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Differences in the stable isotope signatures of seabird egg membrane and albumen – implications for non-invasive studies

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In many bird species, egg membranes can be obtained non-invasively after the chicks have hatched, and stable isotope analysis of egg membranes can be used to study the diet and foraging distribution of these birds during egg formation. It has been suggested that the enrichment factors of albumen and egg membranes differ for ^{13}C, but are similar for ^{15}N. In this study, we compared carbon and nitrogen stable isotopes of the membranes and albumen of individual eggs of three wild seabird species, the Southern Rockhopper penguin *Eudyptes chrysocome*, the Imperial shag *Phalacrocorax atriceps albiventer*, and the Thin-billed prion *Pachyptila belcheri*. We also included chicken eggs for comparison. Egg membranes were generally enriched in ^{13}C, compared with albumen. The difference varied between species, with 2.1‰ in Rockhopper penguins, 1.6‰ in Imperial shags, but only 0.5‰ in Thin-billed prions and 0.4‰ in chicken eggs. Egg membranes were slightly enriched in ^{15}N in Imperial shags (0.9‰) and chickens (0.5‰), compared with albumen, while there was no difference for Thin-billed prions and Rockhopper penguins. The isotopic values of carbon and nitrogen were correlated between albumen and egg membranes of individual eggs, suggesting that egg membranes can be used reliably to investigate trophic differences between individuals, seasons or colonies. Species-specific mathematical corrections could be used to compare results across studies that use different egg components. Copyright © 2009 John Wiley & Sons, Ltd.

Depending on the tissue chosen, dietary information spanning different temporal scales can be obtained by stable isotope analysis.[1–3] In particular, carbon stable isotope ratios are useful indicators mainly of foraging locations,[4] while nitrogen isotope ratios become enriched in ^{15}N with trophic level by approximately 3.0 to 5.0‰,[5,6] and the $\delta^{15}N$ is thus often used to indicate trophic position and can be used to infer dietary composition.[e.g. 7–9]

The stable isotope approach is especially useful for ecologists and conservation biologists if the samples can be obtained non-invasively. To assess diet and foraging areas during the breeding season non-invasively, feathers of dead birds can be sampled opportunistically.[e.g.10] This can be a good way to obtain feathers where large aggregations of birds are subject to a high incidence of predation. Moulted feathers can be searched for systematically in species that moult during the breeding season,[e.g.10] and in birds with a rapid digestion such as grazing geese faecal samples may be useful. However, due to the specific metabolic processes involved, different sample types may differ in their isotopic enrichment factor relative to the diet.[1] While body tissues like blood, feathers and muscle are enriched in ^{15}N, waste products like faeces are depleted compared to the diet.[5]

Other tissues that can be collected non-invasively are eggshells and egg membranes, which are often discarded at or close to the nest after the chick has hatched, or can be obtained from eggs that were eaten by predators or failed to hatch. A diet-switch experiment using quail *Coturnix japonica* indicated that albumen, shell membrane and shell carbonate values reflect diet integrated over 3–5 days prior to laying in this income breeder.[11] However, studies applying this information to wild birds were lacking until recently.[12–14]

Stable isotope studies using egg yolk and/or albumen require the collection of eggs[15–18] or finding deserted eggs.[3,19] The former may not be desirable for conservation projects if the breeding success is decreased or for studies in behavioural ecology, if egg removal influences parental investment. Depending on the species, the collection of egg membranes from hatched eggs can enable a large number of individually known eggs to be sampled, and furthermore enables matching isotope values of the egg with those of the hatched chick. It has recently been shown in King eider *Somateria spectabilis* that the stable isotope signature of egg membranes does not change during embryo development.[13] In contrast, incubation influenced the $\delta^{13}C$ value of albumen and the $\delta^{15}N$ value of yolk protein in domestic hen eggs.[14]

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Thus, these recent studies suggest that egg membranes are particularly suited as an isotopically inert tissue that can be collected non-invasively from hatched eggs.

