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Assessing the condition of juvenile Song Sparrows (Melospiza melodia) using stable nitrogen and carbon isotopes

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Assessing the condition of juvenile Song Sparrows (*Melospiza melodia*) using stable nitrogen and carbon isotopes

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Abstract

Offspring condition may predict a population's future success. We studied the effects of nutritional stress on $\Delta^{15}N$ and $\Delta^{13}C$ in juvenile Song Sparrows (*Melospiza melodia*) hand-reared under *ad libitum* or food-stressed conditions, and compared them to conventional measures of condition. The degree of food stress significantly altered development, causing poor condition and increased corticosterone, glucose, and anemia. Despite this, we could not detect nutritional stress as measured by $\Delta^{15}N$ or $\Delta^{13}C$ and the birds did not display increased developmental instability. ^{15}N enrichment may require greater levels of nutritional stress than used in this experiment. $\Delta^{13}C$ analysis may be complicated by differences in endogenous amino acid formation or by differential digestion of C_3 and C_4 dietary components. We determined $\Delta^{15}N$ and $\Delta^{13}C$ tissue-diet fractionations for blood, feathers, liver, muscle, and excreta. Liver and muscle from which lipids had been removed were significantly enriched in both ^{15}N and ^{13}C.

Key Words: *Melospiza melodia*, nutritional stress, stable isotope, nitrogen, carbon, morphometrics, corticosterone, developmental stability, fractionation, lipid extraction

Co-authorship

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Chapter 1: General Introduction

Stable isotope analysis has developed into a tool with great potential for ecologists. This technique provides a new way to study some of the most complex areas of ecology such as migratory patterns and geographic origin (Marra et al. 1998), diet reconstruction (Hobson and Clark 1992a; Hobson 1995), trophic ecology (Adams and Sterner 2000), and nutritional status (Hobson et al. 1993).

Five elements are used in stable isotope ecology: ^{1}H and ^{2}H, ^{12}C and ^{13}C, ^{14}N and ^{15}N, ^{16}O and ^{18}O, and ^{32}S and ^{34}S (Peterson and Fry 1987). The heavier isotope has additional neutron(s) in the nucleus and forms chemical bonds with lower vibrational frequencies that are slightly stronger and are less likely to undergo chemical reactions than the more reactive light isotope (Adams and Sterner 2000). Because of differences in bond energy and reaction rate, organisms are able to discriminate between the two isotopes, and as a result the isotopic ratio varies between molecules in a process called fractionation (Gannes et al. 1998). This thesis will focus exclusively on the behaviour of stable nitrogen and carbon isotopes in passerine birds.

Carbon isotopes are differentiated mainly through the actions of C_3, C_4, and CAM plants. C_3 plants (most terrestrial plants) are more depleted in ^{13}C relative to C_4 (tropical grasses) and CAM (drought-tolerant) plants (Peterson and Fry 1987; Hobson et al. 1993; Marra et al. 1998). When an animal consumes carbon as lipids, proteins, and carbohydrates, the body preferentially retains ^{13}C and excretes ^{12}C (Voigt and Matt 2004). This effect, however, is not great, and the individual is enriched approximately 1‰ in ^{13}C relative to its diet (Hobson and Clark 1992b), although depletion in ^{13}C is also possible (Voigt et al. 2003).

Differentiation between the two nitrogen isotopes occurs in the metabolic reactions in animals (Voigt and Matt 2004). Animals obtain nitrogen from digested dietary protein. The digested amino acids enter the bloodstream and are taken to cells in the liver and muscle (Kelly 2000). There, the amine group (NH_2) on the amino acids can be moved around through a series of deamination and transamination processes. During transamination, the amine group is removed from an amino acid and added to an α-keto acid to form a new amino acid, an important branching point in the flow of nitrogen through the body (Macko et al. 1986). It is during this process that nitrogen fractionation occurs, because isotopic discrimination is greatest for relatively small molecular fragments. The metabolic enzymes responsible for deamination and transamination preferentially remove anime groups containing the more reactive ^{14}N. The ^{14}N moves more rapidly into the end products (Gannes et al. 1997; Webb et al. 1998; Adams and Sterner 2000), one of which is ammonia, the waste product from protein catabolism. Ammonia, however, is toxic and is not stored in the body. A series of biochemical reactions transform it into urea, which is then excreted by mammals. In birds, amphibians, and reptiles, urea is converted to uric acid (which conserves water) before being excreted by the kidneys (Voet et al. 1999; Kelly 2000; Vanderklift and Ponsard 2003). Since ^{14}N is preferentially converted to ammonia and excreted, ^{14}N leaves the body at a greater rate and the body becomes enriched in ^{15}N (Ponsard and Averbuch 1999). This enrichment has been estimated to be 3-5‰ (Doucett et al. 1999).

