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Wanted: dead or alive? Isotopic analysis ($\delta^{13}C$ and $\delta^{15}N$) of *Pygoscelis* penguin chick tissues supports opportunistic sampling

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RATIONALE: Physiological stress and starvation have been shown to affect $\delta^{13}C$ and $\delta^{15}N$ isotope values and, given that animals often die from starvation, the cause of death may be an important factor to consider in stable isotope analyses of opportunistically collected samples.
METHODS: We addressed this issue by comparing tissue stable isotope values of living and deceased Adélie (*Pygoscelis adeliae*) and Chinstrap Penguin (*P. antarctica*) chicks collected from the same respective populations.
RESULTS: No significant difference was found between living and deceased penguin chick feather, down, and toenail isotope values and both groups displayed similar isotopic trends between tissue types. In addition, similar relationships were observed between both species and across several seasons. Furthermore, sub-dermal adiposity and cause of death (starvation and/or predation) had no significant effect on the $\delta^{13}C$ and $\delta^{15}N$ values.
CONCLUSIONS: Our findings suggest that tissues from deceased penguins can be isotopically representative of tissues obtained from the living population, despite the cause of death, and support the use of opportunistic sampling in stable isotope analyses. Copyright © 2012 John Wiley & Sons, Ltd.

Stable isotope analyses (SIAs) are becoming increasingly important in investigations of the foraging behavior of many animal species due to their ease of collection and informative properties. Stable isotopes (SIs) are predictably and consistently incorporated into the various tissues of organisms through their diet, thus providing a dependable tool for studying these organisms.[1,2] The most common SIs used in biology are carbon (^{13}C) and nitrogen (^{15}N) as they reveal the foraging location and trophic level of an organism, respectively, and thus allow for the assessment of changes in the foraging ecology of the organism. As tissues incorporate SIs at the time of synthesis, tissues grown at different times throughout an organism's life reflect dietary information over many temporal and spatial scales.[3–6] In addition, using heavily keratinized tissues such as feather, hair, and toenails not only allows for less invasive dietary analysis of live animals, but also allows for opportunistic sampling from deceased animal remains due to their ease of collection and preservation.[1]

Many paleontological and historical studies have taken advantage of opportunistic sampling in order to assess any long-term shifts in the foraging behavior of seabird species.[7–10] These studies have conducted SIA on fossilized animal remains as well as preserved museum specimens, but it is not often known if these samples are representative of living populations. Studies have shown that physiological and nutritional stress can affect isotope ratios.[3,5,11–13] Therefore, the cause of death of the organism examined is an important consideration, particularly because many young seabirds may die from starvation. As the physiological status at the time of collection or death is often unknown in paleontological and historical studies, it is necessary to compare living and recently deceased portions of a population in order to assess any differences in isotope ratios.

The purpose of this study was to investigate the use of opportunistic sampling in SIA by (1) comparing the SI ratios of tissues between living and deceased penguin chicks from the same populations, (2) examining the isotopic relationships of living and deceased penguin chicks across multiple tissues from the same bird, and (3) and determining if these relationships are consistent over multiple years. Thus, we address the question as to whether or not opportunistic sampling of tissues from deceased birds provides isotopic information representative of the living population. If so, sampling of deceased birds will facilitate future SIA investigations and mitigate disturbances to live animals.

EXPERIMENTAL

Study site and sampling

Adélie Penguin (*Pygoscelis adeliae*) tissue samples were collected from a colony of approximately 100 000 breeding pairs at Cape Crozier, Ross Island, Antarctica (77°32′S, 169°19′E), in January and February 2010. Tissue samples were taken from 20 live and 20 dead penguin chicks which were approximately 4 to 7 weeks old, corresponding with the crèche period. Feather and down samples were plucked from the breast region of the live and dead penguins. In addition, the middle

