Oecologia (2007) 153:11–18
DOI 10.1007/s00442-007-0717-z

ECOPHYSIOLOGY

Effects of nutritional restriction on nitrogen and carbon stable isotopes in growing seabirds

Cory T. Williams · C. Loren Buck · Justine Sears · Alexander S. Kitaysky

Received: 25 October 2006 / Accepted: 5 March 2007 / Published online: 4 April 2007

Abstract When using stable isotopes as dietary tracers it is essential to consider effects of nutritional state on isotopic fractionation. While starvation is known to induce enrichment of ^{15}N in body tissues, effects of moderate food restriction on isotope signatures have rarely been tested. We conducted two experiments to investigate effects of a 50–55% reduction in food intake on $\delta^{15}N$ and $\delta^{13}C$ values in blood cells and whole blood of tufted puffin chicks, a species that exhibits a variety of adaptive responses to nutritional deficits. We found that blood from puffin chicks fed ad libitum became enriched in ^{15}N and ^{13}C compared to food-restricted chicks. Our results show that ^{15}N enrichment is not always associated with food deprivation and argue effects of growth on diet–tissue fractionation of nitrogen stable isotopes ($\Delta^{15}N$) need to be considered in stable isotope studies. The decrease in $\delta^{13}C$ of whole blood and blood cells in restricted birds is likely due to incorporation of carbon from ^{13}C-depleted lipids into proteins. Effects of nutritional restriction on $\delta^{15}N$ and $\delta^{13}C$ values were relatively small in both experiments ($\delta^{15}N$: 0.77 and 0.41‰, $\delta^{13}C$: 0.20 and 0.25‰) compared to effects of ecological processes, indicating physiological effects do not preclude the use of carbon and nitrogen stable isotopes in studies of seabird ecology. Nevertheless, our results demonstrate that physiological processes affect nitrogen and carbon stable isotopes in growing birds and we caution isotope ecologists to consider these effects to avoid drawing spurious conclusions.

Keywords Diet–tissue fractionation · Physiological condition · Isotopic enrichment · Nitrogen balance

Communicated by Carlos Martinez del Rio.

C. T. Williams (✉)
School of Fisheries and Ocean Sciences,
University of Alaska Fairbanks, Fairbanks, AK 99775, USA
e-mail: fnctw@uaf.edu

C. L. Buck
Department of Biological Sciences,
University of Alaska Anchorage, Anchorage, AK 99508, USA

J. Sears · A. S. Kitaysky
Department of Biology and Wildlife,
University of Alaska Fairbanks, Fairbanks, AK 99775, USA

A. S. Kitaysky
Zoology Department, University of Washington,
Seattle, WA 98195, USA

Introduction

Analysis of $^{12}C/^{13}C$ and $^{14}N/^{15}N$ stable isotopes in animal tissues provides a useful means of delineating dietary sources, determining trophic level of feeding, and tracking migratory movements (reviewed in Hobson 1999; Kelly 2000). This technique has proved particularly useful to estimate foraging location and trophic level of feeding in seabirds (Hobson et al. 1994; Forero et al. 2004; Cherel et al. 2006) where conventional techniques for diet determination are logistically impractical and biased (Wilson et al. 1985; Votier et al. 2003). However, interpretation of isotopic data in studies of animal ecology is complicated by effects of nutritional status on biochemical pathways and physiological processes (Gannes et al. 1998). Despite widespread awareness of physiological processes that may affect diet–tissue fractionation, and a call for more laboratory experiments made nearly a decade ago (Gannes et al. 1997), few experimental studies have been conducted to assess the effects of nutritional status on fractionation of carbon and

nitrogen stable isotopes. The paradigm emerging from studies exposing birds to experimental food deprivation postulates that animal tissues become enriched in ^{15}N following nutritional restriction due to catabolism of endogenous protein stores, recycling of metabolic amino acids, and discrimination against the heavier isotope during formation of nitrogenous wastes (Macko et al. 1986; Hobson et al. 1993; Gannes et al. 1998). Although this effect is well-established in fasting animals that have no access to exogenous nitrogen (Hobson et al. 1993; Oelbermann and Scheu 2002; Cherel et al. 2005a), experimental studies employing moderate food restriction have rarely been undertaken (Kempster et al. 2007).

