Do stable isotopes reflect nutritional stress? Results from a laboratory experiment on song sparrows

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Abstract Stable isotope analysis is an increasingly valuable tool in ecological studies and shows promise as a measure of nutritional stress in wild animals. Thus far, however, the only studies on endotherms that have conclusively shown changes in $\delta^{15}N$ and $\delta^{13}C$ values in response to nutritional stress were conducted on fasting animals and animals growing under extreme levels of food restriction. We conducted a laboratory experiment to test whether $\delta^{15}N$ and $\delta^{13}C$ values provide a general index of nutritional stress. We compared the isotopic composition of whole blood, liver, muscle and feathers between two groups of juvenile song sparrows (*Melospiza melodia*) hand-reared in captivity under identical conditions except for feeding regime. To verify that our experimental treatment induced a biologically meaningful level of nutritional stress, we simultaneously measured the effects on physiology, growth and development at multiple scales. While food-restricted birds were physiologically stressed, physically smaller, and showed poorer growth and brain development compared to ad libitum-fed birds, there was no effect of feeding regime on either $\delta^{15}N$ or $\delta^{13}C$ values in any tissue. Instead of a continuum where the level of change in ^{15}N or ^{13}C contents corresponds to the level of nutritional stress, we suggest there may be a threshold level of nutritional stress below which such isotopic changes are likely to be negligible.

Keywords Corticosterone · Developmental stress · Ecophysiology · Food

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Introduction

Stable isotope analysis is an increasingly used tool in ecological studies, with new papers being published in almost every issue of every major ecological journal (Rubenstein and Hobson 2004; Bearhop et al. 2004; Cherel et al. 2005; Thompson et al. 2005). However, interpretations of stable isotope results are often made without adequate information concerning the constraints on these data and a "call for more laboratory experiments" (Gannes et al. 1997) has been made by several authors (Gannes et al. 1997; Jardine and Cunjak 2005; Phillips et al. 2005). One such constraint is that data concerning dietary, trophic, and nutritional

status may be confounded because these are all facets of the same biochemical process.

Protein in consumer tissue has a higher $^{15}N/^{14}N$ ratio than dietary protein. The tissues of fasting and starving animals may also show a progressive increase in the $^{15}N/^{14}N$ ratio as lean body mass decreases (Hobson et al. 1993; Cherel et al. 2005) because starving animals literally "live on their own meat" (Waterlow 1968). Extrapolating from this relationship between $^{15}N/^{14}N$ ratio and body mass in fasting and starving animals, Hobson et al. (1993) proposed that the $^{15}N/^{14}N$ ratio could be used as a general index of nutritional stress. In a laboratory experiment on Japanese quail chicks (*Coturnix japonica*) hand-reared in captivity under identical conditions except for feeding regime, Hobson et al. (1993) showed that the $^{15}N/^{14}N$ ratio in the blood, liver, muscle and bone of food-restricted chicks was significantly higher than in chicks fed ad libitum. This study has since been cited over 120 times. Despite its importance, we are not aware of any other laboratory experiment on endotherms that has attempted to further explore the relationship between the $^{15}N/^{14}N$ ratio and nutritional stress.

The effects of nutritional stress on juvenile physiology, growth and development may carry-over into adulthood (Ohlsson and Smith 2001; Gil et al. 2004). Experimental studies on both birds and mammals have shown that offspring reared under nutritional stress may mature into adults that are less successful at attracting a mate (Nowicki et al. 2002), and have impaired brain function, learning, and memory retention (Sapolsky 1999; Kitaysky et al. 2003; Pravosudov and Kitaysky 2006). In songbirds, the nestling phase is the most energetically demanding period of life and requires a high volume of energy-rich foods to sustain a rapid rate of development (Lepczyk et al. 1998; Lepczyk and Karasov 2000). Because food demands are so great, nestlings are often hungry, and starvation of some or all of the brood is common (Nowicki et al. 1998; Hovorka and Robertson 2000; Nowicki et al. 2002).

We tested whether nutritional stress during development affected the stable isotope compositions of song sparrows (*Melospiza melodia*). Sparrow nestlings were hand-reared in captivity under identical conditions except for feeding regime. We expected nutritionally stressed young would have higher $\delta^{15}N$ and lower $\delta^{13}C$ values (Hobson et al. 1993; Cherel et al. 2005). We compared our stable isotope results with the effects of nutritional stress on physiology, growth, and development. We are unaware of any other comprehensive laboratory experiment examining the effects of nutritional stress at these multiple scales.