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toenail from the right foot was collected from each dead chick; the fat composition underneath the dead chick's skin was measured and classified into one of two categories: sub-dermal adiposity greater than, or less than, 1 mm in thickness. The cause of death was estimated, based on the condition of the remains, and categorized as either starvation or predation by South Polar Skuas (*Stercorarius maccormicki*). Predation was assigned as the cause of death if there was considerable trauma to the head of the penguin chick.[14]

We collected samples of live Chinstrap Penguin (*Pygoscelis antarctica*) chick tissues from a colony of approximately 4500 breeding pairs at Cape Shirreff, Livingston Island, Antarctica (62°28′S, 60°46′W), from 2007 to 2010. We collected breast feathers from 18 to 30 large, fully feathered chicks during late February of each year when chicks began to enter the sea (fledge) at approximately 7 to 8 weeks of age. Concurrently, we also collected from 9 to 21 chick carcasses each year from beaches near the penguin colony. These carcasses all showed signs of predation by leopard seals (*Hydrurga leptonyx*) and were similar to the live chicks that we sampled in both size and feather development. From each chick carcass we collected samples of breast feathers and the middle toenail from the right foot.

Sample preparation

The feather and down tissue samples were cleaned of surface contaminants using a 2:1 chloroform/methanol rinse (4 mL) and allowed to soak for 24 h. The tissue samples were then rinsed using an additional 1–2 mL of solution and allowed to air dry under a fume hood for 24 h. The feathers were cut into small fragments using stainless steel scissors while the down was left uncut. The toenails were cleaned of tissue, bone and surface contaminants using a stainless steel dental pick and nylon brush and rinsed in a 2:1 chloroform/methanol solution as described above. After being rinsed, the air-dried toenails were moved to an oven to dry for an additional 48 h at 60 °C and were subsequently homogenized into a fine powder using a 110 V Wig-L-Bug grinding mill (model 3110B, International Crystal Laboratories, Garfield, NJ, USA).

Stable isotope analysis

Approximately 0.45 mg each of the above tissue samples was loaded into separate tin cups for isotopic analysis. Samples were flash-combusted using ThermoFinnigan (Thermo Fisher, Bremen, Germany) and Costech ECS4010 elemental analyzers (Costech Analytical Technologies,Valencia, CA, USA) and analyzed for carbon and nitrogen isotope ratios using Delta Plus XL and Delta V Plus (Thermo Fisher) isotope ratio mass spectrometers coupled via a Conflo III open split interface (Thermo Fisher). Stable isotope data were acquired and processed with ISODAT 2.0 software (Thermo Fisher). The isotope ratios are expressed in δ notation in per mil units (‰), according to the following equation:

$$\delta X = \left[\left(R_{sample}/R_{standard}\right) - 1\right] \cdot 1000$$

where X is $\delta^{13}C$ or $\delta^{15}N$ and R is the corresponding ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$. The $R_{standard}$ values were based on the Vienna Pee Dee Belemnite (VPDB) standard for $\delta^{13}C$ and atmospheric N_2 for $\delta^{15}N$ values. Raw δ values were normalized on a two-point scale using depleted and enriched glutamic acid standard reference materials USGS-40 ($\delta^{13}C$: –26.389 ± 0.042; $\delta^{15}N$: –4.5 ± 0.1) and USGS-41 ($\delta^{13}C$: 37.626 ± 0.049; $\delta^{15}N$: 47.6 ± 0.2). The sample precision based on internal repeats and duplicate standard reference materials was 0.1‰ and 0.2‰, for $\delta^{13}C$ and $\delta^{15}N$, respectively.