Seabirds provide their young with prey that varies in its abundance and distribution, often resulting in moderate food restriction during growth when demands for energy and protein are high. Reproductive success and rates of nestling growth of tufted puffins (*Fratercula cirrhata*, hereafter: puffin), for instance, are affected by climate-induced changes in sea surface temperature (Gjerdrum et al. 2003), presumably due to variation in prey availability. Information on trophic level of feeding and foraging location provided by analysis of stable isotopes may therefore be useful for elucidating the mechanistic link between sea surface temperatures and reproductive success. However, effects of nutritional restriction on nitrogen diet–tissue fractionation, assuming it occurs, may produce a spurious correlation between rates of nestling growth and estimates of trophic level of feeding. Because growth rates of puffin chicks vary widely between colonies and years (Piatt and Kitaysky 2002; Gjerdrum et al. 2003) as well as between individuals within a colony (Williams, unpublished data), we set out to determine the effects of moderate food restriction on carbon and nitrogen stable isotopes in growing puffins.

Nutritional status is a function of nutrient intake versus demand, and the physiological and metabolic compensatory mechanisms that minimize the discrepancy between the two (King and Murphy 1985). We hypothesized that the effects of nutritional restriction on isotopic fractionation may be dampened or nullified by physiological and metabolic compensatory responses in species adapted to variable rates of food intake. Puffin nestlings have the physiologically capacity to modulate metabolic rates in response to nutritional limitation (Kitaysky 1999). Furthermore, free-living puffin nestlings are fed diets rich in high-quality protein and growth may therefore be limited by energetic constraints, rather than by protein intake. In this paper, we report the results of two captive-feeding experiments in which we subjected puffin nestlings to nutritional deprivation during growth and measured changes in either whole blood or blood cell concentrations of $\delta^{13}C$ and $\delta^{15}N$.

Methods

We conducted two separate experiments to determine the effects of nutritional restriction on carbon and nitrogen isotopic fractionation in growing tufted puffin nestlings. In the first experiment, we utilized free-living nestlings reared in their burrows on Cliff Island in Chiniak Bay on Kodiak Island, AK, USA. We located 13 free-living puffin nestlings during the early stages of chick-rearing and excavated vertical access holes which were patched with flat rocks to permit later access to the nesting chamber. When chicks were estimated to be approximately ten days old, based on wing chord measurements (Gjerdrum 2001), we blocked burrow entrances to prevent adults from provisioning their young and began feeding them one meal per day. Prior to the experiment, chicks were fed primarily capelin (*Mallotus villosus*) and Pacific sandlance (*Ammodytes hexapterus*) by their parents (Williams, unpublished data). We fed nestlings either 120 g/day (control group; 650 kJ/day, $n = 6$) or 60 g/day (experimental group; 325 kJ/day, $n = 7$) of Pacific herring (*Clupea pallasi*), plus a multivitamin supplement. Blood samples were collected and nestlings were weighed (± 2 g) prior to feeding at ages 10, 19, 28, and 37 days. Blood was collected in heparinized 250-µl Natelson blood-collecting tubes and transferred into 1.5-ml microcentrifuge tubes which were stored on ice until frozen as whole blood samples at -20 °C until analysis. Following collection of the final blood sample, we fed all chicks ad libitum until they reached the age and size of wild fledglings, at which point we removed obstructions from the burrow entrance and allowed them to fledge on their own initiative or released them to the water.

In the second experiment, 14 puffins were reared from hatch in individual nest boxes under thermoneutral conditions with food provided twice daily in dishes placed on the bottom of nest boxes. For the duration of the experiment they were fed exclusively capelin. Nestlings were fed an ad libitum diet until they were ten days old, and then were randomly assigned to one of two diet regimes. The experimental group received 50 g/day (270 kJ/day metabolizable energy) of capelin and the control group received 110 g/day (594 kJ/day). When nestlings approached fledging age (means $\pm$ SE: control group = 49.6 $\pm$ 1.4 days; experimental group = 57.3 $\pm$ 0.6 days), they became restless and jumped from their nest boxes at night. When an individual jumped from their nest box during two consecutive nights, they were housed in a common area with access to a small pool of fresh water and ad libitum capelin supplied twice daily until the end of the experiment. Nestlings were weighed (± 0.1 g) every five days beginning at hatch. Blood samples were collected from post-absorptive nestlings by puncture of the alar vein with a 26 g needle at ages 7, 14, 28, 42, and 75 days. Blood was collected in heparinized 100-µl hematocrit tubes,

transferred into 0.5-ml vials, and stored at 4 °C. Within 2 h of collection, samples were centrifuged, plasma removed, and blood cells stored at −20 °C until analysis.

