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CHICKEN AND EGG: TESTING THE CARBON ISOTOPIC EFFECTS OF CARNIVORY AND HERBIVORY*

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In bone, the spacing between $\delta^{13}C$ in collagen and bioapatite carbonate is greater in herbivores than carnivores, with implications for understanding animal dietary ecology from surviving hard tissues. Two explanations have been proposed: varying diet composition or differences in physiology between herbivores and carnivores. We measured the isotopic effects of carnivorous and herbivorous diets on a single species, to test the effect of diet composition alone. Protein $\delta^{13}C$ and $\delta^{15}N$ and carbonate $\delta^{13}C$ were measured on egg and bone from hens on different diets. Herbivorous hens had a +14.3‰ spacing between egg albumen and shell $\delta^{13}C$, compared to +12.4‰ for omnivorous hens, and +11.5‰ for carnivorous hens. The bioapatite–collagen $\Delta^{13}C$ spacing was measured as +6.2‰ for herbivorous hens, and calculated as +4.3‰ for omnivorous hens, and +3.4‰ for carnivorous hens—similar to observed mammalian herbivore and carnivore bioapatite–collagen $\Delta^{13}C$ differences. We conclude that a shift in diet composition from herbivory to carnivory in a single species does alter the bioapatite–collagen carbon isotopic spacing. Our data strongly suggest that this results from differences in the $\Delta^{13}C_{bioapatite-diet}$ spacing, and not that of $\Delta^{13}C_{collagen-diet}$.

KEYWORDS: BIOMINERAL, ISOTOPE ANALYSIS, PALAEODIET, ISOTOPE ECOLOGY

INTRODUCTION

The relationship between the carbon and nitrogen isotope composition of an individual's diet and their body tissues is now frequently employed as a method of dietary analysis in several different research fields, including ecology, archaeology and physiology (Gannes *et al.* 1998; Lee-Thorp 2008; Boecklen *et al.* 2011). The underlying basis of such work is that there is an isotopic 'fine structure' within the biosphere due to fractionation during natural processes, which results in isotopic differences between food types. An individual's diet can thus be inferred from the isotopic signature, which is transferred to and retained in the body during the absorption and incorporation of food.

The interpretation of diet is predicated on understanding and therefore accounting for any isotopic fractionation resulting from the incorporation of diet into body tissues. Over the decades, there have been a number of empirical studies aimed at elucidating isotopic fractionation during the body's metabolism, yet some observed patterns remain puzzling. One such is the offset between the carbon isotopic values of the biomineral and protein components of bone. This study

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aims to assess two competing hypotheses as to the origin of this signal, that of dietary composition and digestive physiology.

Field observations of carbon isotopic values of bone bioapatite and collagen demonstrate a consistent shift in the spacing between these two values (hereafter referred to as $\Delta^{13}C_{ap-coll}$) with the trophic level of the animal analysed. Krueger and Sullivan (1984) found a $\Delta^{13}C_{ap-coll}$ spacing of +7‰ for modern herbivores ($n = 20$), and +3–4‰ for modern carnivores ($n = 40$). Their analyses of over 200 archaeological humans showed that the majority had $\Delta^{13}C_{ap-coll}$ spacings of +3 to +7‰, as would be predicted for omnivores. In modern southern African wild fauna, Lee-Thorp *et al.* (1989) found that the $\Delta^{13}C_{ap-coll}$ was +6.8 ± 1.4‰ for herbivores ($n = 67$) and +4.3 ± 1.0‰ for carnivores ($n = 49$), with both groups having similar bioapatite $\delta^{13}C$ values of –14‰. In a study of both modern and fossil mammals, Clementz *et al.* (2009) showed a clear and consistent difference in $\Delta^{13}C_{ap-coll}$ between herbivores (+7.6 ± 0.5‰, $n = 46$) and carnivores (+4.8 ± 0.4‰, $n = 27$). Similar $\Delta^{13}C_{ap-coll}$ values have been found in a range of other ungulate herbivores (Schoeninger and DeNiro 1982; Nelson *et al.* 1986; Kellner and Schoeninger 2007). In predominately herbivorous primates, Crowley *et al.* (2010) observed a mean $\Delta^{13}C_{ap-coll}$ of +5.6‰, with some variability (range of +3.6 to +8.6‰). Ambrose (1993) analysed East African historical human bone, and showed that the $\Delta^{13}C_{ap-coll}$ of pastoralists (Kalenjin, Turkana, Pokot and Dasenech) was +3.8 ± 1.2‰ ($n = 8$), whereas the $\Delta^{13}C_{ap-coll}$ of Kikuyu farmers was +5.5 ± 0.5‰ ($n = 16$), supporting the theory that those humans with a diet high in animal protein have a smaller spacing between their collagen and bioapatite $\delta^{13}C$ than predominantly plant-eating farmers. Although based on a limited number of studies, the spacing between bone collagen and bone bioapatite $\delta^{13}C$ values is consistently about +7‰ for herbivores, and about +3–4‰ for carnivores.

The reasons underlying this variation in $\Delta^{13}C_{ap-coll}$ with trophic level are not yet known. It has long been known that different tissues within an individual are isotopically varied in $\delta^{13}C$ (DeNiro and Epstein 1978; Tieszen *et al.* 1983; Nakagawa *et al.* 1985; Tieszen and Boutton 1988). Data from laboratory-based animal feeding experiments have demonstrated that bone collagen $\delta^{13}C$ values primarily reflect the isotopic composition of the dietary protein intake, with some contribution from non-protein, whereas bone bioapatite (carbonate) $\delta^{13}C$ values are isotopically representative of the whole diet consumed (Ambrose and Norr 1993; Tieszen and Fagre 1993; Jim *et al.* 2004; Froehle *et al.* 2010; Fernandes *et al.* 2012). The number of laboratory-based feeding studies that have analysed both bone bioapatite and collagen $\delta^{13}C$ is quite limited. DeNiro and Epstein (1978) measured a mean $\Delta^{13}C_{ap-coll}$ of +6.3‰ in two groups of mice fed proprietary diets ($n = 6$ in total), while Hare *et al.* (1991) found that the mean $\Delta^{13}C_{ap-coll}$ was +9.1‰ for pigs raised on a pure C_3 plant diet ($n = 10$), and +6.8‰ for those consuming a pure C_4 plant diet ($n = 10$). Later feeding studies in which rodents and pigs were fed diets with a variety of isotopic compositions show that the $\Delta^{13}C_{ap-coll}$ can be manipulated to be between +1.3 and +11.3‰, depending on the relative isotopic values of dietary proteins, lipids and carbohydrates (Ambrose and Norr 1993; Tieszen and Fagre 1993; Howland *et al.* 2003; Jim *et al.* 2004; Warinner and Tuross 2009).