When comparing different tissues, tissue-dependent metabolic routing has to be taken into account. The reported isotope fractionation values between lipid-extracted diet and tissue of seabirds for red blood cells (rbc) and whole blood[20,21] are close to $\Delta^{13}C_{diet\text{-}blood} = 1‰$ (range −0.8 to 2.3‰[20]) and $\Delta^{15}N_{diet\text{-}blood} = 3‰$[20,21] (range 2.1 to 4.2‰[20]) and for feathers values commonly average about $\Delta^{13}C_{diet\text{-}feathers} =$ 2‰ (range 0.1 to 3.8%[20–22]) and $\Delta^{15}N_{diet\text{-}feathers} = 4–5‰$ (range 3.0 to 5.3%[20–22]). The isotope fractionation between diet and egg membranes for a range of terrestrial birds was $\Delta^{13}C_{diet\text{-}egg\ membrane} = 3‰$ (range 2.6 to 3.7‰) and $\Delta^{15}N_{diet\text{-}egg\ membrane} = 4‰$ (range 3.5 to 4.4‰).[11] Recent data of captive Gentoo penguins *Pygoscelis papua* provide the first isotope fractionation values between diet and egg tissues for seabirds[12]: $\Delta^{13}C_{diet\text{-}egg\ membrane}$ was in the range 2.8–2.9‰ and $\Delta^{15}N_{diet\text{-}egg\ membrane}$ ranged 4.2 to 4.4‰.[12] Thus, the published data suggest that blood is generally less enriched than feathers in both carbon and nitrogen, while discrimination factors for feathers and egg membranes are in the same range.

The aim of this study is to compare the carbon and nitrogen stable isotope values of albumen and egg membranes of three seabird species in the wild, in order to test for differences in isotopic tissue discrimination between these two egg tissues.

EXPERIMENTAL

Study site, species and sampling

The study was carried out on New Island, Falkland/Malvinas Islands (51°43′S, 61°17′W). Eggs were sampled or collected under licence from the Falkland Islands government, originally for hormonal analyses. The remaining albumen samples were used for the present study.

Around 5000 pairs of the threatened (Vulnerable - IUCN) Southern Rockhopper penguins *Eudyptes chrysocome chrysocome* breed in the Settlement colony on New Island. They lay two eggs, but usually rear only one chick.[23] Thus, by leaving one egg per clutch we assume that we did not significantly affect the breeding success of the colony. The breeding biology of Southern Rockhopper penguins at New Island has been described by Strange[24] and more recently by Poisbleau *et al*.[23] Briefly, males arrive first at the colony (early October) and establish nest sites. Females arrive 2 weeks later, for pairing and copulation. Only the yolk of the eggs may be partly formed when the females arrive, but they fast during the formation of the albumen and egg membrane of the A-egg, and during most of the formation of the B-egg. This fast is made possible by large body stores when they arrive, and the amount of nutrients transferred from the tissues to the eggs is not large compared with the body stores in the closely related Fiordland Crested Penguins *Eudyptes pachyrhynchos*.[e.g.25]

Rockhopper penguin nests were monitored for laying dates. When an A-egg was detected in a study nest, we recorded its laying date, marked it and weighed it to the nearest 0.1 g using a digital balance. Afterwards, we replaced it in its nest and checked its presence daily until the laying of its B-egg sibling. We sampled albumen from eggs as soon as the B-egg was detected in a nest selected to be part of the study. As incubation in Rockhopper penguins typically does not start before clutch completion,[26] neither A- nor B-eggs were incubated for longer than about 24 h at collection. We therefore assumed that embryo development had not yet begun. The laying period lasted from 27 October to 6 November 2007. Only one egg per nest was sampled non-destructively, following an established protocol.[27,28] The acute pole was carefully disinfected with alcohol, and a 22-gauge sterile needle connected to a 1-mL sterile syringe was inserted close to the acute pole of the egg, approximately 6 mm into the albumen and in the direction of the egg's centre. We gently removed 0.5 g of the albumen (i.e. less than 1% of the albumen). Eggshells were patched with a small square of OpSite transparent and breathable wound dressing (Smith & Nephew Medical Ltd., Hull, UK). The hatching success of sampled eggs was determined in a sample of 40 control eggs vs. 20 sampled eggs of each category. Hatching success was slightly smaller in sampled eggs (A-eggs 45%, B-eggs 70%) than in control eggs (A-eggs 50%, B-eggs 75%), but the difference was not statistically significant (A-eggs: $\chi^2 < 0.001$, $P = 0.981$, B-eggs: $\chi^2 = 0.002$, $P = 0.961$). Egg membranes were sampled from marked eggs after hatching. As some egg membranes of hatched eggs were not found, the final sample size in the present stable isotope analysis was seven hatched A-eggs and ten hatched B-eggs.