Ten herring from the batch fed to puffin chicks in the first experiment were collected for analysis of carbon and nitrogen stable isotopes. We also collected ten capelin and thirteen sandlance that were delivered by adult puffins to their chicks at another colony in Chiniak Bay for stable isotope analysis. Capelin used in the second experiment was from a single batch caught commercially in the Atlantic, but none was archived for analysis of stable isotopes. Prior to stable isotope analysis, lipids were extracted from prey samples using a Soxtech apparatus with chloroform solvent. Lipids were not extracted from whole blood or blood cells. All samples were freeze-dried and analyzed using a Costech Elemental Analyzer (ESC 4010; Valencia, CA, USA), and a Finnigan MAT (San Jose, CA, USA) Conflo III interface with a Delta +XP mass spectrometer. Replicate measurements of internal laboratory standards indicated measurement errors to be ±0.20‰ for N, ±0.15‰ for C, and ±0.06 for the C:N ratio. Stable isotope concentrations are reported using "δ" notation according to $\delta X = [(R_{sample}/R_{standard}) - 1] \times 1{,}000$, where X is ^{13}C or ^{15}N and R is the corresponding ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$. Standard values are based on atmospheric N_2 for $\delta^{15}N$, and the Vienna Peedee Belemnite (VPDB) for $\delta^{13}C$.

We performed all statistical analyses using SAS 9.1 (SAS Institute, Cary, NC, USA) and present data as means ± SE. In both experiments, separate repeated-measures mixed models (PROC MIXED) were used to determine the effects of nutritional regime, age, and their interaction on $\delta^{15}N$, $\delta^{13}C$, C:N ratio, and mass. Use of repeated-measures mixed procedures allowed us to model the covariance structure of each data set to account for unevenly spaced sampling dates (Littell et al. 1998). Simple effects tests (LSMEANS/SLICE) were used to examine significant two-way interactions A × B (i.e., treatment × nestling age). This procedure tests for effects of A for each B, which is calculated by extracting the appropriate row from the coefficient matrix for the A × B LSMEANS and using it to form an F test. Finally, we estimate effect sizes of nutritional restriction on mass, $\delta^{15}N$, and $\delta^{13}C$ as the mean difference between treatment groups at ages 37 and 42 days in the first and second experiment, respectively.

Results

Experiment 1: Diet switched to herring at age ten days

Food restriction severely decreased rate of mass growth (Fig. 1a); mass was significantly affected by age ($F_{(3,33)} = 601.09$, $P < 0.0001$), treatment ($F_{(1,11)} = 212.77$, $P < 0.0001$), and the interaction between age and treatment ($F_{(3,33)} = 109.90$, $P < 0.0001$). At age 37 days, the mean difference in body mass between food restricted and control chicks was 227.9 g (95% CI: 205.3, 250.2). Food-restricted chicks were depleted in ^{15}N relative to control chicks (Fig. 1b); values of $\delta^{15}N$ were significantly affected by age ($F_{(3,33)} = 20.30$, $P < 0.0001$) and treatment ($F_{(1,11)} = 22.05$, $P < 0.001$; age × treatment $F_{(3,33)} = 1.04$, $P = 0.39$). Blood $\delta^{13}C$ values were also lower in food-restricted chicks compared to control chicks (Fig. 1c); $\delta^{13}C$ was affected by age ($F_{(3,11)} = 20.41$, $P < 0.0001$), but not by treatment ($F_{(1,11)} = 1.82$, $P = 0.20$). However, the interaction between