Materials and methods

Study system and experimental design

Nestling song sparrows were collected from sites in London, Ontario, Canada, from May to August 2004. Nestlings were brought into the laboratory mostly between ages 3 and 4 days post-hatch (mean $\pm$ SE = 3.75 ± 0.18). A total of 15 broods of 54 young were collected. We randomly assigned each brood to one of our two treatments ("ad libitum" or "food restricted", see below). In total, there were eight broods of 28 young in the ad libitum group, and seven broods of 26 young in the food-restricted group. All birds were maintained in the same room under identical conditions (14 h light–10 h dark photoperiod and a temperature of 26°C at the nestling phase and 21°C after fledging).

From the time of collection until their euthanization (23–26 days post-hatch), we fed nestlings a standard hand-rearing diet of nestling mash (Mazuri Small Bird Maintenance, wheat germ, soy protein powder, hard-boiled chicken eggs with shells removed, and water; blended in a food processor). We mass-produced food 5 times throughout the experiment. The food in each batch was completely consumed before a new batch was prepared and used. Nestlings in each brood received food from a single batch from the time they were brought into the laboratory until they were euthanized. Samples of the food were periodically collected and frozen for stable isotope analysis. The ad libitum birds were fed to satiation at every feeding. We calculated the average amount eaten by ad libitum birds each day and gave the birds in the food-restricted treatment 65% (by volume) of the amount eaten by the ad libitum birds (Searcy et al. 2004).

Stable isotope analyses

For isotopic analyses, we sampled the outermost left tail feather, whole blood, liver, pectoral muscle and randomly selected diet samples. All samples were stored in a freezer at −20°C until analyzed, except for feather, which was sealed in plastic bags and stored at room temperature. The blood, liver, muscle, and diet samples were freeze-dried and powdered. Lipids were removed from the diet using three sequential soakings in a 20:10:8 methanol:chloroform:water solution, and then air-dried (Bligh and Dyer 1959). The lipids were removed from half of each liver and muscle sample using three sequential soakings in a 50:50 methanol:chloroform solution, and then air-dried (Sotiropoulos et al. 2004). The lipid-extracted samples were used for C isotope analysis; lipid-free fractions of liver and

muscle were enriched in ^{13}C by an average of 0.7 ± 0.16 and 0.7 ± 0.10‰, respectively. Lipids were not extracted from the blood (Bearhop et al. 2000). Feathers were cleaned of organic contaminants using a 24-h soak in a 50:50 methanol:chloroform solution, air-dried, and cut into small fragments using scissors.

The relative abundance of stable isotopes of N ($^{15}N/^{14}N$) and C ($^{13}C/^{12}C$) in the samples were determined by continuous-flow mass spectrometry (Costech elemental analyzer, Thermo Finnigan Delta^{plus} XL mass spectrometer). Results were expressed in the standard δ-notation relative to atmospheric N_2 and Vienna Pee Dee Belemnite for $δ^{15}N$ and $δ^{13}C$ values, respectively. All samples were analyzed in duplicate, with an average reproducibility of ±0.03‰. Accuracy of the measurements, as determined using laboratory standards calibrated to international reference samples, was ±0.02‰ for both $δ^{15}N$ and $δ^{13}C$ values. To compensate for small variations in the N (±0.16‰) and C (±0.22‰) isotopic compositions of the diet (five different batches of diet were consumed in this study), the N and C isotope results reported below are provided in terms of the tissue–diet discrimination ($Δ^{15}N_{tissue–diet} = δ^{15}N_{tissue}–δ^{15}N_{diet}$; $Δ^{13}C_{tissue–diet} = δ^{13}C_{tissue}–δ^{13}C_{diet}$).

Physiology, growth and development

Blood analyses provide a reliable method of detecting physiological changes resulting from nutritional stress. Nutritional stress has been shown to have significant impacts on corticosterone and glucose concentrations, and haematocrit in a variety of birds (Heath and Duffy 1998; Clinchy et al. 2004; Pravosudov and Kitaysky 2006). Blood was collected from the ulnar vein into two heparinized capillary tubes just prior to euthanization. Plasma glucose was measured immediately (AccuSoft Advantage blood glucose monitor). The remaining blood was centrifuged, measured for haematocrit, and plasma was drawn off and frozen at $-20°C$ until processing. Corticosterone was measured in samples of 5–21 μl of plasma via radioimmunoassay following extraction in dichloromethane (Wingfield et al. 1992).

