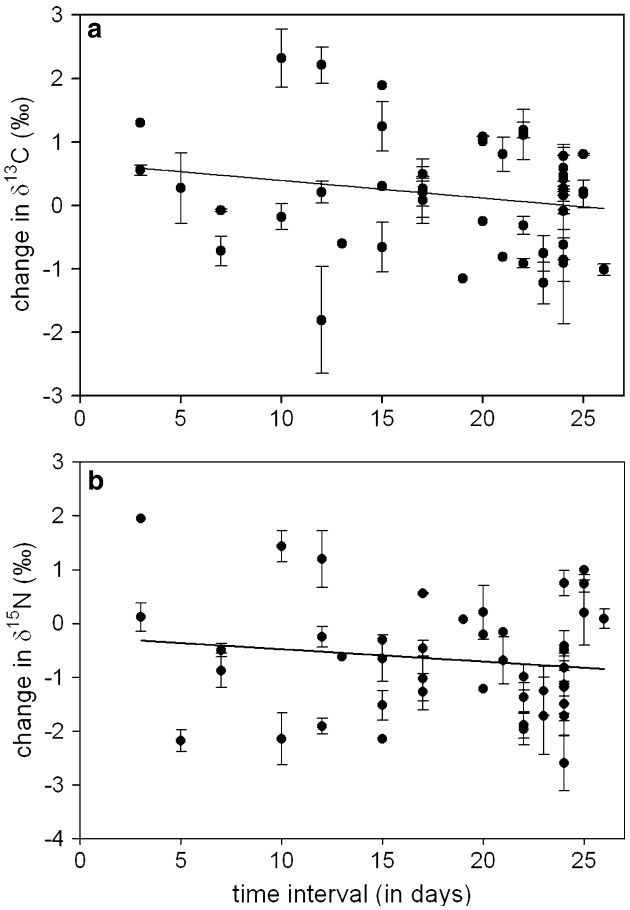

Fig. 2 Change in δ^{13}C (**a**) and δ^{15}N (**b**) over time in king eider eggshell membranes calculated from the difference between complete eggs and remaining membranes of the same clutch. Time interval indicates the number of days between collection of the complete egg and remaining membranes. *Error bars* indicate 1 SD for clutches with more than one remaining membrane. *Solid line* represents linear regression, δ^{13}C: $r^2 = 0.04$, $n = 46$; δ^{15}N: $r^2 = 0.02$, $n = 46$

Discussion

Our study found large variation in both δ^{13}C and δ^{15}N among eggshell membranes, and showed that δ^{13}C of fresh membranes could be predicted from remaining membranes. Fresh membrane δ^{15}N was less predictable from remaining membrane δ^{15}N. Further, δ^{13}C in membranes did not change systematically during incubation. This enables the use of membranes collected from hatched or depredated eggs for estimation of dietary δ^{13}C used for egg synthesis.

The weaker relationship between fresh and remaining membrane δ^{15}N may be due to intra-clutch variability of δ^{15}N resulting from dietary differences, but could be due to unknown effects of δ^{15}N-depleted uric acid accumulation during the growth of an embryo inside an egg (Fiske and Boyden 1926; Packard and Packard 1986). Even though we did not detect a significant decrease of δ^{15}N in remaining membranes, we caution researchers to critically evaluate the utility of δ^{15}N for their study species.

The large variation in both δ^{13}C and δ^{15}N among clutches in our study is consistent with large variation found in pre-breeding blood plasma samples of adult females from the same population (S. Oppel, unpublished data). We interpret this variation as the result of different foraging preferences of individual females (Bolnick et al. 2003), as potential prey items available in the study area also show large isotopic variation (S. Oppel, unpublished data). This variation is most likely a result of different carbon fixation pathways of algal and terrestrial primary producers (Peterson and Fry 1987; Hershey et al. 2006). Within-clutch variation may result from females foraging on different prey items in different water bodies during egg formation, or from isotopic variation within major prey species (Grey et al. 2004).

Membranes comprise only a small fraction ($\sim$1%) of the total amount of nutrients in an egg (Sotherland and Rahn 1987; Burley and Vadehra 1989), and the allocation of nutrients to yolk, albumen, and membranes may differ depending on the breeding strategy of a species (Jönsson 1997; Meijer and Drent 1999; Klaassen et al. 2006). For birds relying mostly on exogenous, recently ingested nutrients for egg formation (income breeders), hatch membranes offer a non-intrusive alternative to the collection of whole eggs to determine important diet components for egg formation. Recent evidence suggests that income breeding may be fairly widespread even at high latitudes (Klaassen et al. 2001, 2006; Bond et al. 2007), offering great potential for the application of the method suggested here.