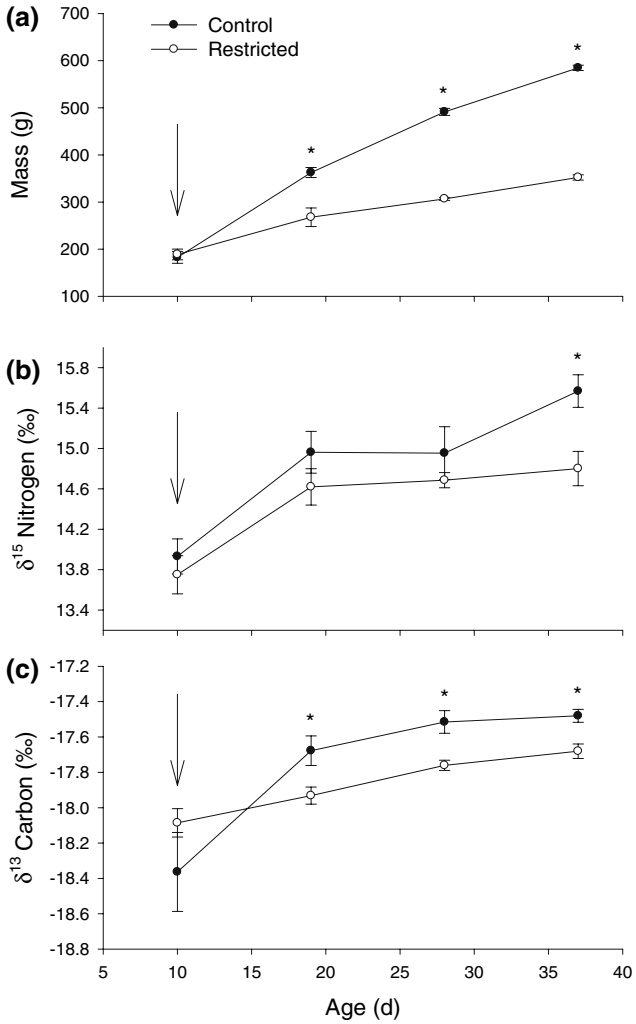

Fig. 1a–c Changes in the values of **a** mass, **b** whole blood $\delta^{15}N$, and **c** whole blood $\delta^{13}C$ (mean ± 1 SE) of tufted puffins (*Fratercula cirrhata*) with age in experiment 1 for control (650 kJ per day, $n = 6$, *filled circles*) and restricted diet (325 kJ per day, $n = 7$, *open circles*). Nestlings in this experiment were fed primarily capelin and sandlance by their parents until age ten days and then switched to a diet consisting exclusively of herring for the duration of the experiment. *Downward arrows* indicate implementation of nutritional restriction and diet switch. *Asterisks* indicate a significant difference (F test, $P < 0.05$) between the control and treatment groups at a given age

age and treatment was significant ($F_{(3,11)} = 4.80$, $P = 0.02$). Post hoc effects tests indicated control chicks had significantly higher $\delta^{13}C$ values at ages 19, 28, and 37 days, but there was no significant difference between groups on the day of the diet switch ($P > 0.20$). At age 37 days, the effect sizes of restriction on $\delta^{15}N$ and $\delta^{13}C$ values were 0.77‰ (95% CI: 0.29, 1.25) and 0.20‰ (95% CI: 0.09, 0.31), respectively. The ratio of carbon to nitrogen was not affected by age, treatment, or the interaction between age and treatment ($P > 0.50$ for all). The mean C:N ratio of whole blood samples was 3.4 ± 0.2 (SE).

The mean $\delta^{15}N$ values of sandlance and capelin fed by adult puffins provisioning their young in Chiniak Bay were 11.41 ± 0.18 (SE) and 11.41 ± 0.13, respectively. The mean $\delta^{13}C$ values of sandlance and capelin fed to puffin chicks by their parents were -18.49 ± 0.19 and -17.72 ± 0.21. The mean $\delta^{15}N$ value of herring fed to chicks was 12.52 ± 0.26 and the mean $\delta^{13}C$ value was -17.18 ± 0.15. At age 37 days, calculated diet–whole blood fractionation factors for nitrogen ($\Delta^{15}N$) were 2.28‰ (95% CI: 1.55, 3.02) and 3.05‰ (95% CI: 2.28, 3.82) for the restricted and control groups, respectively. Diet–whole blood fractionation factors for carbon ($\Delta^{13}C$) at age 37 days were −0.50‰ (95% CI: −0.89, −0.11) and −0.30‰ (95% CI: −0.72, 0.12) for restricted and control groups, respectively.