Two types of explanations can be proposed for the observed difference in $\Delta^{13}C_{ap-coll}$ with trophic level.

(1) Herbivores and carnivores consume fundamentally different diets (herbivorous diets are high in carbohydrate, low in protein and very low in lipid; carnivorous diets are high in protein, moderate in lipid and very low in carbohydrate). The differential use of dietary macronutrients in protein synthesis and energy provision, as well as the relationship between isotopic composition, abundance and differential routing to collagen and bioapatite for each dietary component could result in variation in $\Delta^{13}C_{ap-coll}$ with trophic level. This is the basis for the conceptual schemes proposed by Krueger and Sullivan (1984) and Lee-Thorp *et al.* (1989).

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(2) Differences in overall diet – body isotopic fractionation between herbivores and carnivores may arise from the different types of metabolic processes employed in their digestive physiologies. For example, methane produced during bacterial fermentation in the ruminant digestive system is isotopically very light ($\delta^{13}C$ of –70 to –50‰: Rust 1981; Metges *et al.* 1990). Isotopic mass balance considerations imply that the remaining carbon losses, of which breath CO_2 is by far the most important, will be isotopically heavier than if no methane was produced (Hedges and Van Klinken 2000). Since bone bioapatite carbonate and expired breath CO_2 are isotopically correlated (Tieszen and Fagre 1993), and both are derived from plasma bicarbonate, one could expect methanogenetic digesters to have bone bioapatite carbonate values enriched in ^{13}C relative to non-methanogenetic digesters, so explaining the observed increase in $\Delta^{13}C_{ap-coll}$ spacing between herbivores and carnivores (Hedges 2003). However, detailed estimates of this effect run into complications concerning the equilibration of CO_2 from fermentation with CO_2 from oxidation in herbivores (Hedges 2003), and the hypothesis remains to be demonstrated. Methanogenesis is not the only common physiological difference between herbivores and carnivores, and other processes may also contribute; for example, possible fractionation at the blood plasma – atmosphere interface might vary with energy expenditure (cf., O_2 fractionation during respiration: Kohn 1996).

Observations of variations of $\Delta^{13}C_{ap-coll}$ with trophic level have so far been derived from measurements on different species, hence not controlling for any physiological differences between species. This paper presents the results of the first study, to our knowledge, to assess whether diet macronutrient composition influences the $\Delta^{13}C_{ap-coll}$ spacing within a single species. Using the hen (*Gallus domesticus*) as a model species, we test whether variation in the diet composition (proportions of protein, lipid and carbohydrate) alters the isotopic spacing between body protein and body biomineral. We compare the carbon isotopic values of egg protein (albumen) and eggshell carbonate of hens fed on carnivorous, omnivorous and herbivorous diets. Subsequently, we extrapolate results from our egg data to examine the effect of differences in dietary composition on the spacing between bone collagen and bone bioapatite carbon isotopic values.

Choice of animal model

Given that we would also like to be able to extrapolate any conclusions to mammals (including humans), hens are not the obvious species to study, but there were a number of reasons for this choice. First, they can tolerate a wide range of diets and can survive equally well on an herbivorous or carnivorous diet. Hens can tolerate a wide range of dietary fat intake, from negligible amounts to diets containing up to 30% animal or vegetable fat (Fisher *et al.* 1961; Vermeersch and Vanschoebroek 1968).

Second, hens lay eggs, which can be used as a proxy material for bone. Given the substantial difficulties in getting truly isotopically equilibrated values for collagen and carbonate in most organisms, eggs offer a very useful alternative, since they equilibrate rapidly with the diet (Hobson 1995), and allow measurement of both a biomineral and a protein pool. Egg albumen is mainly composed of protein synthesized in the body (91% of albumen carbon occurs as protein: Johnson 1986), and as such its isotopic value is related to that of bone collagen, as has been demonstrated between hair/nail keratin and bone collagen (DeNiro and Epstein 1978; Tieszen *et al.* 1983; Tieszen and Boutton 1988; O'Connell *et al.* 2001). Eggshell carbon is predominantly in the form of carbonate (over 97%: Johnson 1986), which is derived from dissolved bicarbonate in the blood and in intra-cellular fluid in the uterus (shell gland) (Lörcher *et al.* 1970; Johnson 1986), while the source of bone bioapatite carbonate is blood bicarbonate.

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One difference between egg and bone is that egg production requires a considerably faster deposition of dissolved bicarbonate than bone production or turnover, but both are small in comparison to the respiratory bicarbonate flux: egg production requires typically 3 mmol h^{-1} of carbonate deposition (Sturkie and Mueller 1976) versus a first-order approximation of 200 mmol kg^{-1} h^{-1} of respiratory bicarbonate flux. The regular laying of eggs also means that a number of isotopic measurements can be made over time on the same individual, enabling us to study isotopic change within an individual.

Finally, there are obviously some key differences in physiology and metabolism between birds and mammals (e.g., gut structure, digestive physiology and nitrogen excretory mechanisms). But a number of studies have shown similarities in isotopic patterning between birds and mammals (and other taxa: e.g., Caut *et al.* 2009), such that we consider that physiological differences do not necessarily invalidate our choice of animal model, although they should be borne in mind.

