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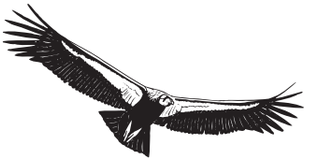

SHORT COMMUNICATIONS

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EMBRYO DEVELOPMENT INFLUENCES THE ISOTOPIC SIGNATURES OF EGG COMPONENTS IN INCUBATED EGGS

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Abstract. Stable isotopes have become an accepted method to track resource acquisition and nutrient utilization by birds. Many studies have used these methods to examine nutrient allocation and utilization during egg formation. None, however, has addressed the potential influence of nutrient utilization and movement during embryo development on the isotopic signature of egg components or its implications for sampling protocols. Such fractionation would distort the isotopic signature in incubated eggs, resulting in conclusions based on eggs that may not reflect resource allocation during formation. Using incubated domestic chicken (*Gallus gallus*) eggs, we examined how embryo development influences $\delta^{13}C$ and $\delta^{15}N$ signatures in albumen, yolk protein, and yolk lipid. Embryo development significantly lowered $\delta^{15}N$ by 1.0‰ in yolk protein after day 15 and depleted $\delta^{13}C$ by 0.2‰ in albumen after just 3 days. Future studies examining resource allocation to eggs must control for the influence of embryonic development on isotopic signatures in eggs.

Key words: embryo development, incubation, nutrient utilization, resource allocation, stable carbon isotope, stable nitrogen isotope.

El Desarrollo del Embrión Influencia las Firmas de Isótopos de los Componentes del Huevo en Huevos Incubados

Resumen. Los isótopos estables se han convertido en un método aceptado para seguir la adquisición de recursos y la utilización de nutrientes por parte de las aves. Muchos estudios han usado estos métodos para examinar la asignación y utilización de nutrientes durante la formación del huevo. Sin embargo, no se ha evaluado la influencia potencial del uso y movilización de los recursos durante el desarrollo embrional sobre la firma isotópica de los componentes del huevo, ni sus implicaciones para los protocolos de muestreo. Dicho fraccionamiento modificaría la firma isotópica en los huevos incubados, conduciendo a conclusiones basadas en huevos que no reflejarían la asignación de recursos durante su formación. Utilizando huevos incubados de gallinas domésticas (*Gallus gallus*), examinamos cómo el desarrollo del embrión influencia las firmas de $\delta^{13}C$ and $\delta^{15}N$ en el albumen, las proteínas del vitelo y los lípidos del vitelo. El desarrollo del embrión redujo significativamente el $\delta^{15}N$ presente en las proteínas del vitelo en un 1.0‰ después del día 15 y redujo el $\delta^{13}C$ del albumen en un 0.2‰ después de sólo tres días. Los estudios futuros que examinen la asignación de recursos en los huevos deberán controlar la influencia del desarrollo embrional sobre las firmas isotópicas de los huevos.

Stable isotopes have become an established method used by numerous avian ecologists to track resource acquisition and nutrient utilization during breeding (Hobson 2006). Many have used these methods to determine nutrient allocation and utilization during egg formation (Hobson et al.1997, 2000, 2004, 2005, Gauthier et al. 2003, Morrison and Hobson 2004, Langin et al. 2005, Klaassen et al. 2006, Schmutz et al. 2006, Bond et al. 2007), incubation (Schmutz et al. 2006), and brood rearing (Cherel et al. 2005). By measuring the isotope ratios in yolk and albumen, researchers are able to determine the origins of the nutrients allocated to the egg by the laying female, whether acquired locally ("income") or transported from staging/wintering areas in the form of muscle and fat ("capital") (Hobson 1995). A common issue among studies dealing with stable isotopes in eggs is the potential for changes in the isotopic signature of the egg components during incubation.