Statistical analysis

Statistical analyses were performed using JMP Statistical Discovery Software 7.0 (SAS Institute, Cary, NC, USA), SAS 9.1 (SAS Institute), and PASW Statistics Data Editor 18.0 (SPSS Inc., Chicago, IL, USA). Two-sample t-tests were performed to assay intraspecific tissue differences in isotope ratios between live and dead penguin chicks from the same species. For each tissue type (feather, down and toenail) collected from dead Adélie Penguin chicks we used separate two-way analysis of variance (ANOVA) with $\delta^{13}C$ or $\delta^{15}N$ values as the dependent variable and the cause of death and sub-dermal adiposity index as independent variables. Pair-wise t-tests with Pearson's correlations were performed to assay interspecific tissue differences in isotope ratios within individual penguin chicks. In addition, the Chinstrap Penguin data were tested for correlations between the isotope ratios of live and dead chicks across multiple years.

To complement the multiple comparisons detailed above we used a unified mixed modeling approach (Proc Mixed, SAS) to identify the relative influence of outside co-factors on comparisons between $\delta^{13}C$ and $\delta^{15}N$ values in live and dead individuals across our entire dataset. In these models we used the $\delta^{13}C$ or $\delta^{15}N$ values as the response variable, live/dead as a fixed effect, and tissue type, year, site and species as either all random effects, or one of the four factors as a fixed effect and the others as random effects. We assessed model fit using Akaike's Information Criterion (AIC).[15] This approach identified that tissue type, year, site and species effects all significantly influence the $\delta^{13}C$ and $\delta^{15}N$ values of penguin chicks (Supplementary Table S1, Supporting Information). However, when controlling for these effects on comparisons between live and dead chicks we obtained results similar to those found using our preliminary multiple comparison approach. Therefore, we provide the results of our mixed modeling approach as Supporting Information and interpret our results based on the more simplistic multiple comparison approach. Data used in the above analyses were examined for normality and all t-tests conducted were two-tailed; significance was assumed at the 0.05 level and means are presented ± standard deviation (SD).

RESULTS

Comparisons between live and dead penguin chicks

We found no significant difference in the $\delta^{13}C$ values of down between live and dead Adélie Penguin chicks (Table 1, Fig. 1). There was a significant difference in the $\delta^{15}N$ values of down between these two groups; however, this difference was at or below the level of instrument precision (0.2‰) and thus not biologically significant. In addition, there was no significant difference in the $\delta^{13}C$ and $\delta^{15}N$ values of feathers between live and dead Adélie Penguin chicks (Table 1, Fig. 1). We found no significant difference in the feather $\delta^{13}C$ and $\delta^{15}N$

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Table 1. *Pygoscelis adeliae* and *P. antarctica*. Intraspecific tissue differences (‰) for carbon and nitrogen stable isotope ratios between live and dead Adélie and Chinstrap Penguin chicks sampled on the Ross and Livingston Islands, Antarctica, during austral summers 2007–2010

		Differences between sample groups (live - dead)			
Species, year	Tissue	$\delta^{13}C$ (‰)	Two-sample t-test	$\delta^{15}N$ (‰)	Two-sample t-test
Adélie Penguin					
2010	Down	0.0	$t = 0.2$, $P = 0.822$	0.2	$t = 2.2$, $P = 0.033$
	Feather	−0.3	$t = 1.4$, $P = 0.182$	0.3	$t = 1.6$, $P = 0.115$
Chinstrap Penguin					
2007	Feather	−0.2	$t = 2.2$, $P = 0.032$	−0.2	$t = 2.5$, $P = 0.017$
2008	Feather	0.0	$t = 0.0$, $P = 0.980$	−0.1	$t = 0.8$, $P = 0.407$
2009	Feather	0.0	$t = 0.0$, $P = 0.966$	−0.1	$t = 1.0$, $P = 0.312$
2010	Feather	0.2	$t = 1.0$, $P = 0.318$	−0.2	$t = 2.7$, $P = 0.010$

values between live and dead Chinstrap Penguin chicks in 2008 and 2009 and no significant difference in the $\delta^{13}C$ values for 2010 (Table 1, Fig. 1). In some years we did observe significant differences between live and dead Chinstrap Penguin chicks in the feather $\delta^{13}C$ (2007) and $\delta^{15}N$ (2007 and 2010) values. However, these differences were also at