MATERIALS AND METHODS

Experimental design

A total of 14 domestic laying hens were divided into three groups (A, B and C) and fed one of three diets: group A received a 100% plant-based diet; group B received a 50% plant-, 50% animal-based diet; and group C received a 10% plant-, 90% animal-based diet. The two diet components were wheat and tinned 'corned beef' (pure beef preserved by cooking in brine), with

Table 1 *Daily dietary intake per hen*

Main components	*Diet*		
	A *100% wheat*	*B* *50% wheat, 50% beef**	*C* *10% wheat, 90% beef**
Wheat (g d^{-1})†	120	60	12
Beef (g d^{-1})†	–	60	108
$CaSO_4$ (g d^{-1})†	14.2	14.2	14.2
$NaPO_4$ (g d^{-1})†	2.6	2.6	2.6
Vitamins and minerals (g d^{-1})†	0.3	0.3	0.3
Percentage protein‡	11	27	56
Percentage lipid‡	3	13	30
Percentage carbohydrate‡	84	57	8
Energy (kJ per 120 g)‡	1620	1370	1170
Total nitrogen (g per 120 g)‡	1.8	3.4	4.6
Bulk $\delta^{13}C$ (‰)*	–26.5	–23.3	–20.8
Bulk $\delta^{15}N$ (‰)*	+1.7	+4.4	+7.1

*Calculated from values of analysis of wheat and corned beef. Bulk beef $\delta^{13}C = -20.2$‰, $n = 2$. Beef fat (50% of carbon) $\delta^{13}C = -22.5 \pm 0.2$‰, $n = 3$.

†Wheat from Seeny's, Oxford, OX2 8HA, UK. The beef was corned beef from Savona Provisions Ltd, Oxford OX4 2SH, UK. Chemicals from BDH Merck, Poole, Dorset, BH15 1TD, UK. Vionate© supplement from Sherley's Ltd, Bishops Stortford CM23 5RG, UK.

‡Derived from analysis of wheat and corned beef by Aspland and James, Chatteris PE16 6QZ, UK. Percentages are of total dry weight. The totals do not add up to 1, as the remaining portion is ash.

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both components chosen to be as isotopically similar as possible and to be C_3 (details given in Table 1). Each hen received at least 120 g of food per day, with water *ad libitum*. Laying hens typically require an energy intake of upwards of 1150 kJ d^{-1} (Leclerq 1986), which was provided by all three diets. The hens were also fed daily supplements of phosphorus and calcium to ensure the continuation of egg laying, grit to aid digestion and a general avian vitamin and mineral supplement (Vionate©). Phosphorus was supplied as sodium phosphate. Calcium was supplied in the form of calcium sulphate rather than the more usual calcium carbonate, to avoid any adulteration of the carbon intake of the bird by non-quantifiable routes. For the same reason, flint was used as grit, rather than limestone or oyster shell. Diet composition and isotopic values are shown in Table 1. This experiment was carried out in 1999, in compliance with UK Home Office Laws on animal experimentation.

The A and B groups were kept on their assigned diets for 27 weeks, Group C were kept on the mixed wheat and corned beef diet (as for Group B) for 17 weeks, then on a diet of 10% wheat, 90% corned beef for 10 weeks. Originally, the experimental design was that Group C should be fed 100% corned beef; however, it proved very difficult to mix the mineral supplements with corned beef in such a way as to ensure that the hens had a homogeneous intake. Eventually, the diet was modified to be 10% wheat, 90% corned beef, with the mineral supplements mixed with the wheat. This problem was responsible for the time lag in the introduction of the carnivorous diet. Prior to the experiment, the hens had been fed a proprietary wheat-based diet.

Sample collection and analysis

During the experiment, eggs were collected for analysis from each group over a 48 h period at the end of each week, beginning in the first week of the experiment. Since all hens in each group were kept in a single enclosure, it was not possible to identify which egg was laid by which hen, but collection over 48 h each week meant that a maximum of two eggs could be collected each week from any one hen. The albumen and shell were separated out from each egg by hand, the shell rinsed in distilled water, and then both samples were stored at –18°C prior to analysis. At the end of the experiment, femoral bone samples were taken from two hens in Group A (wheat-fed) and two in Group C (beef-fed).

Albumen Egg albumen was prepared for isotopic analysis by freeze-drying and then powdering. No attempt was made to separate out the albumen proteins from the albumen carbohydrate and lipid: since total carbohydrate and lipid content of albumen is less than 10% of the total (Sturkie and Benzo 1986), and carbohydrate and lipid within a single individual have not been measured as more than 5‰ different to body protein carbon isotopic values (Tieszen and Fagre 1993), we decided that chemical separation of the protein fraction could possibly result in more alteration of the measured $\delta^{13}C$ than that derived from the carbohydrate and lipid.

Shell Shell samples were prepared for carbonate isotopic analysis by a standard method (Sponheimer and Lee-Thorp 1999). The egg membrane was removed, the shell rinsed in distilled water and crushed. Samples were then defatted in methanol and chloroform (2:1 v/v) for at least 12 h, soaked in a 1.5% solution of sodium hypochlorite until bubbling stopped (usually overnight) to remove organics, soaked for 30 min in 1M acetic acid, and then finally rinsed until neutral and dried.

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Collagen Collagen was extracted from each bone sample for isotopic analysis following a standard method (O'Connell *et al.* 2001). The samples were rinsed in water, defatted by soaking twice in methanol and chloroform (2:1 v/v), first overnight and then for 1 h, then demineralized in 0.5M aq. hydrochloric acid for 2–3 days until the mineral phase was dissolved, gelatinized in water of pH 3 for 24 h at 75°C, and the resulting soluble collagen freeze-dried.

Bioapatite Carbonate was extracted from the bone mineral bioapatite by the same method as for eggshell carbonate (see above).

Diet Samples of wheat, bulk 'corned' beef and fat samples isolated from the 'corned' beef were freeze-dried and ground in preparation for isotopic analysis.