In birds relying partially on endogenous reserves for egg formation (capital and mixed-strategy breeders), these reserves will most likely be used in yolk and, to a lesser extent, in albumen. However, nutrients for membrane formation are most likely derived from the birds' current diet, as the membrane does not require large amounts of nutrients and is synthesized at the end of the egg formation process immediately before the calcified shell is laid down (Taylor 1970; Burley and Vadehra 1989; Schaffner and Swart 1991). Due to this potential for differential allocation of body reserve and dietary nutrients to egg components, the isotopic signature of membranes may not reflect the isotopic signature of other egg components in capital or mixed-strategy breeders. We found this pattern for king eiders, where variation in eggshell membrane δ^{13}C accounted for approximately 70% of the variation in δ^{13}C in lipid-free yolk, indicating that some yolk nutrients were not derived from dietary nutrients (S. Oppel, unpublished data). Researchers therefore need to consider carefully how representative eggshell membrane isotope ratios are for the species under investigation, and we recommend initial

validation of the relationship between membrane and other egg components for each study species.

Membranes may nonetheless offer a valuable tool for the exploration of nutrient allocation to eggs in capital and mixed-strategy breeders. For studies attempting to quantify the proportion of egg nutrients derived from diet assimilated on breeding grounds, an isotopic endpoint reflecting those nutrients is required (Hobson 2006). Birds may exhibit individual dietary preferences (Durell 2000; Bolnick et al. 2003), resulting in variation in isotopic signatures within a population (Klaassen et al. 2004; Bearhop et al. 2006). Due to this individual variability, population-wide averages of diet isotope ratios may not be appropriate endpoints for assessing breeding strategy. An alternative is to obtain isotopic information of current diet from each individual during the egg-laying phase or shortly after the onset of incubation (Gauthier et al. 2003; Schmutz et al. 2007). This approach can, however, be logistically challenging, and will in many cases lead to nest abandonment (Criscuolo 2001; Bourgeon et al. 2006). Membranes provide an isotope signature of recent diet that can be collected non-intrusively. By averaging isotope signatures from several membranes of a clutch, one can derive a reliable estimate of the laying female's average diet during egg formation. Since only a few milligrams of membrane are required for stable isotope analyses, even small fragments remaining in hatched or depredated nests are sufficient for analysis. Membranes therefore provide a non-intrusive alternative for estimating income endpoints for mixed-strategy breeders, especially in populations exhibiting individual diet specialization (Durell 2000; Bolnick et al. 2003). Our results showed that intra-clutch variation of membrane signatures in king eiders was relatively small compared to the variation among clutches, and thus among individual females. Therefore, membrane averages for each clutch provide a more accurate endpoint to quantify the origin of nutrients than a population-wide average of diet isotope ratios.

Eggshell membranes are mostly composed of proteins and contain only small amounts (<5%) of carbohydrates and lipids (Burley and Vadehra 1989). They are isotopically enriched over bulk diet by about 3.2‰ in δ^{13}C and 4‰ in δ^{15}N (Hobson 1995). There is currently very little knowledge about the variability in composition and isotopic differences between macronutrients in membranes, and further experimental studies are required to determine potential sources of variation in isotope ratios of eggshell membranes both among eggs and within individual eggs.

In summary, we suggest that eggshell membranes collected at any stage during or after incubation can be used to determine the δ^{13}C of eggs for the estimation of food sources used during egg formation in income breeders. Care needs to be taken when interpreting δ^{15}N of hatch membranes, as this ratio was not strongly related to that from complete eggs in our study. Another useful application for hatch membranes is their indication of diet signature for individual females: they can provide an estimator of the income endpoint in nutrient allocation studies of capital or mixed-strategy breeders. This technique therefore offers great potential for a wide variety of studies exploring the origin of egg nutrients, especially for vulnerable populations where destructive sampling is not feasible.

Zusammenfassung

Die Schalenmembran als ein nachhaltiges Werkzeug zur Erforschung der Herkunft von Nährstoffen in Vogeleiern

Die Entwicklung von nachhaltigen Methoden zur Erforschung der Nährstoffdynamik in freilebenden Vogelarten ist aus ethischen und naturschutzfachlichen Aspekten angebracht. In diesem Beitrag untersuchen wir wie stabile Isotope in Schalenmembranen die nach dem Schlupf in Nestern zurückbleiben zur Erforschung der Nährstoffherkunft herangezogen werden können. Wir verglichen die Kohlenstoff- (δ^{13}C) und Stickstoffisotope (δ^{15}N) in Schalenmembranen von frischen Prachteiderenten (*Somateria spectabilis*) Eiern mit den Schalenmembranen von geschlüpften oder zerstörten Eiern derselben Gelege. Trotz einer grossen Spannweite der Kohlenstoffisotope (-26 bis -14‰) zwischen Membranen von verschiedenen Gelegen fanden wir eine positive Korrelation zwischen frischen und ausgebrüteten Membranen desselben Geleges. Wir fanden dagegen keine systematische Veränderung der Membran-Isotope während der Bebrütungsphase. Wir schlagen daher vor dass Schalenmembranen zur Erforschung der Herkunft exogener Nährstoffe herangezogen werden können. Darüber hinaus können Schalenmembranen als gelegespezifische Referenz für exogene Nährstoffe in isotopischen Mischmodellen benutzt werden, um die Nährstoffdynamik in Arten zu erforschen die auf endogene Reserven zur Eiformation zurückgreifen.

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trade names or commercial products does not imply endorsement by the U. S. Government.

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