Nitrogen for protein synthesis comes from two sources: (1) amino acids derived from the diet, and (2) amino acids catabolized from body tissues as all animal tissues are continuously being degraded and renewed. The amino acids from tissue breakdown are

mixed with those from the diet and both are incorporated into new tissues (Ayliffe et al. 2004). Under satiated food conditions, amino acids from the diet and from the degradation of body protein are sufficient to meet nitrogen demands, with the amino acids from the diet being preferentially incorporated into the new proteins (Carey 1996; Gaye-Siessegger et al. 2004b; Savidge and Blair 2004). When fasting or food stress occurs, nitrogen intake from the diet drops, and individuals must increasingly rely on stored reserves to meet nitrogen demands. These individuals increasingly deaminate tissue protein to use for energy and as a nitrogen source (Carey 1996). The amino acids released from protein degradation can be deaminated and metabolized as fuel by the body, or used in the synthesis of new proteins (Gannes et al. 1998; Oelbermann and Scheu 2002). The recycled amine groups are transaminated to synthesize new amino acids using carbon skeletons derived from dietary carbohydrates and lipids (Webb et al. 1998; Gaye-Siessegger et al. 2004a; Savidge and Blair 2004). The amines liberated from body tissue and incorporated into new proteins are already ^{15}N-enriched relative to those from the diet (Doucett et al. 1999). As a result, the $\delta^{15}N$ value of the newly synthesized proteins increases, while the lean body mass decreases (Hobson et al. 1993; Kelly 2000). Meanwhile, ^{14}N loss through metabolic waste products continues during starvation (Gannes et al. 1997; Adams and Sterner 2000). As the demand for nitrogen increases, protein turnover escalates, resulting in greater ^{15}N enrichment in the new proteins (Gaye-Siessegger et al. 2004b).

Because the $\delta^{15}N$ values for proteins synthesized under nutritional stress are higher than those formed under satiated conditions, the physical condition of an animal should be detectable through increases in $\delta^{15}N$. Hobson et al. (1993) were the first to test

this idea of ^{15}N-enrichment and they demonstrated that nutritionally stressed animals have an increase in $\delta^{15}N$ relative to animals fed *ad libitum*. In their experiment, juvenile Japanese Quail were provided with enough food to maintain but not enlarge their mass, while the birds fed *ad libitum* tripled in mass. After two weeks, the food-stressed birds showed enriched ^{15}N in muscle, liver, blood, and bone. Similarly, fasting Ross's geese showed enriched ^{15}N in muscle and liver with concomitant decreases in liver and muscle mass.

Most of the theory to date has focused on the enrichment of ^{15}N, with little or no attention paid to ^{13}C. Since heavy isotopes preferentially remain in a bonded state and accumulate in the body, nutritional stress should show enrichment in both ^{15}N and ^{13}C (Hatch et al. 1995; Haramis et al. 2001). Enrichment in ^{13}C has been shown only in food-stressed spiders (Oelbermann and Scheu 2002) and in the blood proteins in food-stressed chicks and chickens (Hatch et al. 1995).

All work done in this thesis was conducted on juvenile Song Sparrows (*Melospiza melodia*). Nestlings were collected from the wild between 2 and 7 days post hatch, and hand-reared in captivity under identical conditions except for feeding regime; half of the nestlings were fed *ad libitum*, the other half food restricted to mimic food stress that wild nestlings might experience (Searcy et al. 2004). The nestlings were euthanized between 23 and 26 days post hatch. In Chapter 2, we examined the potential of stable isotope analysis as a measure of food stress and body condition in passerines. Hobson et al.'s 1993 study has been cited widely, and their hypothesis that ^{15}N-enrichment is the result of increased protein catabolism has been applied to a wide variety of bird species. That hypothesis, however, has not been further tested except for one related report that

examined the $\delta^{13}C$ of a blood protein in food-stressed domestic chickens (Hatch et al. 1995). This is the first study to test the simultaneous enrichment of ^{15}N and ^{13}C in a passerine species. The results generated from nitrogen and carbon stable isotope analyses of blood, feather, liver, and muscle were then compared to those generated using more conventional measures of condition: morphometrics, fluctuating asymmetry, feather fault bars, and blood analyses.