Isotopic analysis Carbon and nitrogen isotopic analyses of egg albumen, bone collagen and diet (wheat and beef) were performed using an automated carbon and nitrogen analyser coupled to a continuous-flow isotope ratio monitoring mass spectrometer (a Carlo Erba elemental analyser coupled to a Europa Geo 20/20 mass spectrometer at the RLAHA, University of Oxford). Typical replicate measurement errors are ±0.2‰ for $\delta^{13}C$ and $\delta^{15}N$, calculated from repeat measurements of in-house standards (nylon and alanine). Carbon isotopic analyses of eggshell carbonate and bone bioapatite carbonate were performed by acidification of samples with 100% H_3PO_4 at 90°C on an ISOCARB 1 preparation system, with subsequent analyses of the evolved CO_2 carried out on a VG PRISM isotope ratio mass spectrometer (in the Department of Earth Sciences, University of Oxford). Reproducibility was better than ±0.1‰ for $\delta^{13}C$. All isotopic results are reported using the standard 'δ' notation in units of permil (‰), with $\delta^{13}C$ relative to VPDB and $\delta^{15}N$ relative to AIR (Coplen 2011).

RESULTS

The isotopic composition of the eggs of the three groups in this experiment showed little variability during the course of the experiment, of less than 2‰ from start to finish (Fig. 1). For the

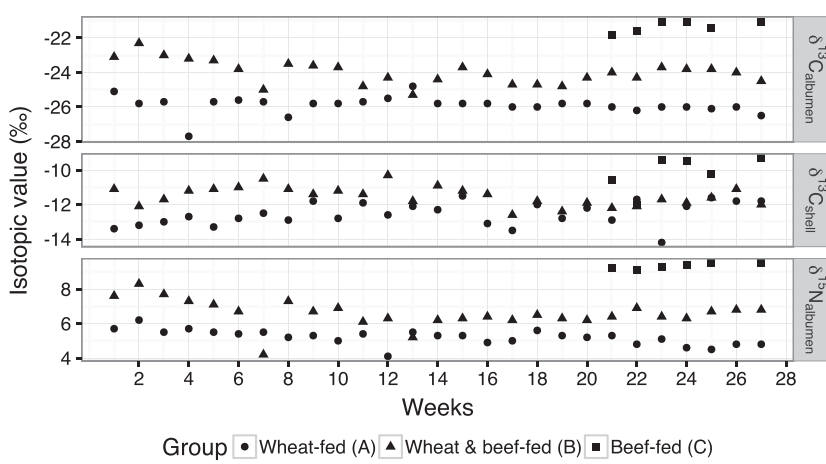

Figure 1 *The variation in eggshell $\delta^{13}C$ and egg albumen $\delta^{13}C$ and $\delta^{15}N$ over the time course of the experiment. Each point represents the mean of the analyses of three eggs.*

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Table 2 *Mean isotopic values of diet, egg albumen and shell, and bone collagen and bioapatite*

Group	*n, egg*	*Diet* $\delta^{13}C$ (‰)	*Diet* $\delta^{15}N$ (‰)	*Shell* $\delta^{13}C^{*}$ (‰)	*Albumen* $\delta^{13}C^{*}$ (‰)	*Albumen* $\delta^{15}N^{*}$ (‰)	*Albumen C/N ratio*	*n, bone*	*Bone bioapatite* $\delta^{13}C^{\dagger}$(‰)	*Bone collagen* $\delta^{13}C^{\dagger}$ (‰)	*Bone collagen* $\delta^{15}N^{\dagger}$(‰)	*Bone collagen C/N*† *ratio*
A, hens‡	12	–26.5	2.2	–11.8 ± 0.8	–26.1 ± 0.3	4.7 ± 0.3	4.0 ± 0.1	2	–15.7	–21.6	5.0	3.2
									–14.8	–21.3	5.2	3.2
B, hens‡	12	–23.3	4.9	–11.6 ± 0.8	–24.0 ± 0.4	6.7 ± 0.2	4.0 ± 0.1	–				
C, hens‡	9	–20.8	7.1	–9.7 ± 1.0	–21.2 ± 0.3	9.4 ± 0.3	4.0 ± 0.1	2	–15.0	–21.1	5.2	3.2
									–14.1	–21.2	5.2	3.2

*Mean of values from each group's eggs from the final 4 weeks of the experiment, giving mean ± 1 SD.

†Data from each individual reported, as $n = 2$.

‡Group A hens fed on 100% wheat; group B hens fed on 50% wheat, 50% corned beef; group C hens fed on 10% wheat, 90% corned beef.

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overall isotopic comparison of egg albumen and eggshell, the results of the eggs sampled from each group were averaged over the final 4 weeks of collection, which was a total of 12 eggs for both the A and B groups, and nine eggs for the C group (Table 2).

Eggs from hens fed on the wheat diet (A) had a mean $\Delta^{13}C_{shell-albumen}$ spacing of +14.3 ± 0.8‰ (SD), compared to a mean $\Delta^{13}C_{shell-albumen}$ spacing of +11.5 ± 0.5‰ (SD) for those hens fed on the diet of corned beef (C). Those fed on the mixed diet (B) had an intermediate mean $\Delta^{13}C_{shell-albumen}$ spacing of +12.4 ± 0.7‰ (SD) (Fig. 2). Analysis of variance provides strong evidence that the $\Delta^{13}C_{shell-albumen}$ values of these three groups are significantly different (SPSS ANOVA: $F = 34.29$, 2/30*df*, $p < 0.001$). Post-hoc Bonferroni tests show that group A is significantly different at the 95% level from both B and C ($p < 0.001$), but groups B and C are not significantly different from each other ($p = 0.071$). In view of the unavoidable pseudo-replication in the experimental design (more than one egg laid by each hen in the final 4 weeks of egg collection), the probability values are very likely to be overestimates. However, it is obvious graphically that the $\Delta^{13}C_{shell-albumen}$ of eggs from A and C hens are very different, and that those of A and B hens are probably different (Fig. 2).

Isotopic data of bone from two group A hens (wheat-fed) and two group C hens (beef-fed) are shown in Table 2. For the group A hens, the $\Delta^{13}C_{collagen-diet}$ is +5.1‰, and the $\Delta^{15}N_{collagen-diet}$ is +2.9‰, and for the C hens, the $\Delta^{13}C_{collagen-diet}$ is –0.3‰, and the $\Delta^{15}N_{collagen-diet}$ is –1.9‰ (Table 3). Both groups had very similar bone collagen $\delta^{15}N$ values (5.1 versus 5.2‰: Table 2).