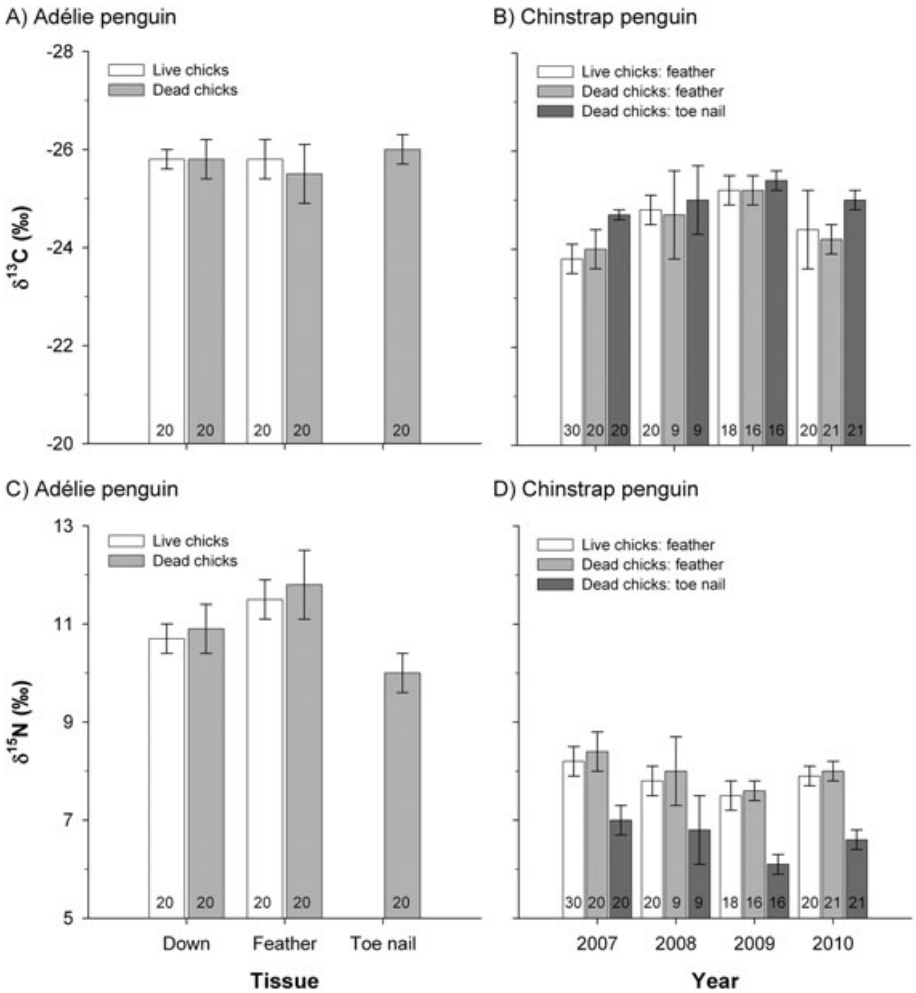

Figure 1. *Pygoscelis adeliae* and *P. antarctica*. (A) Carbon and (C) nitrogen stable isotope ratios (mean ± SD) of down, feather, and toe nails sampled from live and dead Adélie Penguin chicks at Cape Crozier, Ross Island, Antarctica, during austral summer 2010. (B) Carbon and (D) nitrogen stable isotope ratios (mean ± SD) of feather and toe nails sampled from live and dead Chinstrap Penguin chicks at Cape Shirreff, Livingston Island, Antarctica, during austral summers 2007–2010. Numbers in bars designate the sample size.

or below the level of instrument precision and therefore not biologically significant (Table 1, Fig. 1).In addition, inter-annual variations in the isotope ratios of live and dead Chinstrap Penguin chick feathers were positively correlated across the four years of sampling ($\delta^{13}C$: $R = 0.960$, $P = 0.040$; $\delta^{15}N$: $R = 0.989$, $P = 0.010$).