In Chapter 3, we determined the nitrogen and carbon tissue-diet discrimination factors for blood, feather, liver, muscle, and excreta in the juvenile Song Sparrows and compared them with those reported for other avian species in the literature. It was previously assumed that isotopic changes from diet to tissue were similar across all species (Gannes et al. 1997). It is now clear, however, that these tissue-diet discrimination factors are not only species-specific but tissue-specific as well (Hobson and Bairlein 2003). In passerines, tissue-diet discrimination factors are known only for blood and feathers in two warbler species. We also examined the effects of solvent lipid extraction techniques on $\delta^{15}N$ and $\delta^{13}C$. Lipid extraction is expected to increase $\delta^{13}C$ by removing ^{13}C-depleted lipids, but its effects on $\delta^{15}N$ remain unknown (Gannes et al. 1997; Kelly 2000).

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Chapter 2: Stable isotopes do not reflect food stress in juvenile Song Sparrows

2.1 Introduction

Food availability during the early stages of growth can have life-long consequences in birds (Metcalfe and Monaghan 2001). The condition of an individual as a juvenile may carry-over into adulthood to affect fitness (Ohlsson and Smith 2001; Gil et al. 2004). Experimental studies have shown that offspring reared under nutritional stress mature into adults that are less successful at attracting a mate (Nowicki et al. 2002), have impaired brain function, learning, and memory retention (Kitaysky et al. 2003), and have difficulties obtaining and maintaining breeding territories (McCarty 2001). Because the growth and survival of young offspring can affect future success, the condition of young birds may be used as a predictor of a population's prospect for future growth or stability (Podlesak and Blem 2001).

In passerines, 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; McCarty 2001). The quality and quantity of food provided by the parents ultimately determines the nestlings' growth and survival (Lepczyk et al. 1998; Lepczyk and Karasov 2000). While some avian species can significantly adjust their developmental patterns to their nutritional status, this is not a characteristic displayed by many altricial nestlings. Altricial nestlings under food stress continue to grow rapidly and thus experience the full impact of nutritional stress (Konarzewski et al. 1996; Konarzewski and Starck 2000). Poor

nutrition in nestlings can cause increased mortality, lower body weights, abnormal growth, and permanent stunting (Nowicki et al. 1998; Konarzewski and Starck 2000; Saino et al. 2003; Jovani and Blas 2004). Because food demands are so great during the nestling period and little can be done to mitigate the effects of food shortage (i.e. nestlings cannot significantly lower their growth or metabolic rates), nestlings are often hungry and starvation of some or all of the nestlings within a nest is common (Nowicki et al. 1998; Hovorka and Robertson 2000; Nowicki et al. 2002).

Because offspring condition is affected by nutrition during growth, there is much interest in measuring condition. Determining body condition in passerine nestlings is complex because each of the many methods currently used examines only one specific physical aspect. Common measures include (1) morphometrics, where poor nutrition can produce permanent morphological effects in both nestlings and adults such as lower mass (Hovorka and Robertson 2000); (2) fluctuating asymmetry (FA) or random deviations from bilateral symmetry, where malnourished individuals have poorer control of developmental precision, compromising the maintenance of developmental stability (Lens et al. 2002); (3) feather fault bars, areas of absent or abnormal barbs on the feather, where stress causes a lag in keratin deposition during feather formation (Bortolotti et al. 2002; Jovani and Blas 2004; Jovani and Tella 2004); and (4) blood indicators including corticosterone, hematocrit, and glucose. Corticosterone, the primary stress hormone in birds, rises in response to poor body condition (Heath and Duffy 1998). Hematocrit (packed red blood cell volume) reflects nutritional state, with a lower hematocrit indicating poor condition and anaemia (Ben-David et al. 1999; Dubiec and Cichoñ 2001),

and food stress causes an increase in endogenous glucose production (Kubíková et al. 2001; Eeva et al. 2003).

A more recently developed indicator of nutritional stress is stable isotope composition, measured through an increase in either $\delta^{15}N$ or $\delta^{13}C$ in the body tissues (Hobson et al. 1993; Hatch et al. 1995; Oelbermann and Scheu 2002). This enrichment arises, in theory, because during nutritional stress the body preferentially retains the heavy isotopes liberated during protein catabolism. Very little, however, is known about the isotopic response to food stress in birds. It has been rigorously examined only in juvenile captive Japanese Quail and incubating Ross' Geese (Hobson et al. 1993) and has never been studied in passerine birds. While theory would predict that stable isotope analysis has tremendous potential as a measure of condition, more empirical studies are needed before it can be applied with confidence to wild birds.