Experiment 2: Nestlings fed capelin from hatch

Food restriction severely decreased rate of mass growth (Fig. 2a); mass was significantly affected by age ($F_{(13,156)} = 129.62$, $P < 0.0001$), treatment ($F_{(1,12)} = 63.81$, $P < 0.0001$), and the interaction between age and treatment ($F_{(13,156)} = 6.69$, $P < 0.0001$). Effects tests revealed significant differences between treatment groups by age 20 days. At age 40 days (30 days of restriction) the effect size of restriction on body mass was 166.7 g (95% CI: 146.4, 187.0). Blood cells of food-restricted chicks were depleted in ^{15}N relative to control chicks (Fig. 2b); $\delta^{15}N$ was significantly affected by age ($F_{(4,48)} = 83.39$, $P < 0.0001$) and treatment ($F_{(1,12)} = 8.47$, $P = 0.013$). The age and treatment interaction was not significant ($F_{(4,48)} = 2.47$, $P = 0.057$). Nutritionally restricted chicks had blood cells depleted in ^{13}C compared to control chicks (Fig. 2c); stable isotopes of carbon were significantly affected by age ($F_{(4,12)} = 15.63$, $P = 0.0001$), but not by treatment ($F_{(1,12)} = 4.47$, $P = 0.056$). However, $\delta^{13}C$ values were significantly affected by the interaction between age and treatment ($F_{(4,12)} = 24.59$, $P < 0.0001$). Post hoc effects tests revealed significant differences in $\delta^{13}C$ values between control and experimental animals at ages 28 and 42 days. At age 42 days (32 days of restriction), the effect sizes of restriction on $\delta^{15}N$ and $\delta^{13}C$ values were 0.41‰ (95% CI: 0.18, 0.64) and 0.25‰ (95% CI: 0.19, 0.32), respectively. At age 75 days (~18 days after restriction ended), there was no difference in $\delta^{15}N$ or $\delta^{13}C$ values between control and treatment groups. The C:N ratio of blood cells was affected by age ($F_{(4,48)} = 7.54$, $P < 0.0001$), but not by treatment ($F_{(1,12)} = 0.08$, $P = 0.779$; age × treatment: $F_{(4,48)} = 0.59$, $P = 0.671$). The C:N ratio of blood cells was slightly, but significantly, higher at age seven days (mean ± SE: 3.34 ± 0.03) and then did not change for the remainder of the experiment (3.25 ± 0.01).