Sub-dermal adiposity and cause of death

The isotope ratios of deceased Adélie Penguin chick tissues did not differ by the sub-dermal adiposity index (greater or less than 1 mm) for feather ($\delta^{15}N$: $F_{3,19} = 1.01$, $P = 0.331$; $\delta^{13}C$: $F_{3,19} = 1.36$, $P = 0.261$), down ($\delta^{15}N$: $F_{3,19} = 0.30$, $P = 0.594$; $\delta^{13}C$: $F_{3,19} = 0.60$, $P = 0.451$) or toenails ($\delta^{15}N$: $F_{3,19} = 0.21$, $P = 0.656$; $\delta^{13}C$: $F_{3,19} = 1.83$, $P = 0.195$). We also observed no differences in feather ($\delta^{15}N$: $F_{3,19} = 0.88$, $P = 0.361$; $\delta^{13}C$: $F_{3,19} = 0.37$, $P = 0.550$), down ($\delta^{15}N$: $F_{3,19} = 0.27$, $P = 0.608$; $\delta^{13}C$: $F_{3,19} = 0.08$, $P = 0.783$) and toenail ($\delta^{15}N$: $F_{3,19} = 1.20$, $P = 0.289$; $\delta^{13}C$: $F_{3,19} = 0.01$, $P = 0.928$) isotope ratios between chicks that were predated and those that probably died of starvation. There was no significant interactive effect between sub-dermal adiposity and cause of death on the isotope ratios of chick feather ($\delta^{15}N$: $F_{3,19} = 0.16$, $P = 0.692$; $\delta^{13}C$: $F_{3,19} = 0.22$, $P = 0.649$), down ($\delta^{15}N$: $F_{3,19} = 0.53$, $P = 0.475$; $\delta^{13}C$: $F_{3,19} = 0.00$, $P = 0.967$), or toenails ($\delta^{15}N$: $F_{3,19} = 1.45$, $P = 0.245$; $\delta^{13}C$: $F_{3,19} = 1.45$, $P = 0.245$).

Comparisons across tissues

There was no significant difference or correlation between the feather and down $\delta^{13}C$ values in either live or dead Adélie Penguin chicks (Table 2, Fig. 2). While the down $\delta^{13}C$ values were significantly higher than the toenail values in dead Adélie Penguin chicks, these values were not correlated. In contrast, there was both a significant difference and positive correlation between feathers and toenails $\delta^{13}C$ values in dead Adélie Penguin chicks, with feathers having higher values. Similarly, in all but one season (2008), the feather $\delta^{13}C$ values were significantly higher than those of the toenails in dead Chinstrap Penguin chicks. In addition these tissues were positively correlated in all but one season (2007; Table 2, Fig. 2).

The feather $\delta^{15}N$ values were significantly higher and positively correlated with down values in both live and dead Adélie Penguin chicks (Table 2, Fig. 2). A similar trend was observed between the down and toenails $\delta^{15}N$ values, with the down values being significantly higher than the toenail values. In addition, the feather $\delta^{15}N$ values were significantly higher and positively correlated with the toenail values in dead Adélie Penguin chicks. The dead Chinstrap Penguin chick feather $\delta^{15}N$ values were significantly higher than the toenail values in all years, and with a positive correlation between these two tissues in all but one season (2009; Table 2, Fig. 2).

DISCUSSION

As we found no difference in $\delta^{13}C$ and $\delta^{15}N$ values between live and dead penguin chick tissues, our results support the use of opportunistic sampling of dead chick tissues for SIA in modern Adélie and Chinstrap Penguin populations. In addition, these isotopic similarities and inter-tissue relationships were generally consistent between species and across several breeding seasons.

