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Black and white – does melanin change the bulk carbon and nitrogen isotope values of feathers?

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Bird feathers are employed in a wide range of carbon and nitrogen isotope studies relating to diet and migration. Feathers are chemically inert with respect to carbon and nitrogen, after synthesis. It has always been assumed that feathers show isotope values characteristic of keratin, a fibrous structural protein from which they are formed. Little attention has been paid to other components of feathers such as melanin or carotenoids. Melanin is synthesized from tyrosine, which is depleted in both ^{13}C and ^{15}N. We compared isotope values of coeval black and white feathers in four different species. Black feather parts were in all cases significantly depleted in ^{13}C relative to white feather parts but in most species no clear trend was discernable for ^{15}N. We suggest that additional evaluation may be required to characterize the carbon and nitrogen isotope contribution of feather pigments like carotenoids. Care should be taken in future stable isotope studies when comparing differently coloured feathers. Copyright © 2010 John Wiley & Sons, Ltd.

Currently, stable isotope analyses of nitrogen and carbon are widely used in ecological food-web studies as these isotope ratios are believed to change in a predictable manner[1–3] – but see Eggers and Jones.[4] Carbon mainly indicates the feeding place, whereas nitrogen indicates differences in trophic level.[5–7] In avian research these stable isotope techniques are often used to investigate behaviour in the non-breeding season, when birds are not accessible or cannot be monitored by techniques such as transmitters.[7,8] In such cases, feather is the tissue chosen for isotope studies because feathers provide an indication of dietary sources and trophic position during the moulting period.[9–11] Furthermore, they can be sampled non-destructively from living birds,[12] can be collected at moulting places or taken from historical collections in museums.[13,14]

A number of studies comparing carbon and nitrogen isotope discrimination factors between different tissues from the same animal (e.g. blood and feathers) have been conducted[15,16] but little attention has been paid to differences within feathers.[17,18] Within other tissues, different components are known to have different isotope values. Feathers mainly consist of keratin, a fibrous structure protein. Colour pigments such as melanin and carotenoids are also incorporated into their structure, but any carbon or nitrogen isotope variation resulting from these components has still to be characterized. Melanin, for example, is synthesized from tyrosine which is known to be depleted in ^{13}C as well as in ^{15}N compared with average muscle tissue.[19,20] Here, we seek to test the hypothesis that black feathers are more depleted in both ^{13}C and ^{15}N than white feathers. In this study we, therefore, compare the carbon and nitrogen isotope values of coeval black and white feathers from four bird species.

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EXPERIMENTAL

Study areas, species and sampling

Feathers from Imperial Shags *Phalacrocorax atriceps albiventer* were collected in three consecutive breeding seasons from 2006/2007 to 2008/2009 at New Island, Falkland Islands. In the last year, immature birds were also sampled. We took white breast feathers and black or (in the case of the immatures) dark brownish back feathers from the same individuals, 50 adults and 20 immatures.

Feathers from all other species contained black and white parts removed from within the same feather, to allow pairwise comparison. We analyzed white and black parts of the vane grown at the same time, by using opposite areas from the same distance to the feather tip. In this way five feathers each from Wilson's Storm Petrels *Oceanites oceanicus*, Oystercatchers *Haematopus ostralegus* and Common Shelduck *Tadorna tadorna* were analyzed (see Table 1 for further details).

Sample preparation and stable isotope analysis

Feathers collected from the drift line were cleaned with distilled water and dried at 60°C. Feathers taken from live birds were not cleaned as isotope ratios change little.[9]

Table 1. Study species, collected feathers and sampling locations from samples analyzed in this study including species abbreviations. Feathers from colony originated from caught birds, whereas moulted feathers were collected at the coast

Species	Abbreviation	Feathers	Age and sample locations
Imperial Shag	PA ad	black back feathers	adults, colony New Island
Phalacrocorax atriceps		white breast feathers	Falkland Islands
Imperial Shag	PA immat	brown back feathers	immatures, colony New Island
Phalacrocorax atriceps		white breast feathers	Falkland Islands
Wilson's Storm Petrel	OO	black and white	adults, colony King George Island,
Oceanites oceanicus		tail feathers	South Shetland Islands
Oystercatcher	HO	black and white	adults, moulted feathers,
Haematopus ostralegus		wing feathers	Schleswig Holstein, Germany
Common Shelduck	TT	black and white	adults, dead birds and moulted feathers,
Tadorna tadorna		body feathers	Schleswig Holstein, Germany

Around 0.7 mg aliquots of each sample were weighed into tin capsules.