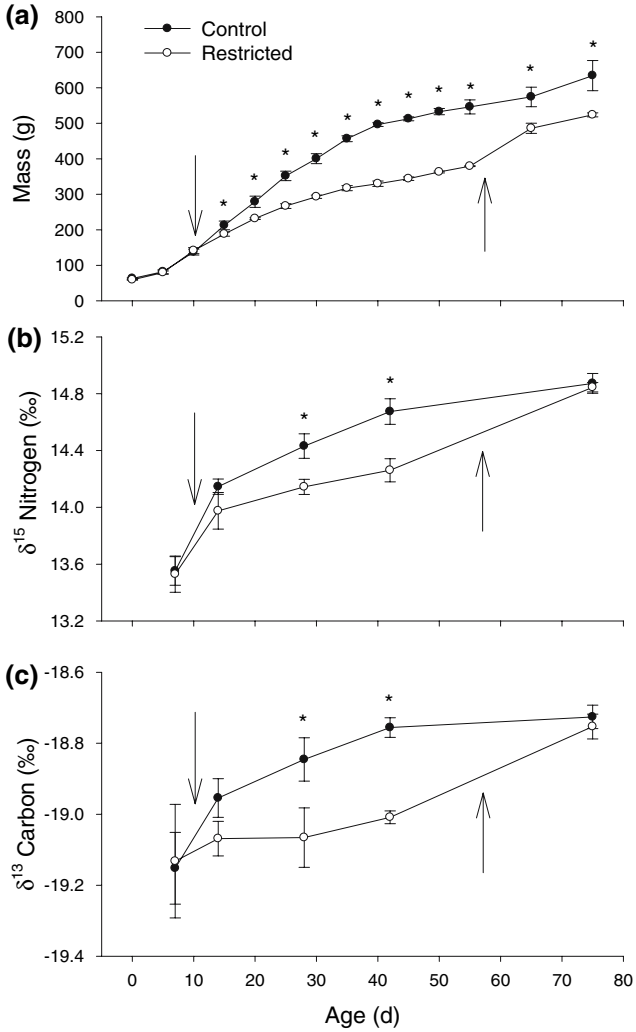

Fig. 2a–c Changes in the values of **a** mass, **b** blood cell $\delta^{15}N$, and **c** blood cell $\delta^{13}C$ (mean ± 1 SE) with age in experiment 2 for control (594 kJ per day, $n = 7$, *filled circles*) and restricted diet (270 kJ per day, $n = 7$, *open circles*). Chicks in this experiment were fed exclusively capelin from hatch. *Downward arrows* indicate implementation of nutritional restriction and *upward arrows* indicate the mean age that nestlings resumed an ad libitum diet regimen. *Asterisks* indicate a significant difference (F test, $P < 0.05$) between the control and treatment groups at a given age

Discussion

Nitrogen stable isotopes

Whereas studies of fasting animals support the hypothesis that tissues become enriched in ^{15}N following nutritional restriction (Hobson et al. 1993; Oelbermann and Scheu 2002; Cherel et al. 2005a), we found whole blood and blood cells from nutritionally restricted puffin nestlings were significantly depleted in ^{15}N compared to well-fed conspecifics. Our study is not the first to question the effects of nutritional restriction on $\delta^{15}N$ values of consumer tissues. Studies of ectothermic invertebrates have failed to detect changes in whole-animal $\delta^{15}N$ values (Frazer et al. 1997; Schmidt et al. 1999) following several months of fasting. Ben-David et al. (1999) did not find any relationship between body condition and $\delta^{15}N$ in free-living arctic ground squirrels (*Spermophilus parryii plesius*), although they acknowledge that physiological effects may have been masked by ecological processes such as diet selection. Additionally, changes in body condition are sometimes attributed primarily to catabolism of fat stores (Niizuma et al. 2002) and enrichment of ^{15}N in body tissues is only expected if poor body condition is associated with protein loss (Martinez del Rio and Wolf 2005).

Nutrient dynamics are complicated during growth as nutritionally restricted birds may be simultaneously protein-limited and in positive nitrogen balance. Kempster et al. (2007) found that a 35% reduction in food intake had no effect on $\delta^{15}N$ values of tissues in song sparrows (*Melospiza melodia*). Contrary to our study and to that of Kempster et al. (2007), Hobson et al. (1993) determined that food-restricted Japanese quail (*Coturnix japonica*) chicks become enriched in ^{15}N compared to growing birds fed ad libitum. The degree of nutritional restriction may explain the apparently contradictory results: chicks in the study of Hobson et al. (1993) were restricted to the extent that they did not gain body mass, whereas nestlings in Kempster et al. (2007) and in our study continued to grow, albeit at a greatly reduced rate. Protein content of the diet may also play a role and was likely much higher in our study: quail chicks were fed commercial turkey starter, whereas puffins were fed a fish diet. With a decrease in content of dietary protein, the ratio of nitrogen assimilation to nitrogen loss increases and thus diet–tissue fractionation of nitrogen stable isotopes is predicted to decrease (Pearson et al. 2003; Martinez del Rio and Wolf 2005). However, when exogenous amino acids are insufficient to meet demands for protein synthesis, recycling of metabolic amino acids should increase, producing the opposite effect (Voigt and Matt 2004). Taken further, food restriction may also produce an increase in diet–tissue fractionation of nitrogen isotopes in cases where growth is limited by dietary protein rather than energy intake.

Finally, species-specific differences in physiological response to nutritional restriction may be responsible for differences between studies. Puffin chicks are adapted to intermittent provisioning and are able to adjust their rates of development in response to food deprivation (Kitaysky 1999). Puffin nestlings subjected to nutritional restriction also down-modulate the hypothalamus–pituitary–adrenal (HPA) axis, resulting in decreased plasma levels of corticosterone (Kitaysky et al. 2005), an anti-anabolic stress steroid that functions to mobilize stored protein reserves. In contrast, the nutritional state of other seabird chicks is negatively correlated with activity of the HPA axis (Nunez-de la Mora et al. 1996; Kitaysky et al. 2001). Thus, food-deprived puffin chicks are able to spare endogenous protein stores and maintain a positive nitrogen balance despite a 50% reduction in food intake, which may explain the lack of ^{15}N enrichment in restricted chicks. However, song sparrow chicks subject to moderate food restriction exhibited numerous symptoms of nutritional stress, including elevated levels of corticosterone, yet $\delta^{15}N$ values were unaffected (Kempster et al. 2007).