Imperial shags *Phalacrocorax atriceps albiventer* (the Falkland population is also called King shag) were also sampled at the Settlement colony, where about 3000 pairs breed. Imperial shags arrived at the colony during early October, when courtship and nest building commenced. Egg laying took place between early November and the end of December. The modal clutch size was three in our study area, with a laying gap between consecutive eggs from 1 to 5 days. We monitored shag nests on a daily basis for laying dates. We marked all new eggs with nest number and laying order (A, B or C) on laying, measured the length and width to 0.1 mm, and weighed them to the nearest 0.1 g. We collected eggs laid between 19 November and 7 December 2007. Imperial shags experience strong brood reduction and, as we left one egg per clutch in the nest, we assume that we did not significantly affect the breeding success of the colony.

After collection, the eggs of Imperial shags were weighed and frozen whole at −20°C for at least 4 days. We removed the shell while the egg was still frozen. We then separated the yolk from the albumen by taking advantage of the fact that albumen thaws more quickly than yolk. A small quantity of albumen was transferred to a 1.5-mL Eppendorf tube and stored at −20°C for transport. A piece of egg membrane was separated, washed in distilled water and dried. Twenty-six eggs were collected from 16 different nests, including one egg of 6 nests and two eggs of each of 10 nests. For the latter, we checked if the nest identity influenced the isotope signatures. As the data were independent within nests (Kruskal-Wallis-ANOVA for $\delta^{13}C_{albumen}$: $H = 11.0$, d.f. 9, $P = 0.273$, for $\delta^{15}N_{albumen}$: $H = 12.3$, d.f. 9, $P = 0.196$), we included both eggs.

Four abandoned eggs of Thin-billed prions *Pachyptila belcheri* were collected opportunistically during ongoing

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studies at New Island exploring variability in provisioning and parent-chick interactions.[e.g.19,29–37] The life cycle and basic biology of Thin-billed prions have been described by Strange.[38] Adults arrive at the colonies in September to occupy the nest burrows and for the courtship. The copulations take place in the second half of October, after which the females leave for a pre-laying exodus flight of 3 weeks duration. Eggs are laid in the first three weeks of November, and incubated for 7 weeks. Thin-billed prions lay a single egg per season, but, during our study, some active nests contained two eggs each, one warm and one cold egg. Strange[34] suggested that this may occur if a female returns from the pre-laying exodus at the point of laying and finds that the nest has been taken over by another pair. We collected four cold, and thus most probably abandoned, eggs from such nests.

Hens' eggs were purchased in Radolfzell, Germany, and samples of albumen and egg membranes were taken from fresh eggs, without prior freezing.

Sample preparation and stable isotope analysis

For the stable isotope analyses, egg membrane pieces were cut into small fragments using stainless steel scissors. Small amounts of albumen were dried at 60°C overnight. Carbon and nitrogen isotope analyses were carried out on 0.65–0.7 mg aliquots of sample, weighed into tin cups.

Carbon and nitrogen isotope ratios were measured simultaneously by continuous-flow isotope ratio mass spectrometry (CF-IRMS) using a Costech (Milan, Italy) elemental analyser (EA) linked to a ThermoFinnigan Delta Plus XP mass spectrometer (Thermo, Bremen, Germany). Two laboratory standards were analysed for every ten unknown samples, allowing any instrument drift over a typical 14-h run to be corrected. Stable isotope ratios were expressed in δ notation as parts per thousand (‰) deviation from the international standards V-Pee Dee Belemnite (carbon) and AIR (nitrogen), according to the following equation $\delta X = [(R_{sample}/R_{standard}) - 1] \times 1000$, where X is ^{15}N or ^{13}C and R is the corresponding ratio $^{15}N/^{14}N$ or $^{13}C/^{12}C$. The measurement precision of both $\delta^{15}N$ and $\delta^{13}C$ was <0.3‰. Statistical analysis was carried out using SigmaStat 2.03 (SPSS Inc., Chicago, IL, USA). Before carrying out paired t-tests, we ascertained that the assumptions of normality and homogeneous variance between groups were met.