The $\Delta^{13}C_{ap-coll}$ spacing was measured as +6.2‰ for hens on the wheat-based diet (A), and as +6.6‰ for hens on the beef-based diet (C). We also calculated the bone bioapatite–collagen spacings for those on a mixed wheat and beef diet (B) and beef diet (C) from the measurements of egg albumen–shell spacings for these groups. We assumed that the eggshell carbonate – bone bioapatite $\Delta^{13}C$ spacing is the same for all hens, as is the egg albumen – bone collagen $\Delta^{13}C$ spacing, independent of diet, and used the spacings measured in the wheat-fed hens ($\Delta^{13}C_{shell-bioapatite}$ of 3.4‰ and $\Delta^{13}C_{albumen-collagen}$ of –4.7‰). The $\Delta^{13}C_{ap-coll}$ spacing is calculated as +4.3‰ for those on a mixed wheat and beef diet (B), and +3.4‰ for those on the beef diet (C) (Table 3).

DISCUSSION

Egg results from this study

The lack of isotopic variability of eggs of the three groups during the course of the experiment (Fig. 1) implies that the eggs of all groups rapidly achieved isotopic equilibrium with the diet (a matter of a few days), supporting Hobson's conclusions from dietary switching experiments that eggs of laying birds are isotopically equilibrated to a new diet within a short period (10 days) after a change in diet (Hobson 1995). Similarly fast isotopic changes in rapidly turning over body pools have been documented in mammals and birds (Ayliffe *et al.* 2004; Podlesak *et al.* 2005; Sponheimer *et al.* 2006). Both the egg albumen values and the offsets between egg albumen and diet in both carbon and nitrogen isotopic values show some degree of variability (Table 3), but are similar to those found in large-scale studies of eggs and egg-laying hens in the Netherlands, New Zealand and Brazil (Madeira *et al.* 2015; Rogers *et al.* 2015).

Hens fed on the wheat diet (A) had a mean $\Delta^{13}C_{shell-albumen}$ spacing of +14.3‰, compared to a mean spacing of +12.4‰ for those hens on a mixed diet (B), and +11.5‰ for those hens fed on beef (C), showing that within a single species, the $\Delta^{13}C_{shell-albumen}$ spacing is affected by the diet composition, and that an herbivorous diet results in a greater spacing between body protein and

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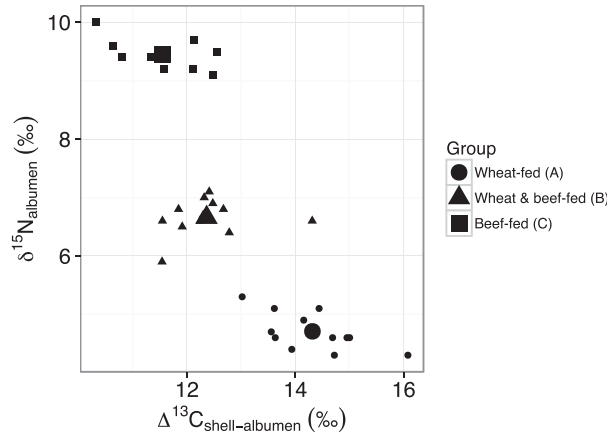

Figure 2 *The egg albumen $\delta^{15}N$ versus $\Delta^{13}C_{shell-albumen}$ spacing for the final 4 weeks of egg collection from each group of hens, showing the data for each egg (smaller symbols) as well as the mean for each group (large symbols).*

carbonate $\delta^{13}C$ than does a carnivorous diet (Fig. 2). Data from other species are in agreement with these results: the $\Delta^{13}C_{shell-albumen}$ spacing for the wheat-fed hens (+14.3‰) is similar to that observed by Hobson in mallards and quails fed on a wheat-based diet (+14.0‰, +13.1‰), and the observed $\Delta^{13}C_{shell-albumen}$ for the beef-fed hens (+11.5‰) is similar to that observed in (carnivorous) falcons (+10.4‰) (Hobson 1995: data shown in Table 3). And while shell and albumen $\delta^{13}C$ values cannot be directly equated with the $\delta^{13}C$ of bone bioapatite carbonate and collagen, the difference between the observed $\Delta^{13}C_{shell-albumen}$ values of the herbivorous and carnivorous hens is 2.8‰ (+14.3‰ versus +11.5‰), very similar to the typical 3‰ difference observed between the $\Delta^{13}C_{ap-coll}$ spacings of herbivores (about +7‰) and carnivores (about +4‰) (Krueger and Sullivan 1984; Lee-Thorp *et al.* 1989; Clementz *et al.* 2009).

It appears that most of the difference in spacing with trophic level is due to a difference in the carbonate–diet spacing: the $\Delta^{13}C_{albumen-diet}$ varies within 1‰ for the three groups of hens, but the $\Delta^{13}C_{shell-diet}$ differs by 3.6‰ from +14.7‰ for the wheat-fed hens to +11.1‰ for the beef-fed hens. Other studies have similarly found that the $\Delta^{13}C_{collagen-diet}$ spacing is a constant value between different species, while the $\Delta^{13}C_{biopatite-diet}$ spacing is variable (Sullivan and Krueger 1981; Krueger and Sullivan 1984; Lee-Thorp *et al.* 1989; Hobson 1995; Cerling and Harris 1999; Passey *et al.* 2005).

Bone results from this study

This study aimed to test whether the observed differences in $\Delta^{13}C_{ap-coll}$ in herbivorous and carnivorous animals could result from differing dietary composition (in terms of macronutrients). The $\Delta^{13}C_{ap-coll}$ spacing was measured as +6.2‰ for hens on the wheat-based diet (A), and as +6.6‰ for hens on the beef-based diet (C). However, although the results indicate that the eggs of all groups are isotopically equilibrated with the diet within the time frame of the experiment, this was not the case for the bone samples: there are considerable discrepancies in the offsets between bone and diet isotopic values for the samples analysed from the wheat-fed (A) and beef-fed (C) hens. For the wheat-fed hens, the $\Delta^{13}C_{collagen-diet}$ is +5.1‰, and the $\Delta^{15}N_{collagen-diet}$ is +2.9‰, as would be expected based on other animal feeding experiments (the enrichment in small animals between diet and collagen is typically +5‰ for $\delta^{13}C$, and +3‰ for $\delta^{15}N$: DeNiro and Epstein 1978, 1981; Ambrose and Norr 1993; Tieszen and Fagre 1993; Caut *et al.* 2009).