Although we found significantly higher $\delta^{15}N$ values in well-fed (control) chicks compared to food-restricted animals, we do not believe that this was due to nutritional restriction per se. It is possible that this effect is due to incomplete turnover of blood cells from hatch and slower turnover in restricted birds. However, this seems unlikely given $\delta^{15}N$ values of restricted chicks appeared to be approaching a different asymptotic level compared to control birds (Fig. 2b). Instead, we suggest the increase in $\delta^{15}N$ with body mass observed in our study is an artifact of growth and results from recycling of a continually expanding pool of metabolic nitrogen and/or from changes in nitrogen use efficiency. During growth, the size of endogenous protein stores and metabolic amino acid pools increased, while the amount of exogenous protein available remained unchanged. The metabolic amino acid pool is enriched relative to diet, so a proportional increase in amino acids drawn from the metabolic pool would produce progressive whole-body enrichment as body size increases. Additionally, growth of control chicks slowed as chicks aged, whereas food intake did not change, indicating protein was being used as a metabolic substrate to a greater extent during the latter half of the nestling stage. Thus, the ratio of nitrogen assimilation to nitrogen loss decreased as well-fed chicks grew, which is predicted to result in higher diet–tissue fractionation (Martinez del Rio and Wolf 2005). Finally, the specific balance of essential amino acids needed for protein accretion differs from that needed to replace obligatory losses (reviewed in Klasing 1998), although the effect this has on diet–tissue fractionation is unknown.

Carbon stable isotopes

Although nutritional status is thought to primarily affect diet–tissue fractionation of nitrogen, a small and variable diet–tissue fractionation of ^{13}C occurs (see references in Kelly 2000), yet the cause of the variability is not well understood. Hatch et al. (1995) determined that protein lysate (95% hemoglobin) from growing roosters and adult hens subjected to nutritional restriction became enriched in $\delta^{13}C$ and suggested its use as an indicator of catabolic state. Contrary to these findings, Hobson et al. (1993) found food restriction had no effect on $\delta^{13}C$ in muscle and liver tissue of quail chicks and fasting geese, whereas Cherel et al. (2005a) found ^{13}C was depleted in blood plasma, but not cells, from fasted birds.

Body lipids are depleted in ^{13}C relative to proteins and carbohydrates (DeNiro and Epstein 1977). Effects of food deprivation on the lipid content of tissues may consequently produce lower $\delta^{13}C$ values in lipid-rich tissues with a higher C:N ratio (Cherel et al. 2005a). However, in our second experiment, $\delta^{13}C$ values of blood cells were affected by nutritional restriction even though they have very little lipid content. Furthermore, the C:N ratios of whole blood and blood cells were unaffected by treatment, yet birds became progressively enriched in ^{13}C when fed ad libitum. Increased metabolism of exogenous or endogenous lipids associated with nutritional restriction will alter diet–tissue fractionation if carbon isotopes from these lipids are incorporated into other molecules during anabolic processes. The isotopic composition of body protein generally reflects the composition of dietary protein (Ambrose and Norr 1993), a process known as isotopic routing. However, carbon from oxidized fatty acids may be incorporated into the carbon skeletons of some nonessential amino acids because they are synthesized from intermediates of the carboxylic acid cycle (Klasing 1998). Thompson et al. (2000) suggested that incorporation of carbon from ^{13}C-depleted lipids into proteinaceous tissues was responsible for the high variability in $\delta^{13}C$ signatures they observed between several free-living species of albatross. Cherel et al. (2005b) hypothesized that this mechanism was responsible for variability in diet–tissue fractionation observed in captive penguins. Furthermore, by manipulating the concentrations and isotopic signatures of macronutrients in the diets of song birds, Podlesak and McWilliams (2006) determined that birds fed a low-protein diet incorporated dietary carbon from other macronutrients into proteins to a greater extent. We hypothesize that nutritional restriction may increase the amount of carbon from ^{13}C-depleted lipids incorporated into proteins as dietary and endogenous lipids are metabolized to meet energetic demands.