Preferential utilization or movement of macronutrients during incubation could result in changes in the isotopic signature of egg components. During embryo development significant amounts of protein are transferred from the shell and albumen to the yolk, potentially affecting the isotopic signature of the yolk (Romanoff 1967, Deeming 2002). There is also evidence supporting the preferential uptake of fatty acids in the yolk during embryo development (Ding and Lilburn 1996, Speake et al. 1998). If utilization and movement of macronutrients during embryo development influence the isotopic signatures of egg components, then results based on incubated eggs may not reflect actual resource allocation during egg formation.

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Those who have used stable isotopes in eggs have addressed this issue in one of two ways: (1) they have assumed that incubation does not affect the isotopic signature of egg components or (2) they collect eggs before incubation begins and prior to any embryo development. The collection of eggs before incubation begins can present many challenges. In numerous species, incubation begins before the entire clutch is laid (e.g., raptors, gulls, and many passerines). Although clutches may not be complete for several days into incubation, embryos in the first-laid eggs can begin developing (Clark and Wilson 1981, Mock 1984, Ricklefs 1993). Finding nests of ground-nesting species before incubation can also be difficult because these nests are typically found by flushing the incubating bird.

It remains unknown whether embryo development influences the isotopic signatures of egg components. Several authors (Gannes et al. 1997, Bearhop et al. 2002, Hobson 2006, Barnes et al. 2008) have indicated the need for laboratory experiments to test common assumptions to ensure the proper interpretation of stable-isotope data. Therefore, we used captive-bred poultry to test whether embryo development influences the carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) signatures of egg components (albumen, yolk protein, and yolk lipid).

METHODS

Domestic chicken (*Gallus gallus*) eggs provide an ideal model for understanding nutrient dynamics within eggs. Chickens have the best-understood biology of any avian species; knowledge of all their life stages, including embryogenesis and nutrient utilization, is comprehensive (Deeming 2002). Laying hens providing eggs for our experiment were raised under strictly controlled conditions (light:dark cycle 14:10 hours, 21–24° C) and fed a nutritionally complete broiler–breeder ration (16% protein, 2.8% fat). The 40 fertilized eggs were obtained from laying hens identical in age and laying cycle (all laying hens hatched the same day) and originated from the same commercial laying barn. The eggs were selected randomly and all were laid on the same day. They were held at 17° C for 3 days prior to the beginning of incubation. All were incubated at 37.5° C and approximately 50% relative humidity.

The mean incubation period for domestic chickens is 21 days (Romanoff 1967). We sampled 10 eggs selected at random before incubation and five eggs selected every 3 days during incubation until the 18th day of incubation (days 3, 6, 9, 12, 15, 18). Samples were not taken from one egg incubated for 15 days because it had not developed beyond day 6. We sampled yolk from all eggs. We sampled albumen only from eggs up to 15 days of incubation because the albumen sac typically disappears by day 16 (Deeming 2002). One egg incubated 15 days was not sampled for albumen because of potential contamination by yolk.

We removed yolk and albumen samples from each egg with a 3-ml syringe. Embryos older than 9 days were euthanized by decapitation (Trent University animal-care protocol: 08017). Yolk and albumen samples were freeze-dried to a constant mass and homogenized with a mortar and pestle. Yolk samples were separated into yolk lipid and yolk protein (lipid-free yolk) by 2:1 chloroform:methanol fat extraction (Bligh and Dyer 1959).

All samples were weighed (0.30 ± 0.05 mg) into tin capsules for isotope analysis at Trent University's Worsfold Water Quality Centre. We determined the carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) composition of the albumen and yolk-protein samples. As lipids contain only trace amounts of nitrogen, we measured only carbon for yolk lipids. The isotopic composition of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) was determined with an elemental analyzer (EuroVector) interfaced with an IsoPrime continuous-flow isotope ratio mass spectrometer (GV Instruments). We expressed the isotopic composition of carbon and nitrogen in the conventional delta notation relative to international standards (Pee Dee Belemnite for ^{13}C and atmospheric N_2 for ^{15}N). Typical precision of replicate analyses was usually within 0.2 and 0.4‰ for carbon and nitrogen, respectively. Standard deviations for repeated yolk-protein samples analyzed across runs ranged between 0.05 and 0.17 with a mean standard deviation of 0.10 for $\delta^{13}C$ and between 0.05 and 0.81 with a mean standard deviation of 0.33 for $\delta^{15}N$.