We measured the stable isotope ratios of carbon and nitrogen using continuous-flow isotope-ratio mass spectrometry (CF-IRMS) with a Costech ECS 4010 elemental analyzer (Costech Analytical Technologies, Milan, Italy) linked to a Thermo Fisher Scientific (Bremen, Germany) Delta Plus XP mass spectrometer. The stable isotope ratios are expressed in δ-values as parts per thousand (‰). Three lab standards (gelatine and two isotopically distinct alanines) were used to correct for instrumental drift over a 12 h run. Errors calculated from replicate measurements of tryptophan (run as unknown, 4 samples each run in 5 runs) were small and the standard deviation (SD) was less than 0.1‰ for carbon and 0.3‰ for nitrogen.

Data analysis

We had dependent samples from the same individual or feather for all tests, and therefore used paired t-tests that were carried out separately for each species and age group. To test for differences between years in Imperial Shags we used an analysis of variance (ANOVA) after controlling for the assumptions of homoscedasticity and normality of errors. All statistical analyses were performed using the free software R (R Development Core Team, 2009[21]).

RESULTS

Black feathers or black areas of feathers were in all cases significantly depleted in $\delta^{13}C$ compared to white areas (Table 2, Fig. 1). The difference ranged between 0.24‰ and 0.69‰ for carbon. For adult shags this difference was consistent and did not differ between years (ANOVA, $F_{1,49} = 0.6$, $p = 0.455$).

For nitrogen in most cases no significant differences occurred. The black feathers of shags were, however, enriched in ^{15}N, a result which also did not differ between years (ANOVA, $F_{1,49} = 0.1$, $p = 0.747$), whereas black feathers of Shelduck tended to be depleted in ^{15}N (Table 2, Fig. 1).

The C/N ratios of black feathers or black areas of feathers were between 0.03 and 0.12 higher (two-way ANOVA, $F_{1,81} = 34.9$ $p < 0.001$) than those of white ones, independent of species and age groups ($F_{3,81} = 0.7$, $p = 0.539$). In addition, differences in the C/N ratio were negatively correlated with differences in carbon isotope values (linear regression, $p < 0.001$, $r^2 = 0.29$).

DISCUSSION

Melanin influenced the bulk carbon isotope value of feathers, by decreasing $\delta^{13}C$ by 0.2–0.7‰ (Table 2), although the melanin content of feathers is only up to 2% of weight.[22–24] The carbon-to-nitrogen ratio gives an indication of the

Table 2. Isotope values of white and black feathers ± SE of paired samples from one bird (Imperial Shag) or one feather (all others). For abbreviations, see Table 1

Species	$\delta^{13}C$ white feather	$\delta^{13}C$ black feather	paired differences	pairwise t-test
PA ad (N = 51)	−16.1 ± 0.1	−16.7 ± 0.1	0.6 ± 0.1	t = 9.9, p < 0.001
PA immat (N = 20)	−15.2 ± 0.1	−15.9 ± 0.1	0.7 ± 0.1	t = 7.0, p < 0.001
OO (N = 5)	−17.8 ± 0.4	−18.2 ± 0.4	0.5 ± 0.0	t = 20.9, p < 0.001
HO (N = 5)	−15.1 ± 0.3	−15.3 ± 0.3	0.2 ± 0.2	t = 7.4, p = 0.002
TT (N = 5)	−14.7 ± 0.3	−15.3 ± 0.4	0.6 ± 0.2	t = 3.4, p = 0.028
	$\delta^{15}N$ white feather	$\delta^{15}N$ black feather	paired differences	pairwise t-test
PA ad (N = 51)	16.0 ± 0.8	16.3 ± 0.6	−0.3 ± 0.1	t = −2.9, p = 0.005
PA immat (N = 20)	15.6 ± 0.6	15.7 ± 0.7	−0.1 ± 0.2	t = −0.9, p = 0.379
OO (N = 5)	15.4 ± 0.4	15.5 ± 0.4	−0.1 ± 0.0	t = −0.9, p = 0.435
HO (N = 5)	17.6 ± 0.4	17.4 ± 0.3	0.1 ± 0.4	t = 1.1, p = 0.334
TT (N = 5)	17.6 ± 0.8	17.3 ± 0.3	0.3 ± 0.4	t = 2.4, p = 0.071

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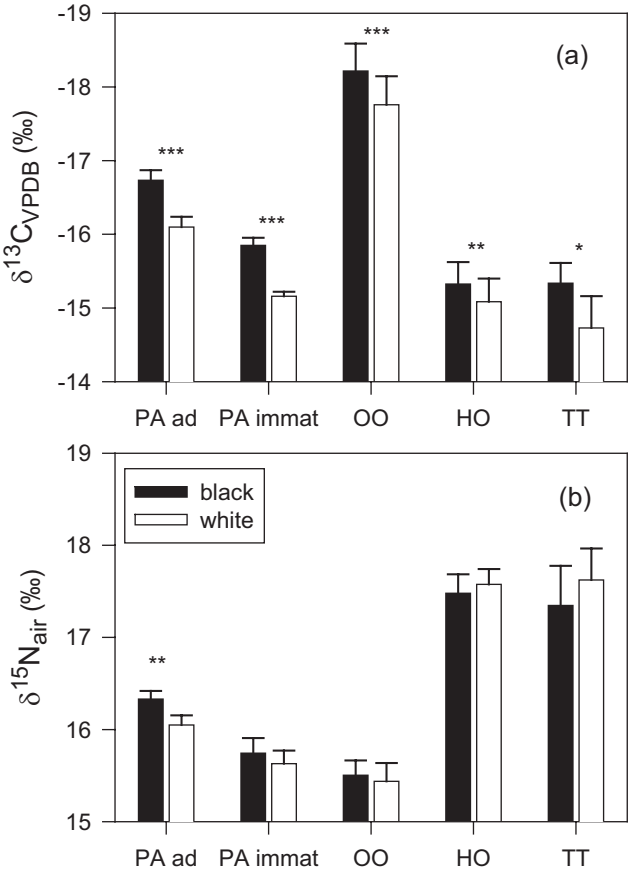