We measured mass, fat deposits, breast muscle mass and keel length. The birds were weighed every day to the nearest 0.25 g using a spring scale. We scored the amount of subcutaneous fat in the furcular deposit from 0 to 5 (Wingfield et al. 2003). The left breast muscle was weighed immediately following removal from the keel. The keel was then measured to the nearest 0.1 mm. Condition was calculated as mass/tarsus length[3] (Saino et al. 2003).

We measured brain development in three song-control regions (high vocal center, area X, and robust

nucleus of the acropallium; MacDonald et al. 2006), and evaluated four other indicators of anomalous development. Measures of asymmetry are a common, but controversial, measure of developmental stress, reflecting nutritional stress in some instances (Grieco 2003; Clinchy et al. 2004; Pravosudov and Kitaysky 2006) but not others (Hovorka and Robertson 2000; Searcy et al. 2004). We measured asymmetry in the tarsi and seventh primaries. The muscle was removed from the tarsi prior to measurement. The right and left tarsi were each measured 3 times, and the right and left seventh primaries were each measured twice. All measurements were made by just one person (B. K.). Asymmetry was calculated as $|R - L|$ and analyzed following Palmer and Strobeck (2003). Asymmetry was significantly larger than measurement error in both the tarsi ($P < 0.001$, $R^2 = 0.999$) and seventh primaries ($P < 0.001$, $R^2 = 0.999$) with repeatabilities [$(ME5)_{rA}$] of 0.840 and 0.847. Fault bar analyses were conducted on the second and third outer left tail feathers and the sixth and seventh right and left primary wing feathers (Searcy et al. 2004). Fault bars are areas where barbs are absent or abnormal due to stress-induced lags in keratin deposition during feather formation (Bortolotti et al. 2002).

Statistical analyses

We compared the effect of nutritional stress between the ad libitum and food-restricted brood means using t tests (STATISTICA 6.0, StatSoft, 2001). The data were checked for normality and homogeneity of variances and were square root-transformed when necessary. In all cases, the tests were one-tailed and α was set at 0.05. All means are presented $\pm$SEM. The statistical examination of the $Δ^{15}N_{tissue–diet}$ values for liver and muscle was performed on tissues containing lipids, while analyses of the $Δ^{13}C_{tissue–diet}$ values for liver and muscle were done on tissues from which lipids had been extracted (Sotiropoulos 2004). Average brood residuals were used when analyzing glucose (covariate body weight) (Ben-David et al. 1999; Christensen et al. 2000), corticosterone (covariate bleeding time), and haematocrit (covariate hatch date).

Results

Stable isotopes

In contrast to the effects shown in Japanese quail chicks by Hobson et al. (1993), there was no evidence of ^{15}N enrichment in response to nutritional stress in

any tissue in the song sparrows. There were no significant differences in $\Delta^{15}N_{tissue-diet}$ values between food-restricted and ad libitum-fed birds in blood ($t_{13} = 1.03$, $P = 0.160$), liver ($t_{12} = 0.85$, $P = 0.207$), muscle ($t_{12} = 1.16$, $P = 0.135$) or feathers ($t_{13} = 0.63$, $P = 0.271$; Table 1). There were also no significant differences in $\Delta^{13}C_{tissue-diet}$ values between food-restricted and ad libitum-fed birds in blood ($t_{13} = 0.24$, $P = 0.406$), liver ($t_{12} = 1.02$, $P = 0.164$), muscle ($t_{12} = 0.56$, $P = 0.292$) or feathers ($t_{13} = 0.25$, $P = 0.402$; Table 1).

Physiology

While there was no significant effect of food restriction on stable isotope compositions, food restriction affected all three of the physiological indices examined. Consistent with their being physiologically stressed (Clinchy et al. 2004), food-restricted birds had significantly higher corticosterone (22.7 ± 2.7 vs. 10.8 ± 2.7 ng/ml; treatment, $F_{1,11} = 8.47$, $P = 0.007$; covariate, $F_{1,11} = 7.67$, $P = 0.018$), higher plasma glucose levels (24.4 ± 0.87 vs. 20.7 ± 0.95 mmol/l; treatment, $t_{12} = 2.03$, $P = 0.032$), and lower haematocrit (0.59 ± 0.01 vs. 0.63 ± 0.01; treatment, $t_{12} = 2.62$, $P = 0.011$) than did ad libitum-fed birds.