STATISTICAL ANALYSES

To determine the influence of incubation duration on the isotopic signature of egg components, we used analysis of variance (ANOVA) for each tissue type (albumen, yolk protein, and yolk lipid) for both carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) with developmental age of the embryo being the main factor. Although linear regression also would have been an appropriate statistical technique (Cottingham et al 2005), we chose ANOVA because it allowed us to examine differences between treatments (days of incubation). Values of $\delta^{13}C$ and $\delta^{15}N$ were normally distributed (Kolmogorov–Smirnov tests, $P > 0.05$) and had equal variances (Levene's test for homogeneity of variances, $P > 0.20$) for all tissue types). All statistical analyses were done in STATISTICA version 6.1 (StatSoft 2003). Results were considered significant at a P-value of 0.05.

RESULTS

ALBUMEN

Embryo development resulted in a significant decline in albumen carbon ($\delta^{13}C$) values ($F_{5,27} = 9.40$, $P < 0.001$) but did not influence albumen nitrogen ($\delta^{15}N$) values ($F_{5,27} = 5.05$, $P = 0.77$) (Fig. 1). $\delta^{13}C$ values were significantly depleted after day 6 in comparison to values in unincubated eggs and in eggs incubated for 3 days (Tukey HSD test for unequal n, $P < 0.05$; Fig 1).

YOLK

Embryo development did not have a significant effect on $\delta^{13}C$ values ($F_{6,32} = 0.70$, $P = 0.62$) in yolk protein but did have a significant influence on $\delta^{15}N$ values ($F_{6,32} = 5.05$, $P < 0.001$) (Fig. 2).

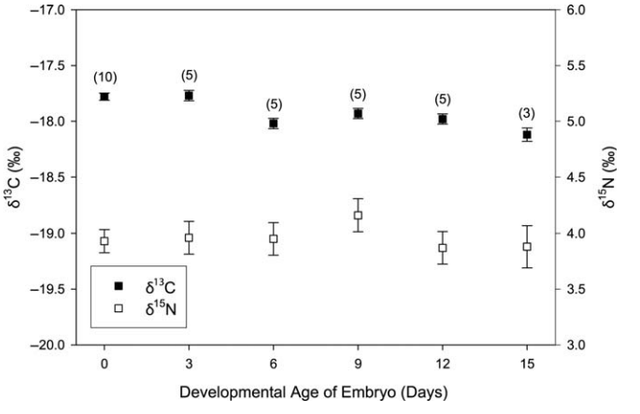

FIGURE 1. Mean (± SE) values for $\delta^{13}C$ and $\delta^{15}N$ in the albumen of fertilized chicken (*Gallus gallus*) eggs from before incubation (day 0) to day 15 of embryo development. Sample sizes are indicated in parentheses.

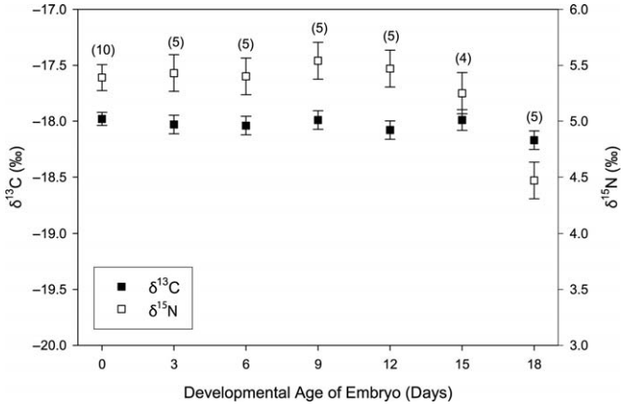

FIGURE 2. Mean (± SE) values for $\delta^{13}C$ and $\delta^{15}N$ in the yolk protein of fertilized chicken (*Gallus gallus*) eggs from before incubation (day 0) to day 18 of embryo development. Sample sizes are indicated in parentheses.