Figure 1. Stable carbon (a) and nitrogen (b) isotope values (mean ± SE) of different black/brownish and white feathers of the same bird (PA) and of opposite black and white areas of the same feather (OO, HO, TT). For abbreviations, see Table 1.

melanin content as the C/N ratio of eumelanin is, at 8 to 9, much elevated compared with that of keratin of around 3. The fact that black feathers showed higher C/N ratios, and that the differences in C/N ratio correlated with differences in carbon isotope values, gives further support to the hypothesis that melanin is responsible for the different isotope values. The significant correlation between differences in C/N ratio and differences in $\delta^{13}C$ gives an indication that the isotope values change with the amount of melanin in feathers.

While many studies have found much larger differences in $\delta^{13}C$,[25] the differences between black and white feathers seen here are of the order of magnitude of the differences observed in some stable isotope studies.[14] Thus, these effects should be taken into account in future analyses of feathers.

For Imperial Shags in both age groups we cannot exclude a different timing in the moult of black and white feathers and consequently differences between black and white feathers may be due to behavioural changes or nutritional stress. Nevertheless, the differences were consistent among years, which is a first indication that changes in diet, foraging locations or such intrinsic factors as nutritional stress are not responsible unless they occur stereotypically each year. We can control this variable for Wilson's Storm Petrels, Oystercatchers and Common Shelduck as here we used black and white parts of the feathers that are grown opposite each other along the shaft of the same feather and therefore are coeval. This excludes differences caused by behavioural or metabolic changes since the black and white areas are grown under exactly the same conditions and any variability has therefore to be linked with colouration and melanin content.

Lower $\delta^{13}C$ values in melanized feathers were expected, as tyrosine, the amino acid from which melanin is derived, is depleted in ^{13}C.[20] Surprisingly, the predicted shift in nitrogen isotope ratios due to the influence of melanin was not detected, although tyrosine is also depleted in ^{15}N.[19] Perhaps additional fractionation processes occur during melanin synthesis and tyrosine which is relatively ^{15}N-enriched is preferentially used in this pathway. ^{15}N-depleted tyrosine may also be catabolized preferentially, as uric acid is known to be depleted in ^{15}N, which causes ^{15}N enrichment with each trophic level.[26] Furthermore, each metabolic step within one organism has been described as causing enrichment in ^{15}N and therefore, for example, high nitrogen isotope values of starving individuals can feign a higher trophic level,[27] and feathers synthesized from endogenous resources can also be enriched in ^{15}N.[15,16]

The variability in isotope value differences seen here between groups may be due to differences in the baseline tyrosine values or it may be that components are stored to different extents before feathers are synthesized, thus causing different fractionation factors.

CONCLUSIONS

The measured ^{13}C depletion in black feathers takes us towards an explanation of some of the variability between feathers. This may be important in isotope studies within a community between species with differently coloured plumages. Even if the biological significance of these differences is small, it may help to include the influence of melanin in order to calculate fractionation factors more precisely when estimating the relative proportions of food items in diet, as calculated, for example, in Phillips and Gregg.[28]

Although the influence of melanin of feathers is obvious, in respect to the biochemical side, this work is only preliminary and component-specific studies on feather melanin would be needed to understand how the carbon and nitrogen isotope ratio of this component is changed both during synthesis and by metabolic or environmental factors. Future studies should look at other components with varying proportions in feathers (e.g., carotenoids) and at how they influence isotope values. It may be somewhat premature to agree with Norris *et al.*[29] that different carotenoid contents do not change isotope values.

Although stable isotope analyses remain a powerful tool, these results show once again that great caution should be exercised when comparing isotope values derived from bulk tissue samples. Not only do different tissues show different isotope fractionation factors, but the isotope value of the same tissue may depend on its chemical composition[4] and, more specifically, on the pigmentation of that tissue. Thus, careful sampling of feather material, or compound-specific stable isotope analysis, may be required in future studies, especially when small differences between experimental or empirical groups are expected.

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