Table 2. *Pygoscelis adeliae* and *P. antarctica*. Interspecific tissue differences (‰) ± SD for carbon and nitrogen stable isotope ratios between live and dead Adélie and Chinstrap Penguin chicks sampled on the Ross and Livingston Islands, Antarctica, during austral summers 2007–2010. n = sample size

Species	Group	*n*	Tissue comparison	Isotope	Pairwise difference (‰)	Paired t-test	Correlation
Adélie Penguin	Live, 2010	20	Feather – Down	C	0.1 ± 0.4	$t = 1.1$, $P = 0.299$	$R = 0.360$, $P = 0.119$
				N	0.8 ± 0.3	$t = 10.8$, $P < 0.001$	$R = 0.625$, $P = 0.003$
	Dead, 2010	20	Feather – Down	C	0.3 ± 0.7	$t = 1.8$, $P = 0.096$	$R = 0.073$, $P = 0.760$
				N	0.8 ± 0.3	$t = 10.8$, $P < 0.001$	$R = 0.625$, $P = 0.003$
			Down – Toenail	C	0.2 ± 0.4	$t = 2.2$, $P = 0.043$	$R = 0.334$, $P = 0.149$
				N	0.8 ± 0.3	$t = 10.8$, $P < 0.001$	$R = 0.625$, $P = 0.003$
			Feather – Toenail	C	0.5 ± 0.5	$t = 4.1$, $P < 0.001$	$R = 0.593$, $P = 0.006$
				N	1.8 ± 0.5	$t = 16.8$, $P < 0.001$	$R = 0.775$, $P < 0.001$
Chinstrap Penguin	Dead, 2007	20	Feather – Toenail	C	0.7 ± 0.3	$t = 9.3$, $P < 0.001$	$R = 0.382$, $P = 0.097$
				N	1.4 ± 0.3	$t = 20.1$, $P < 0.001$	$R = 0.635$, $P = 0.003$
	Dead, 2008	9	Feather – Toenail	C	0.3 ± 0.4	$t = 1.9$, $P = 0.099$	$R = 0.869$, $P = 0.002$
				N	1.1 ± 0.6	$t = 6.2$, $P < 0.001$	$R = 0.691$, $P = 0.039$
	Dead, 2009	16	Feather – Toenail	C	0.2 ± 0.3	$t = 3.3$, $P = 0.005$	$R = 0.562$, $P = 0.024$
				N	1.4 ± 0.3	$t = 17.9$, $P < 0.001$	$R = 0.168$, $P = 0.535$
	Dead, 2010	21	Feather – Toenail	C	0.8 ± 0.2	$t = 19.1$, $P < 0.001$	$R = 0.756$, $P < 0.001$
				N	1.5 ± 0.2	$t = 31.4$, $P < 0.001$	$R = 0.609$, $P = 0.003$
	Dead, all years	66	Feather – Toenail	C	0.6 ± 0.4	$t = 11.7$, $P < 0.001$	$R = 0.824$, $P < 0.001$
				N	1.4 ± 0.3	$t = 31.4$, $P < 0.001$	$R = 0.745$, $P < 0.001$