Eggs incubated for 18 days had $\delta^{15}N$ values significantly lower than those of all other eggs (Tukey HSD test for unequal n, $P < 0.01$; Fig. 2). Incubation did not have a significant effect on $\delta^{13}C$ values in yolk lipid ($F_{6,32} = 2.3$, $P = 0.06$; Figure 3).

DISCUSSION

These results demonstrate that embryo development during incubation can have a significant effect on the isotopic signature of egg components. More specifically, incubation significantly lowered $\delta^{15}N$ in yolk protein and $\delta^{13}C$ in albumen. Development had no effect on the $\delta^{13}C$ values of yolk protein or yolk lipid. There was no change in $\delta^{15}N$ values in albumen throughout the first 15 days of incubation. The assumption that the isotopic signatures of egg components remain constant throughout incubation is not valid.

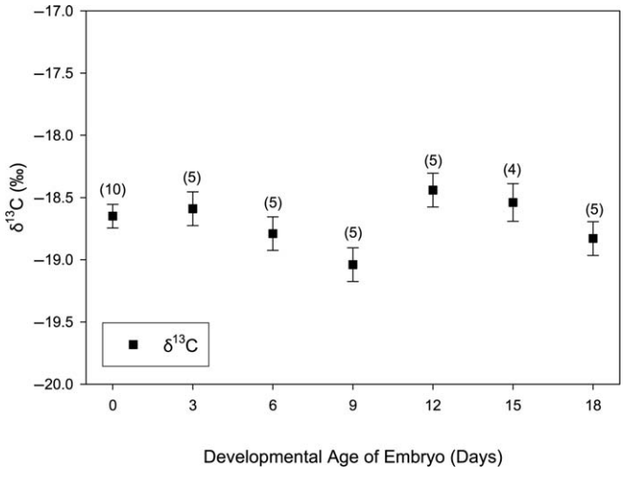

FIGURE 3. Mean (± SE) values for $\delta^{13}C$ in the yolk lipid of fertilized chicken (*Gallus gallus*) eggs from before incubation (day 0) to day 18 of embryo development. Sample sizes are indicated in parentheses.

The shift in $\delta^{13}C$ values in albumen between eggs in their first 3 days of incubation and eggs incubated 6 days or longer was significant, although the mechanism for the shift is unclear. In several species of birds, early in incubation there is substantial movement of water from albumen to the subembryonic fluid and yolk (Romanoff 1967). During the first 6 days of development, the wet weight of albumen of chicken eggs decreases by 20 g (Romanoff 1967), potentially initiating the 0.2‰ fractionation in albumen $\delta^{13}C$.

The change in $\delta^{15}N$ in yolk protein is driven by the approximately 1‰ shift following day 15 of incubation. This shift in isotope signature corresponds with an inflow of proteins from the shell and albumen into the yolk (Romanoff 1967, Deeming 2002) and is a plausible mechanism for the change in isotopic signature. Romanoff (1967) indicated that in chickens protein from the shell is transported to the yolk beginning at day 11 of incubation. At day 12 of incubation there is also a transfer of albumen proteins into the amniotic fluid through the ruptured sero-amniotic connection (Deeming and Ferguson 1991). The albumen–amniotic mixture is consumed orally by the developing embryo, and albumen proteins are subsequently transferred into the yolk sac through the gastrointestinal tract (Moran 2007). As a result of the infiltration of proteins from outside the yolk, the yolk's protein content is higher at 18 days of incubation than in a fresh egg (Romanoff 1967). The influx of albumen was not detectable in the $\delta^{13}C$ signature of yolk protein because in our study the $\delta^{13}C$ of albumen and yolk protein were very similar (Figs. 1 and 2).