Growth

Like the effects on physiology, the effects of food restriction on growth were consistent with expectations. In terms of body mass, the ad libitum-fed birds grew at a greater rate (Fig. 1) and weighed 15% more at the end of the experiment than the food-restricted birds (20.0 ± 0.4 vs. 16.7 ± 0.3 g, $t_{13} = 6.45$, $P < 0.001$). In terms of body size, the ad libitum-fed birds were also significantly larger at the end of the experiment, as measured by keel length (16.0 ± 0.2 vs. 14.5 ± 0.3 mm; $t_{10} = 4.15$, $P = 0.001$), wet muscle mass (1.09 ± 0.04 vs.

Table 1 $\Delta^{15}N_{tissue-diet}$ and $\Delta^{13}C_{tissue-diet}$ values for blood, liver, muscle and feather in ad libitum-fed and food-restricted juvenile song sparrows

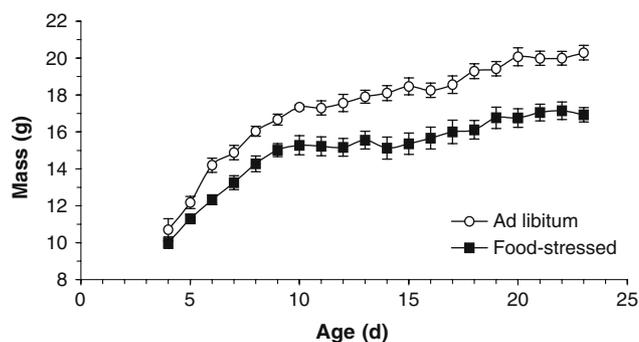| Tissue	Treatment	$\Delta^{15}N_{tissue-diet}$ (‰)		$\Delta^{13}C_{tissue-diet}$ (‰)	
		Mean	SE	Mean	SE
Blood	Ad libitum	2.15	0.14	−1.55	0.20
	Food restricted	1.96	0.10	−1.62	0.19
Liver	Ad libitum	3.02	0.06	−0.50	0.10
	Food restricted	2.94	0.07	−0.66	0.13
Muscle	Ad libitum	2.19	0.08	−0.84	0.08
	Food restricted	2.04	0.10	−0.94	0.16
Feather	Ad libitum	2.85	0.15	0.25	0.14
	Food restricted	2.74	0.08	0.20	0.15

Fig. 1 Body mass (mean ± SE) of song sparrow (*Melospiza melodia*) nestlings 4–23 days (*d*) after hatching with ad libitum (*open circle*) and food-restricted (*filled square*) diets

0.79 ± 0.07 g; $t_{10} = 4.20$, $P = 0.001$), dry breast muscle mass (0.30 ± 0.01 vs. 0.22 ± 0.02 g; $t_{10} = 4.38$, $P < 0.001$) and seventh primary length (44.8 ± 1.3 vs. 43.2 ± 2.0 mm; $t_{13} = 1.79$, $P = 0.048$). Tarsus length was the sole measure not significantly greater in ad libitum-fed birds (20.3 ± 0.2 vs. 20.0 ± 0.2 mm; $t_{10} = 0.79$, $P = 0.222$). Correcting for body size (mass/tarsus^3) at the end of the experiment, ad libitum-fed birds were significantly heavier ($t_{13} = 6.79$, $P < 0.001$), and they had significantly higher fat scores (2.08 ± 0.16 vs. 1.10 ± 0.13; $t_{13} = 4.59$, $P < 0.001$) than the food-restricted birds.

Indicators of anomalous development

Food restriction significantly affected brain development as reported by MacDonald et al. (2006). Food restriction also significantly increased the asymmetry in the seventh primary feathers but none of the other three indices of anomalous development were significantly affected (food restricted vs. ad libitum; seventh primary, 0.09 ± 0.02 vs. 0.04 ± 0.02, $t_{13} = 1.98$, $P = 0.034$; tarsi, 0.17 ± 0.04 vs. 0.12 ± 0.02, $t_{13} = 1.24$, $P = 0.119$; tail fault bars, 0.84 ± 0.23 vs. 0.81 ± 0.18, $t_{11} = 0.11$, $P = 0.457$; wing fault bars, 0.06 ± 0.02 vs. 0.11 ± 0.04, $t_{13} = 1.01$, $P = 0.166$).