RESULTS

In all species, egg membranes were enriched in ^{13}C, compared with albumen (Table 1, Fig. 1). The difference varied between species, from 0.4‰ in chicken eggs to 2.1‰ in Rockhopper penguins (Table 1).

Egg membranes were slightly enriched in ^{15}N in Imperial shags (0.9‰) and chickens (0.5‰), compared with albumen, while there was no difference for Rockhopper penguins or Thin-billed prions (Table 1, Fig. 2).

The isotopic values of carbon and nitrogen were correlated between albumen and egg membranes of individual eggs in Rockhopper penguins, Thin-billed prions and Imperial shags (Table 1).

There are two possibilities for species-specific mathematical corrections. The first consists of adding the factor derived as pairwise difference in Table 1. However, a better correction can be achieved using equations derived by linear regression for $\delta^{13}C$ (Fig. 1; Rockhopper penguins $\delta^{13}C_{membrane} = -0.652 + 0.862 \times \delta^{13}C_{albumen}$; $\delta^{13}C_{albumen} = -4.529 + 0.862 \times$

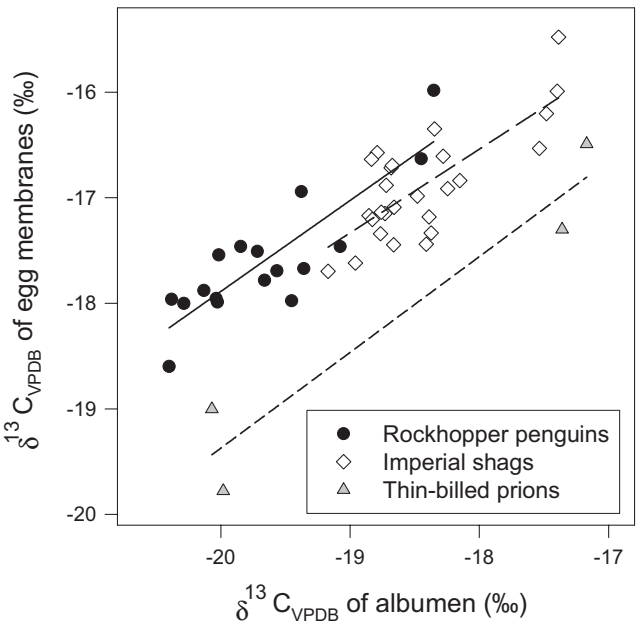

Figure 1. Carbon stable isotopes of individual eggs of Southern Rockhopper penguins, Thin-billed Prions and Imperial shags from New Island, Falkland/Malvinas Islands, sampled for albumen and egg membrane.

Table 1. Carbon and nitrogen stable isotopes (means ± standard error (S.E.) in ‰) of seabird egg albumen and egg membrane, and individual differences between the tissues (means ± S.E.). Statistically significant results are marked in bold

Species	Isotope	Albumen	Egg membrane	Pairwise difference	Pairwise test	Correlation
Southern Rockhopper penguin (N = 17)	$\delta^{13}C$	−19.7 ± 0.1	−17.6 ± 0.1	2.1 ± 0.1	**$t = 26.7$, $P < 0.001$**	**$R = 0.861$, $P < 0.001$**
	$\delta^{15}N$	14.7 ± 0.3	14.6 ± 0.3	0.1 ± 0.2	$t = 0.8$, $P = 0.441$	**$R = 0.821$, $P < 0.001$**
Imperial shag (N = 26)	$\delta^{13}C$	−18.4 ± 0.1	−16.9 ± 0.1	1.6 ± 0.1	**$t = 22.6$, $P < 0.001$**	**$R = 0.760$, $P < 0.001$**
	$\delta^{15}N$	14.8 ± 0.2	15.7 ± 0.1	0.9 ± 0.1	**$t = 8.3$, $P < 0.001$**	**$R = 0.719$, $P < 0.001$**
Thin-billed prion (N = 4)	$\delta^{13}C$	−18.6 ± 0.8	−18.1 ± 0.8	0.5 ± 0.2	$t = 2.2$, $P = 0.118$	**$R = 0.957$, $P = 0.043$**
	$\delta^{15}N$	10.4 ± 1.6	10.2 ± 1.3	0.2 ± 0.5	$t = 0.3$, $P = 0.791$	**$R = 0.969$, $P = 0.031$**
Chicken (N = 5)	$\delta^{13}C$	−22.3 ± 0.1	−21.9 ± 0.1	0.4 ± 0.1	**$t = 3.9$, $P = 0.017$**	$R = 0.627$, $P = 0.258$
	$\delta^{15}N$	4.6 ± 0.2	5.2 ± 0.1	0.5 ± 0.2	$t = 2.4$, $P = 0.074$	$R = -0.781$, $P = 0.119$