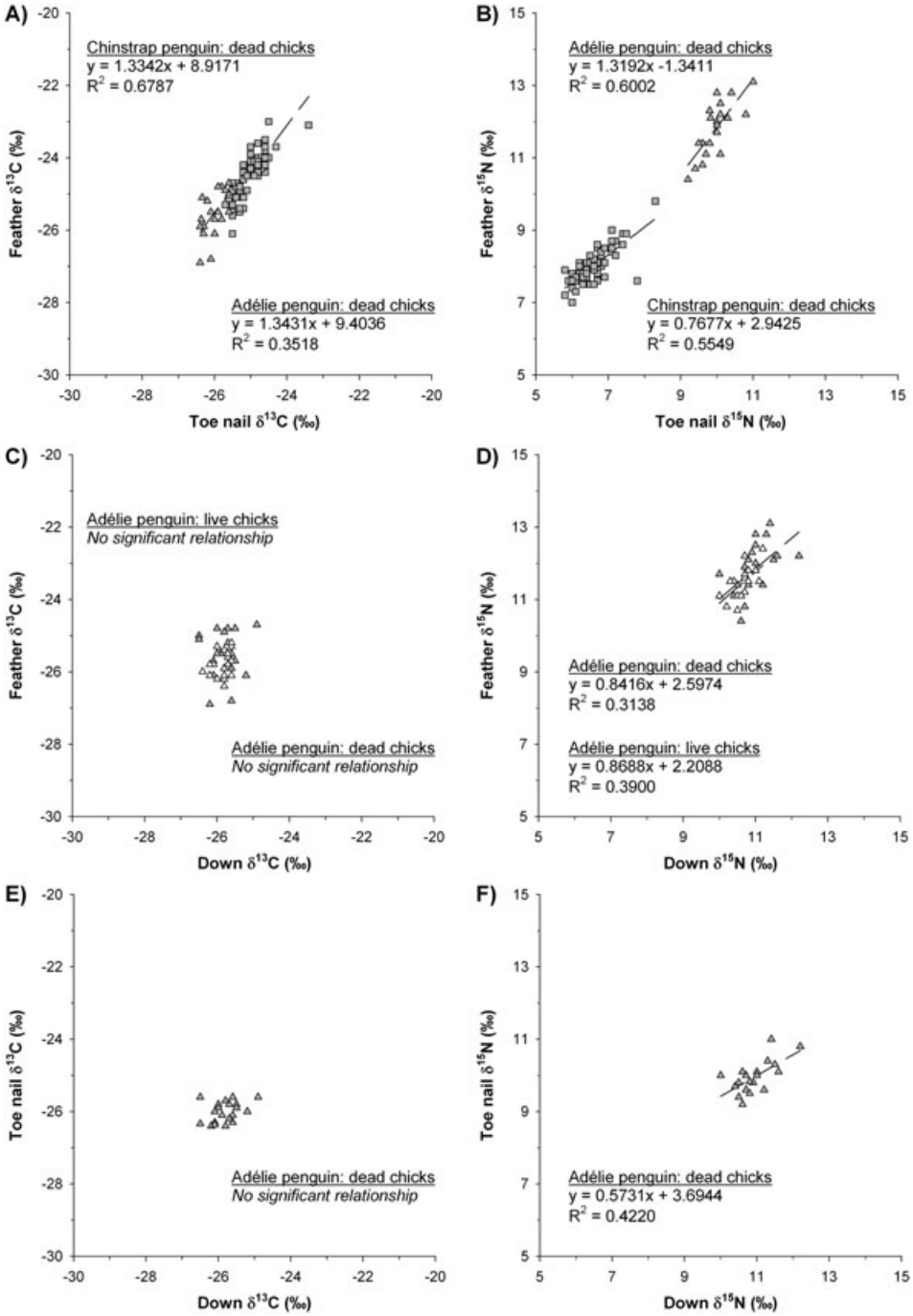

Figure 2. *Pygoscelis adeliae* and *P. antarctica*. Scatter plot of pairwise correlations between tissue carbon and nitrogen stable isotope ratios from Adélie and Chinstrap Penguin chicks sampled at Cape Crozier, Ross Island, and Cape Shirreff, Livingston Island, Antarctica, during austral summers 2007–2010 showing tissue-tissue isotopic relationships for feather and toe nails (A) carbon and (B) nitrogen; feather and down (C) carbon and (D) nitrogen; toe nail and down (E) carbon and (F) nitrogen stable isotope ratios. Triangles and squares represent Adélie and Chinstrap Penguins, respectively; non-shaded and shaded represent live and dead birds, respectively. Significant correlations are indicated with a line.