Uptake of lipids by the developing embryo across the yolk-sac membrane is accomplished largely by phagocytosis (Speake et al. 1998). As a result of the bulk uptake of lipids, the fatty-acid profile of the yolk remains constant throughout embryo development (Donaldson 1964, Ding and Lilburn 1996, Speake et al. 1998, Moran 2007). Ding and Lilburn (1996) and Speake et al. (1998), however, presented evidence suggesting the preferential uptake of specific fatty acids during embryo development, although the exact pathways are unknown. While there is some evidence of preferential uptake of lipids during embryo development, we found no significant influence on $\delta^{13}C$ of yolk lipids, possibly because there is little or no difference in the isotopic signatures of different fatty acids.

As embryonic development can influence the $\delta^{13}C$ in albumen by the 6th day of incubation, one must weigh on the side of caution when collecting eggs that have been incubated. The magnitude of the shift is equivalent to the precision typically achieved during replicate analysis. In these situations, a sensitivity analysis could be used to determine the relative importance of correcting for the changes in $\delta^{13}C$ of albumen early in incubation (Phillips and Koch 2001). In most situations where endogenous and exogenous nutrient sources are very distinct isotopically (e.g., a shift from C-3 to C-4 isoscapes; marine vs. terrestrial), the relatively small fractionation in $\delta^{13}C$ in albumen may not have a significant effect on the end results and could be disregarded. However, in systems where nutrient sources are isotopically close (forbs vs. graminoids; Gauthier et al. 2003), a 0.2‰ effect of incubation must be accounted for if eggs have been incubated longer than 3 days since estimates of nutrient allocations could be influenced.

The resulting shift in $\delta^{15}N$ following protein transfer from albumen and shell limits the window in which researchers can gather eggs for isotope analysis. Our results suggest that in studies in which isotopic signatures are used to trace nutrient origin, eggs must be collected before the inflow of proteins from the shell and albumen into the yolk. Eggs collected after protein transfer may not have yolk-protein signatures representative of the fresh egg, making it difficult to assemble conclusions about nutrient

allocation during egg formation. Although we did not find a shift in $\delta^{13}C$ following protein transfer, in eggs whose $\delta^{13}C$ signatures of albumen and yolk are very different, a shift in $\delta^{13}C$ would also be expected following the movement of proteins into the yolk. In wild populations, the direction and magnitude of the shift seen in yolk protein will depend on the isotopic composition of albumen, shell, and yolk proteins; therefore, it may be impossible to correct for protein influxes in eggs late in incubation.

Although domestic poultry have proven to be useful models for understanding embryo development for most birds, differences across the altricial–precocial spectrum and in egg composition and size may influence patterns of development (Deeming 2002). Witschi (1949, cited in Deeming 2002) demonstrated in sparrows (*Passer* sp.); that protein transfer to the yolk begins about 70% through incubation (from day 9 through 11 of the 13-day incubation period), somewhat later than in the chicken. In the chicken, a precocial species, this transfer occurs approximately 60% through incubation (Romanoff 1967), but isotopic shifts were not evident until more than 70% of the way through incubation (after day 15 of 21). To our knowledge, Witschi (1949) and Romanoff (1967) are the only studies to examine the mobilization and transfer of albumen and shell proteins to the yolk. Further study is required to determine how nutrient utilization and movement varies with different developmental modes and how this variation affects isotopic changes during incubation. Also, it is unclear how egg size and egg composition might influence protein mobilization during incubation. The mechanisms responsible for isotopic changes seen during embryo development also demand further study.

Stable isotopes are undoubtedly an important tool for quantifying resource allocation during egg formation (Hobson 2006). To ensure that data are properly interpreted, however, it is important that researchers determine the developmental age of the egg when eggs are collected during incubation. For eggs collected >25% of the way through incubation, researchers should evaluate the effect of a potential 0.2‰ fractionation on a case-by-case basis. In wild populations, the collection of eggs for isotope analysis should be limited to the first 60% of the incubation period because it may be difficult to correct for protein influxes in eggs collected in the last 40% of incubation.

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