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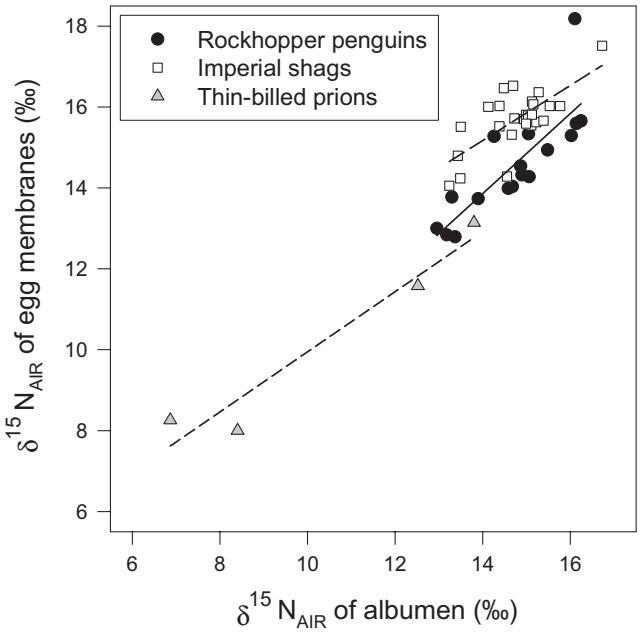

Figure 2. Nitrogen stable isotopes of individual eggs of Southern Rockhopper penguins, Thin-billed prions and Imperial shags from New Island, Falkland/Malvinas Islands, sampled for albumen and egg membrane.

$\delta^{13}C_{membrane}$; Imperial shags: $\delta^{13}C_{membrane} = -2.256 + 0.794 \times \delta^{13}C_{albumen}$; $\delta^{13}C_{albumen} = -6.191 + 0.725 \times \delta^{13}C_{membrane}$).

Correction for $\delta^{15}N$ is only necessary for Imperial Shags: $\delta^{15}N_{membrane} = 5.637 + 0.681 \times \delta^{15}N_{albumen}$ and $\delta^{15}N_{albumen} = 2.916 + 0.755 \times \delta^{15}N_{membrane}$.

DISCUSSION

Southern Rockhopper penguins fast during the formation of albumen and membrane and, thus, the dietary sources are constant in this species. Female Imperial shags and Thin-billed Prions, in contrast, stay at sea during the formation of the eggs, and have the possibility to take different food sources. However, the time of formation of albumen and shell membrane is only 3–5 days prior to laying[11] and, thus, no major diet switch is expected. We also included eggs of hens *Gallus domesticus* for comparison, which presumably have been fed on a constant diet. We, therefore, assume that any differences observed between the stable isotope values of albumen and egg membranes are due to different metabolic routing, rather than reflecting a change in diet.

In all species, egg membranes were enriched in ^{13}C, compared with albumen, the difference not being statistically significant in Thin-billed prions. The difference varied between species, with 2.1‰ in Rockhopper penguins, 1.6‰ in Imperial shags, but only 0.5‰ in Thin-billed prions and 0.4‰ in chicken eggs. Egg membranes were slightly enriched in ^{15}N in Imperial shags (0.9‰) and chickens (0.5‰), compared with albumen, while there was no difference for Thin-billed prions or Rockhopper penguins.

For Thin-billed prions, the differences between albumen and membrane were small compared with the differences between eggs. A larger sample size is required to obtain a reliable equation for correction for the small differences observed in this species.