Discussion

Our food-restriction treatment significantly affected the physiology, growth and development of juvenile song sparrows without inducing morbidity or mortality. Nutritionally stressed birds had higher corticosterone and glucose levels and lower haematocrit, grew more slowly, weighed less, were physically smaller, in poorer condition, showed some signs of anomalous development, and had smaller song-control regions in the brain

(McDonald et al. 2006). Despite the consistent evidence of nutritional stress shown at these multiple scales there was no indication that this biologically meaningful level of nutritional stress resulted in significant changes in $\Delta^{15}N_{tissue-diet}$ or $\Delta^{13}C_{tissue-diet}$ values.

Hobson et al.'s (1993) laboratory experiment is one of the most frequently cited studies regarding ^{15}N enrichment and nutritional stress. In contrast to Hobson et al.'s results, numerous correlational studies on a wide variety of organisms have failed to find ^{15}N enrichment in association with nutritional stress. Because these results are inconsistent with those of Hobson et al.'s (1993) manipulation, the authors of these correlational studies have frequently attributed their negative findings to idiosyncracies in their study, or the organisms they examined (e.g., Pfeiler et al. 1998; Hobson and Schell 1998; Gloutney et al. 1999; Gorokhova and Hansson 1999; Doucett et al. 1999; Ben-David et al. 1999). Our goal was to replicate Hobson et al's (1993) experiment on Japanese quail chicks using song sparrow nestlings and a less extreme level of nutritional stress. The results from our laboratory experiment differed from Hobson et al's indicating that ^{15}N enrichment in response to nutritional stress may not be a universally applicable pattern across all circumstances.

We suggest the contrast between our stable isotope results and Hobson et al.'s (1993) is largely a function of the level of nutritional stress induced in the two studies. Hobson et al. (1993) described their study as testing "the hypothesis that fasting or nutritional stress" can cause elevation of ^{15}N. It may not be the case, however, that a continuum exists wherein the level of ^{15}N enrichment corresponds to the level of nutritional stress. Our results suggest that there may be a threshold level of nutritional stress below which changes in isotopic composition are likely to be negligible. Ross' geese (Hobson et al. 1993) and king penguins (Cherel et al. 2005) that fast during incubation are clearly experiencing extreme nutritional stress. Likewise, the juvenile quail studied by Hobson et al. (1993) were also subjected to extreme nutritional stress that severely retarded their growth: food-restricted birds grew to only one-third of the size of ad libitum-fed birds (40 vs. 120 g, respectively). The song sparrows nestlings in our study were subjected to a less extreme though biologically meaningful level of nutritional stress that affected their physiology, growth and brain development to an extent that may have life-long consequences (Pravosudov and Kitaysky 2006). Yet, there were no significant changes in the N (or C) isotopic composition of the song sparrows' tissues. Hence, the efficacy of the stable isotope method for detection of nutritional stress in wild populations may be more restricted (e.g., to extreme situations such as experienced by fasting or dying animals) than previously considered.

Cherel et al. (2005) recently reported a decrease in the $\delta^{13}C$ value in the plasma of fasting king penguins. They attributed this outcome to an increase in lipid content in the plasma. All of our samples had low natural concentrations of lipids (whole blood and feather) or had been treated to remove lipids prior to C isotope analyses (liver and muscle). Other authors have proposed that nutritional stress should result in higher $\delta^{13}C$ values (Hatch et al. 1995; Oelbermann and Scheu 2002) on the basis that the same mechanisms that may cause $\delta^{15}N$ values to increase would affect $\delta^{13}C$ values in a similar fashion. More studies are required to determine if $\delta^{13}C_{tissue}$ and $\Delta^{13}C_{tissue-diet}$ can be used in the field as reliable indicators of nutritional stress.

We examined whether stable isotope analysis can identify nutritional stress in song sparrows as measured through increases in $\delta^{15}N$ or $\delta^{13}C$ values in their body tissues. This enrichment arises, in theory, because during nutritional stress the body preferentially retains heavy isotopes liberated during protein catabolism. While theory would predict that stable isotope analysis has tremendous potential as an indicator of nutritional stress, our study in conjunction with Hobson et al. (1993) suggests that the cycling of N from body protein stores will lead to significant enrichment only when the catabolic process is substantial. Animals that are fasting or starving do appear to "live on their own meat" and consequently exhibit ^{15}N enrichment, while animals that are malnourished do not. The stable N and C isotope compositions of animal tissues are the result of complex interactions among diet, ecology, physiology and biochemistry. As others have emphasized, more laboratory experiments are needed to fully test the assumptions and limitations inherent in the interpretation of these data as they relate to nutritional stress.

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