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Table 3 *Isotopic offsets between diet and egg albumen and shell, and diet and bone collagen and bioapatite, in comparison to other published data*

Group	$\Delta^{13}C_{shell-diet}$ (‰)	$\Delta^{13}C_{albumen-diet}$ (‰)	$\Delta^{15}N_{albumen-diet}$ (‰)	$\Delta^{13}C_{shell-albumen}$ (‰)	$\Delta^{13}C_{bioapatite-diet}$ (‰)	$\Delta^{13}C_{collagen-diet}$ (‰)	$\Delta^{15}N_{collagen-diet}$ (‰)	$\Delta^{13}C_{measured\ bioapatite-collagen}$ (‰)	$\Delta^{13}C_{calculated\ bioapatite-collagen}$* (‰)
A, hens†	14.7	0.4	2.5	14.3 ± 0.8	11.3	5.1		6.2	
B, hens†	11.7	–0.7	1.7	12.4 ± 0.7					**4.3**
C, hens†	11.1	–0.4	2.4	11.5 ± 0.5	*6.3*‡	*–0.3*‡		*6.6*‡	**3.4**
Mallards§	14.5	1.4	2.9	14.0					
Quails§	15.6	1.6	2.4	13.1					
Falcons§	11.2	0.9	3.1	10.4					

*Calculated using the $\Delta^{13}C_{shell-bioapatite}$ value of 3.4‰ and the $\Delta^{13}C_{albumen-collagen}$ value of –4.7‰ derived from analyses of group A hens.

†Group A hens fed on 100% wheat; group B hens fed on 50% wheat, 50% corned beef; group C hens fed on 10% wheat, 90% corned beef.

‡Italics denote that bone collagen and bioapatite are not isotopically equilibrated with the diet.

§Data from Hobson (1995).

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Prior to this experiment, all the hens had been on a proprietary wheat-based diet, so we assume that the wheat-based diet of this experiment did not constitute a significant change (either isotopically or compositionally) and therefore the bone collagen of the wheat-fed hens can be considered as isotopically equilibrated. However, for the beef-fed hens, the $\Delta^{13}C_{collagen-diet}$ is –0.3‰, and the $\Delta^{15}N_{collagen-diet}$ is –1.9‰. Both A and C hens also had similar collagen $\delta^{15}N$ values of 5.1 and 5.2‰. This implies that the bone collagen of group C hens has not yet isotopically equilibrated with the diet, which is to be expected, given that quail bone collagen was not equilibrated 212 days (30 weeks) after a change in diet from C_3 to C_4 protein (Hobson and Clark 1992). The turnover rate for bone bioapatite carbonate is poorly quantified, but is probably similar to that of collagen (in rats, mean 99% turnover time of collagen calculated as 6.9 years, mean 99% turnover time of apatite carbonate calculated as 4.1 years: Jim 2000). Because of the lack of isotopic equilibration, we consider that the measured $\Delta^{13}C_{ap-coll}$ spacing of +6.6‰ for hens on the beef-based diet (C) is not a true representation of what would be expected from birds raised long-term on such a diet.

Assuming that the both bones and eggs of the wheat-fed hens were in isotopic equilibrium with the diet, and that the eggs of the other two groups were also in isotopic equilibrium with the diet, then from the measured $\Delta^{13}C_{shell-bioapatite}$ and $\Delta^{13}C_{albumen-collagen}$ of the wheat-fed hens, we can calculate the expected $\Delta^{13}C_{ap-coll}$ values for the other two groups (Table 3). The measured or calculated $\Delta^{13}C_{ap-coll}$ spacings of +6.2‰, +4.3‰ and +3.4‰ for groups A, B and C are similar in magnitude to those observed in analyses of modern wild herbivorous and carnivorous fauna (Krueger and Sullivan 1984; Lee-Thorp *et al.* 1989). However, further work is needed to demonstrate incontrovertibly that for hens on different diets the $\Delta^{13}C_{ap-coll}$ spacing is as we have calculated, based on the egg data from this experiment.

CONCLUSIONS

This experiment documents that there is a clear difference in the $\Delta^{13}C_{shell-albumen}$ spacing in egg, depending on the type of diet consumed by the laying hen (herbivorous, omnivorous or carnivorous). This difference is in the same direction and magnitude as expected from observations of herbivore and carnivore $\Delta^{13}C_{ap-coll}$ spacings.

Although both protein and carbonate in egg require isotopic 'corrections' in order to calculate bone collagen and bioapatite carbonate, these have been measured accurately for hens on one of the three diets, and, when applied to the egg results of the other two groups of hens, indicate that differences in diet composition (herbivorous versus carnivorous) are sufficient to bring about differences of similar magnitude in the $\Delta^{13}C_{ap-coll}$ spacing to that observed in mammals. From our results, it appears that most of the variation in the protein-carbonate spacing is due to differences in the $\Delta^{13}C_{carbonate-diet}$ spacing, rather than the $\Delta^{13}C_{protein-diet}$ spacing, a result also suggested by other studies (Sullivan and Krueger 1981; Krueger and Sullivan 1984; Lee-Thorp *et al.* 1989; Hobson 1995; Cerling and Harris 1999; Passey *et al.* 2005).

We conclude that the differences in diet composition with trophic level are likely to affect bioapatite–collagen carbon isotopic spacing. More research is required to assess whether differences in gut physiology with trophic guild also have any effect on the $\Delta^{13}C_{ap-coll}$ spacing.

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REFERENCES

Ambrose, S. H., 1993, Isotopic analysis of paleodiets: methodological and interpretive considerations, in *Investigations of Ancient Human Tissue* (ed. M. K. Sandford), Gordon & Breach Science Publishers, Langhorne, PA.