Therefore, it appears that opportunistic sampling is a promising, non-invasive method for studying species-specific and inter-annual variation in the diets and foraging ecology of *Pygoscelis* penguins as their breeding colonies contain abundant remains such as carcasses, bones, and feathers.[16] As similar remains are often plentiful around colonies of other colonial breeding seabirds, these opportunistic sampling methods may be broadly applicable in other studies, thereby reducing the need to handle live animals, particularly when dealing with threatened or endangered species.[17–19]

Our study also helps to validate paleontological and historic studies that use SIA of seabird remains, waste products, and museum specimens to examine changes in diets over time.[8,9,20–25] A general concern in these studies is whether the isotope ratios of sub-fossilized remains and museum specimen tissues are representative of living populations. While these studies must still account for the effects of diagenesis and other processes that can degrade tissues,[8,24] our study indicates that these seabird remains can be reflective of their living counterparts at the time of deposition.

Studies of the effects of nutritional stress on SI ratios in avian tissues have been somewhat contradictory, especially for $\delta^{15}N$ values.[3,5,11–13,26–28] When examining the condition of dead chicks we found no effect of sub-dermal adiposity or the cause of death (predation or starvation) on the SI ratios of tissues. There is evidence that severe nutritional stress can lead to ^{15}N enrichment in tissues due to utilization of endogenous protein stores.[5,11,28] In these studies, however, experimental groups were chronically food deprived over several weeks or months and experienced body mass reductions of between 33 and 53 %, providing a relatively large timeframe for these effects to be seen in developing tissues.[5,11] It is likely that the chicks used in our study starved over a considerably shorter timeframe prior to their death,[14] and therefore any ^{15}N enrichment due to starvation is underrepresented in the sampled tissues. In addition, seabird chicks under moderate dietary restrictions have relatively low tissue $\delta^{15}N$ values, possibly due to increased efficiency in nitrogen use during rapid growth.[12,13] It also appears likely that in many cases there is a critical threshold, where the growth of developing animals is severely retarded or adults are chronically food stressed before the SI ratios of tissues are affected.[5,11,26,27] This threshold effect may explain why we found no difference in SI ratios between living and deceased chicks or between deceased chicks that differed in sub-dermal adiposity or cause of death. The lack of difference between live and dead chicks suggests that either starvation effects were underrepresented in tissues, that the chicks in our study did not reach a critical threshold prior to death or that the effects of growth offset any use of endogenous protein stores.

The SI ratios generally differed across chick tissues, but were often positively correlated with one another. There are several possible explanations for this relationship. Tissues reflect diets during synthesis and the three tissues that we examined were grown over slightly different time periods.[1,6] Toenails are grown continuously starting at the egg stage, down is grown during the middle stage of chick development, while the feathers are grown late in chick development.[14] Any temporal variation in diets across these time periods would lead to differences in SI ratios. In addition, tissue-specific differences in amino acid content and composition can lead to differences in SI ratios.[29,30] Differences in metabolic routing can also affect the isotope ratios as each tissue develops from unique carbon and nitrogen stores at different rates during tissue synthesis.[31] When exogenous protein is available, it is the main source of nitrogen for newly synthesized tissues and this common source probably explains the correlations in nitrogen ratios between the tissues. In contrast, carbon sources may be exogenous or endogenous,[12,31] and differential use of these two sources during growth may confound inter-tissue comparisons. For example, the lack of correlation between stable carbon ratios of down and feather and down and toenails may be due in part to temporal differences in the timing of growth and the relative importance of carbon from the maternal yolk sac vs. dietary carbon sources.[13,31–33]

CONCLUSIONS

Our study confirms that the SI ratios of recently deceased *Pygoscelis* penguin chicks provide an accurate representation of these same ratios in the living population. The opportunistic sampling of chick remains can provide reliable isotopic data that can be used to study species-specific and inter-annual variation in penguin diets. These results also support opportunistic sampling in modern and paleontological studies of species that have similar ecological behavior. However, when using such samples it is important to have an understanding of the temporal integration of each tissue, the relative role of exogenous or endogenous sources during tissue synthesis, and how physiological condition before or at the time of death may affect SI ratios.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

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