Tissue turnover

One limitation in our experiments is that the effects of growth and tissue turnover are necessarily confounded because both processes are occurring simultaneously. Furthermore, observed isotopic turnover in growing animals reflects a combination of metabolic turnover and accretion of new tissue. Although turnover rates of tissues in slow-growing chicks are expected to be slower than control chicks, the asymptotic levels of $\delta^{15}N$ and $\delta^{13}C$ both appear to be different between treatment groups in both experiments, indicating that turnover alone cannot explain the observed pattern. We selected whole blood and blood cells for isotopic analyses because these tissues are commonly used in ecological studies. However, use of plasma, a tissue with a much higher turnover rate than blood cells (Hobson and Clark 1993), may have allowed us to disentangle growth and turnover effects. Future studies may benefit by selecting plasma as a target tissue; however, lipids must be extracted prior to analysis because ^{13}C-depleted triglycerides are affected by nutritional restriction (Alonso-Alvarez and Ferrer 2001).

Implications for ecological studies

In this study, we tested the hypothesis that nutritional restriction affects $\delta^{15}N$ and $\delta^{13}C$ concentrations of blood in growing seabird chicks. Our primary objective was to determine if variation in nestling growth rates observed in wild populations would potentially confound estimates of trophic level of feeding and/or foraging location based on stable isotopes. Therefore, it is critical to first assess whether levels of food supplied to chicks in our experiments are comparable to food provisioned in wild populations. In our study, mass gain of nestlings during the "linear growth phase" (Gjerdrum 2001), was 17.15 g/day (control) versus 6.55 g/day (restricted) in the first experiment and 13.16 g/day versus 7.59 g/day in the second experiment. Piatt and Kitaysky (2002) review 49 colony years of nestling growth data and report an overall mean growth rate of 10.9 g/day ± 4.7 SD (range −0.6 to 19 g/day). Thus, growth rates of our food restricted birds in our experiments were less than 1 SD below the overall mean and well within the range reported for wild birds. Control groups in our experiments grew faster than average, and in our first experiment, approached the maximum growth rate observed in the wild. At 38–40 days old, the minimum age required to fledge, the masses of control and restricted chicks in both experiments were within the range of fledging masses reported for wild birds (range 270–609 g; overall mean: 477 g ± 99.2 SD; Piatt and Kitaysky 2002). Thus, we conclude that levels of food

supplied to chicks in our experiments are within the range received by free-living nestlings.

The effect size of nutritional restriction on $\delta^{15}N$ in our experiments ranged from 0.41 to 0.77‰. Assuming that $\delta^{15}N$ increases 3.4–3.8‰ per trophic level in marine systems (Minigawa and Wada 1984; Hobson and Welch 1992), nutritional restriction during growth would produce a maximum error in estimates of trophic level of approximately 0.23 trophic levels. The mean effect size of nutritional restriction on $\delta^{13}C$ was also relatively small in our study (0.20–0.25‰). Hobson et al. (1994) found that $\delta^{13}C$ values from tissues of seabird species known to forage inshore versus offshore differed by 2–3‰. Therefore, despite effects of nutritional restriction on diet–tissue fractionation, stable isotopes of nitrogen and carbon should still provide a useful measure of trophic level of feeding and foraging location in free-living animals. Nevertheless, our results demonstrate that physiological processes affect stable isotope signatures and we caution isotope ecologists to consider these effects to avoid drawing spurious conclusions.

Acknowledgments Funding was provided by NOAA/NMFS (NA16FX1270) to CLB, by NPRB to CLB and CTW, by EVOS Trustees Council and NPRB to ASK, and by fellowships from AK EPSCoR to CTW and JS. We thank Rachael Orben, Travis Cooper, John Brewer, and Erin Whidden for assistance with feeding experiments and lab work and two anonymous reviewers for helpful comments. All procedures were approved by Institutional Animal Care and Use Committees at the University of Washington (IACUC #2212-26) and University of Alaska Fairbanks (#05-43) and authorized under state and federal permits.

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