Ambrose, S. H., and Norr, L., 1993, Experimental evidence for the relationship of the carbon isotope ratios of whole diet and dietary protein to those of bone collagen and carbonate, in *Prehistoric human bone—archaeology at the molecular level* (eds. J. B. Lambert and G. Grupe), 1–37, Springer-Verlag, Berlin.

Ayliffe, L. K., Cerling, T. E., Robinson, T., West, A. G., Sponheimer, M., Passey, B. H., Hammer, J., Roeder, B., Dearing, M. D., and Ehleringer, J. R., 2004, Turnover of carbon isotopes in tail hair and breath CO_2 of horses fed an isotopically varied diet, *Oecologia*, **139**, 11–22.

Boecklen, W. J., Yarnes, C. T., Cook, B. A., and James, A. C., 2011, On the use of stable isotopes in trophic ecology, in *Annual review of ecology, evolution, and systematics* (eds. D. J. Futuyma, H. B. Shaffer, and D. Simberloff), Vol. **42**, Annual Reviews, Inc., Palo Alto, CA.

Caut, S., Angulo, E., and Courchamp, F., 2009, Variation in discrimination factors ($\Delta^{15}N$ and $\Delta^{13}C$): the effect of diet isotopic values and applications for diet reconstruction, *Journal of Applied Ecology*, **46**, 443–53.

Cerling, T. E., and Harris, J. M., 1999, Carbon isotope fractionation between diet and bioapatite in ungulate mammals and implications for ecological and paleoecological studies, *Oecologia*, **120**, 347–63.

Clementz, M. T., Fox-Dobbs, K., Wheatley, P. V., Koch, P. L., and Doak, D. F., 2009, Revisiting old bones: coupled carbon isotope analysis of bioapatite and collagen as an ecological and palaeoecological tool, *Geological Journal*, **44**, 605–20.

Coplen, T. B., 2011, Guidelines and recommended terms for expression of stable-isotope-ratio and gas-ratio measurement results, *Rapid Communications in Mass Spectrometry*, **25**, 2538–60.

Crowley, B. E., Carter, M. L., Karpanty, S. M., Zihlman, A. L., Koch, P. L., and Dominy, N. J., 2010, Stable carbon and nitrogen isotope enrichment in primate tissues, *Oecologia*, **164**, 611–26.

DeNiro, M. J., and Epstein, S., 1978, Influence of diet on the distribution of carbon isotopes in animals, *Geochimica et Cosmochimica Acta*, **42**, 495–506.

DeNiro, M. J., and Epstein, S., 1981, Influence of diet on the distribution of nitrogen isotopes in animals, *Geochimica et Cosmochimica Acta*, **45**, 341–51.

Fernandes, R., Nadeau, M.-J., and Grootes, P. M., 2012, Macronutrient-based model for dietary carbon routing in bone collagen and bioapatite, *Archaeological and Anthropological Sciences*, **4**, 291–301.

Fisher, H., Feigenbaum, A. S., and Weiss, H. S., 1961, Requirement of essential fatty acids and avian atherosclerosis, *Nature*, **192**, 1310.

Froehle, A. W., Kellner, C. M., and Schoeninger, M. J., 2010, FOCUS: effect of diet and protein source on carbon stable isotope ratios in collagen: follow up to Warinner and Tuross (2009), *Journal of Archaeological Science*, **37**, 2662–70.

Gannes, L. Z., del Rio, C. M., and Koch, P., 1998, Natural abundance variations in stable isotopes and their potential uses in animal physiological ecology, *Comparative Biochemistry and Physiology*, *Part A, Molecular & Integrative Physiology*, **119**, 725–37.

Hare, P. E., Fogel, M. L., Stafford, T. W. Jr., Mitchell, A. D., and Hoering, T. C., 1991, The isotopic composition of carbon and nitrogen in individual amino acids isolated from modern and fossil proteins, *Journal of Archaeological Science*, **18**, 277–92.

Hedges, R. E. M., 2003, On bone collagen – apatite–carbonate isotopic relationships, *International Journal of Osteoarchaeology*, **13**, 66 –79.

Hedges, R. E. M., and Van Klinken, G. J., 2000, 'Consider a spherical cow ...'—on modelling and diet, in *Biogeochemical approaches to paleodietary analysis* (eds. S. H. Ambrose and M. A. Katzenberg), 211–41, Kluwer Academic/Plenum Press, New York.

Hobson, K. A., 1995, Reconstructing avian diets using stable carbon and nitrogen isotope analysis of egg components: patterns of isotopic fractionation and turnover, *The Condor*, **97**, 752–62.

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Hobson, K. A., and Clark, R. G., 1992, Assessing avian diets using stable isotopes I: turnover of ^{13}C in tissues, *The Condor*, **94**, 181–8.

Howland, M. R., Corr, L. T., Young, S. M. M., Jones, V., Jim, S., van der Merwe, N. J., Mitchell, A. D., and Evershed, R. P., 2003, Expression of the dietary isotope signal in the compound-specific $\delta^{13}C$ values of pig bone lipids and amino acids, *International Journal of Osteoarchaeology*, **13**, 54–65.

Jim, S, 2000, The development of bone cholesterol $\delta^{13}C$ values as a new source of palaeodietary information: qualitative and quantitative models of its use in conjunction with bone collagen and apatite $\delta^{13}C$ values, Ph.D. thesis, University of Bristol.

Jim, S., Ambrose, S. H., and Evershed, R. P., 2004, Stable carbon isotopic evidence for differences in the dietary origin of bone cholesterol, collagen and apatite: Implications for their use in palaeodietary reconstructions, *Geochimica et Cosmochimica Acta*, **68**, 61–72.

Johnson, A. L., 1986, Reproduction in the female, in *Avian physiology* (eds. P. D. Sturkie and C. A. Benzo), 403–31, 4th edn, Springer-Verlag, New York.

Kellner, C. M., and Schoeninger, M. J., 2007, A simple carbon isotope model for reconstructing prehistoric human diet, *American Journal of Physical Anthropology*, **133**, 1112–27.

Kohn, M. A., 1996, Predicting animal $\delta^{18}O$: accounting for diet and physiological adaptation, *Geochimica et Cosmochimica Acta*, **60**, 4811–29.