Although Hobson[11] reported no difference in $\delta^{15}N$ between yolk, albumen and membranes in four species of captive-raised birds, a re-analysis of the data indicated differences as great as 1.7‰ in quail eggs (Table 2). The enrichment in ^{15}N relative to diet for albumen ranged from 2.8 to 3.1‰, a value typical of processes associated with protein synthesis. For membranes, the enrichment in ^{15}N relative to diet ranged from 3.2 to 4.4‰ for the five species, and was different from albumen for quail and mallard *Anas platyrhynchos* eggs (Table 2). Thus, while all these fractionation data are within the 3–5‰ enrichment in ^{15}N between trophic steps seen in terrestrial and marine food webs,[39–41] and the $\delta^{15}N$ of all egg components is comparable with feather $\delta^{15}N$, there are some systematic differences to be taken into account for fine-scale analyses such as comparisons within species.

Tissue-specific differences are even more important with respect to $\delta^{13}C$. For quail and mallard ducks, albumen, membranes, and shell carbonate were enriched in ^{13}C relative to diet by 1.5‰, 3.6‰, and 14.9‰,[10] respectively, thus suggesting a difference between albumen and membranes of 2.1‰. Falcons *Falco* spp. showed lower carbon diet-tissue fractionation for all tissues (albumen: +0.9‰, membranes: +2.7‰, carbonate: +11.2‰), possibly due to a greater reliance on dietary lipids vs. carbohydrates for the synthesis

Table 2. Data from Hobson[11] and Polito *et al.*[12] and M. Polito (unpublished data) of diet-tissue fractionation factors for nitrogen and carbon stable isotopes (means ± S.E. in ‰) for egg albumen and egg membranes of five captive-raised bird species, and differences between the means (independent samples t-tests). Statistically significant results are marked in bold

Species	Isotope	Albumen	Egg membrane	Difference (‰)	t-test
Quail (N = 9)[11]	C	1.6 ± 0.1	3.5 ± 0.1	+1.9	$\mathbf{t_{16} = 11.4,\ P < 0.001}$
	N	2.4 ± 0.1	4.1 ± 0.1	+1.7	$\mathbf{t_{16} = 11.4,\ P < 0.001}$
Mallard, first clutch (N = 8)[11]	C	1.4 ± 0.2	3.7 ± 0.1	+2.3	$\mathbf{t_{14} = 9.7,\ P < 0.001}$
	N	3.0 ± 0.1	4.4 ± 0.1	+1.4	$\mathbf{t_{14} = 7.9,\ P < 0.001}$
Mallard, second clutch (N = 8)[11]	C	1.4 ± 0.2	3.4 ± 0.1	+2.0	$\mathbf{t_{14} = 7.8,\ P < 0.001}$
	N	2.8 ± 0.1	4.0 ± 0.1	+1.2	$\mathbf{t_{14} = 6.8,\ P < 0.001}$
Peregrine falcon (N = 6)[11]	C	0.9 ± 0.2	3.5 ± 0.2	+2.6	$\mathbf{t_{10} = 9.9,\ P < 0.001}$
	N	3.1 ± 0.2	3.5 ± 0.2	NS	$t_{10} = 1.7,\ P = 0.114$
Prairie falcon (N = 2)[11]	C	0.9	3.0		
	N	3.1	3.2		
Gentoo Penguins vs. whole fish (N = 5, 20)[12]	C	0.8 ± 0.3	2.8 ± 0.1	+2.0	$\mathbf{t_{23} = 7.8,\ P < 0.001}$
	N	4.7 ± 0.2	4.4 ± 0.1	NS	$t_{23} = 1.3,\ P = 0.198$

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of egg components.[10] The difference between albumen and membranes here was 1.8‰. According to that study, the $\delta^{13}C$ of albumen could be best compared with that of feathers. In comparison, significant enrichment of ^{13}C has to be taken into account for the shell membrane, while shell carbonate may be very difficult to use due to a high fractionation factor.

The isotopic values of carbon and nitrogen were correlated between the albumen and the egg membranes of individual eggs, suggesting that egg membranes can be used reliably to investigate trophic differences between individuals, between seasons or between colonies. Species-specific mathematical corrections could be used to compare results across studies which differ in the use of these two egg tissues.

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