Krueger, H. W., and Sullivan, C. H., 1984, Models for carbon isotope fractionation between diet and bone, in *Stable isotopes in human nutrition* (eds. J. S. Turnlund and P. E. Johnson), 205–22, American Chemical Society, Washington, DC.

Leclerq, B., 1986, Energy requirements of avian species, in *Nutrient requirements of poultry and nutritional research* (eds. C. Fisher and K. N. Boorman), 125–39, Butterworths, London.

Lee-Thorp, J. A., 2008, On isotopes and old bones, *Archaeometry*, **50**, 925–50.

Lee-Thorp, J. A., Sealy, J. C., and van der Merwe, N. J., 1989, Stable carbon isotope ratio differences between bone collagen and bone apatite, and their relationship to diet, *Journal of Archaeological Science*, **16**, 585–99.

Lörcher, K., Zscheile, C. H., and Bronsch, K., 1970, Transfer of continuously i.v. infused $NaHC^{14}O_3$ and $Ca^{47}Cl_2$ to the hen's egg-shell, *Annales de Biologie animale*, **10**, 193–8.

Madeira, L. A., Denadai, J. C., Ducatti, C., Pezzato, A. C., de Araujo, P. C., Pereira Sartori, M. M., Pizzolante, C. C., and Sartori, J. R., 2015, Assessment of low amounts of meat and bone meal in the diet of laying hens by using stable isotopes, *Semina-Ciencias Agrarias*, **36**, 1155–67.

Metges, C., Kempe, K., and Schmidt, H.-L., 1990, Dependence of the carbon isotope contents of breath carbon dioxide, milk serum and rumen fermentation products on the $\delta^{13}C$ value of food in dairy cows, *British Journal of Nutrition*, **63**, 187–96.

Nakagawa, A., Kitagawa, A., Asami, M., Nakamura, K., Schoeller, D. A., Slater, R., Minagawa, M., and Kaplan, I. R., 1985, Evaluation of isotope ratio (IR) mass spectrometry for the study of drug metabolism, *Biomedical Mass Spectrometry*, **12**, 502–6.

Nelson, B. K., DeNiro, M. J., Schoeninger, M. J., and De Paolo, D. J., 1986, Effects of diagenesis on strontium, carbon, nitrogen and oxygen concentration and isotopic composition of bone, *Geochimica et Cosmochimica Acta*, **50**, 1941–9.

O'Connell, T. C., Healey, M. A., Hedges, R. E. M., and Simpson, A. H. W., 2001, Isotopic comparison of hair, bone and nail: modern analyses, *Journal of Archaeological Science*, **28**, 1247–55.

Passey, B. H., Robinson, T. F., Ayliffe, L. K., Cerling, T. E., Sponheimer, M., Dearing, M. D., Roeder, B. L., and Ehleringer, J. R., 2005, Carbon isotope fractionation between diet, breath CO_2, and bioapatite in different mammals, *Journal of Archaeological Science*, **32**, 1459–70.

Podlesak, D. W., McWilliams, S. R., and Hatch, K. A., 2005, Stable isotopes in breath, blood, feces and feathers can indicate intra-individual changes in the diet of migratory songbirds, *Oecologia*, **142**, 501–10.

Rogers, K. M., van Ruth, S., Alewijn, M., Philips, A., and Rogers, P., 2015, Verification of egg farming systems from the Netherlands and New Zealand using stable isotopes, *Journal of Agricultural and Food Chemistry*, **63**, 8372–80.

Rust, F., 1981, Ruminant methane $\delta(^{13}C/^{12}C)$ values: relation to atmospheric methane, *Science*, **211**, 1044–6.

Schoeninger, M. J., and DeNiro, M. J., 1982, Carbon isotope ratios of apatite from fossil bone cannot be used to reconstruct diets of animals, *Nature*, **297**, 577–8.

Sponheimer, M., and Lee-Thorp, J. A., 1999, Oxygen isotopes in enamel carbonate and their ecological significance, *Journal of Archaeological Science*, **26**, 723–8.

Sponheimer, M., Robinson, T. F., Cerling, T. E., Tegland, L., Roeder, B. L., Ayliffe, L., Dearing, M. D., and Ehleringer, J. R., 2006, Turnover of stable carbon isotopes in the muscle, liver, and breath CO_2 of alpacas (*Lama pacos*), *Rapid Communications in Mass Spectrometry*, **20**, 1395–9.

Sturkie, P. D., and Benzo, C. A., 1986, *Avian physiology*, Springer, New York.

Sturkie, P. D., and Mueller, W. J., 1976, Reproduction in the female and egg production, in *Avian physiology* (eds. P. D. Sturkie and T. B. Bolton), 302–30, 3rd edn, Springer, New York.

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Sullivan, C. H., and Krueger, H. W., 1981, Carbon isotope analysis of separate chemical phases in modern and fossil bone, *Nature*, **292**, 333–5.

Tieszen, L. L., and Boutton, T. W., 1988, Stable carbon isotopes in terrestrial ecosystem research, in *Stable isotopes in ecological research* (eds. P. W. Rundel, J. R. Ehleringer, and K. A. Nagy), 167–95, Springer-Verlag, Berlin.

Tieszen, L. L., Boutton, T. W., Tesdahl, K. G., and Slade, N. A., 1983, Fractionation and turnover of stable isotopes in animal tissues: implications for $\delta^{13}C$ analysis of diet, *Oecologia*, **57**, 32–7.

Tieszen, L. L., and Fagre, T., 1993, Effect of diet quality and composition on the isotopic composition of respiratory CO_2, bone collagen, bioapatite, and soft tissues, in *Prehistoric human bone—archaeology at the molecular level (eds. J. B. Lambert and G. Grupe)*, 121–55, Springer-Verlag, Berlin.

Vermeersch, G., and Vanschoebroek, F., 1968, The quantification of the effect of increasing levels of various fats on body weight gain, efficiency of food conversion and food intake of growing chicks, *British Poultry Science*, **9**, 13–30.

Warinner, C., and Tuross, N., 2009, Alkaline cooking and stable isotope tissue–diet spacing in swine: archaeological implications, *Journal of Archaeological Science*, **36**, 1690–7.

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