In this study, we examine the potential of stable isotope analysis as a measure of food stress and body condition in passerine juveniles. Song Sparrow (*Melospiza melodia*) nestlings were collected from the wild, and hand-reared in captivity under identical conditions except for feeding regime; some nestlings were fed *ad libitum*, others were food-restricted to mimic feeding stress that wild nestlings might experience (Searcy et al. 2004). The results generated from stable isotope analysis were then compared to those generated using more conventional measures of condition. We expected that birds experiencing nutritional stress would be in poor physical condition, show symptoms of developmental stress including increased fluctuating asymmetry and expression of fault bars, be more anemic with higher corticosterone and glucose concentrations, and show increases in both $\delta^{15}N$ and $\delta^{13}C$ values.

2.2 Methods and Materials

2.2.1 Collection and Housing

Nestling Song Sparrows were collected from London, Ontario, Canada, during the spring and summer of 2004. Nests were located using adult behavioural cues and they were monitored every 3 to 4 days. We removed the nestlings from the nest between 2 and 7 days post-hatch. We removed 83% of 54 nestlings between the ages of 2 and 5 days post-hatch, with the remainder brought in 6-7 d post-hatch. Nestlings were aged based on our observations of hatch day and feather tract development.

A total of 15 broods of 54 young were collected. Each brood was transferred from its nest into an artificial nest (a plastic bowl lined with paper towels). To identify individuals, we applied non-toxic marker in unique combinations to the legs of nestlings under 6 d post-hatch, and switched to plastic colour leg bands thereafter. We randomly assigned each brood to one of our two treatments ("*ad libitum*" or "food-stressed", see below). In total, there were 8 broods of 28 young (14 male and 14 female) in the *ad libitum* group, and 7 broods of 26 young (11 male and 15 female) in the food-stressed group. Sexing was done by direct gonad observation during dissection.

Broods were raised in artificial nests until fledging (the age when they left the nest and began moving around freely), at which time each brood was housed in a 35 cm x 25 cm x 30 cm cage containing a source of drinking water. At all stages, all birds were maintained in the same room under identical conditions including a photoperiod of 14 light:10 dark, and a temperature of 26°C at the nestling phase and 21°C after fledging.

2.2.2 Treatments

From the time of collection until their euthanization (23-26 d post-hatch), we fed nestlings a standard hand-rearing diet of nestling mash (470 mL Mazuri® Small Bird Maintenance (see Appendix 1 for ingredients), 120 mL wheat germ, 30 g soy protein powder, 2 hard-boiled chicken eggs with shells removed, and 950 mL water, blended in a food processor). We mass-produced food five times throughout the experiment. The food in each batch was completely consumed before switching to the next batch. Each batch was stored frozen in small containers and thawed when needed, with samples of the food periodically collected and frozen for stable isotope analysis. We fed the nestlings using 1cc Bectin-Dickinson syringes, which allowed the amount fed to be controlled and measured to the nearest 0.1 mL. All nestlings were fed from 7:00 am until 9:00 pm. Nestlings between the ages of 2 and 13 d were fed every 30 minutes, while those 14 d and older were fed every 45 minutes. The *ad libitum* birds were fed to satiation at every feeding. We calculated the average amount fed to the *ad libitum* birds for each day post-hatch, and fed the birds in the food-stressed treatment 65% of this amount. We chose this level of food restriction to mimic food stress occurring in the wild without inducing starvation (Searcy et al. 2004). The survival rate was excellent for the birds. Three nestlings (one at two days post-hatch, and two at four days post-hatch) died within 18 hours of being brought in from the field. There were no other fatalities. The birds were euthanized between the ages of 23 d to 26 d, via decapitation with surgical scissors after anaesthetisation with isofluorine. The birds were then frozen.

2.2.3 Morphological Measurements

Morphological measurements were carried out on the birds' including mass, tarsus and feather length, fat deposits, breast muscle mass, and keel length. The birds were weighed to the nearest 0.25 g using a spring scale every day. Every second day, we measured the right tarsometatarsus (hereafter "tarsus") from the notch at the back of the tibia-tarsus joint to the distal point obtained by bending the toes 90° to the tarsus (Anciães and Marini 2000) using calipers accurate to 0.1 mm. Once the nestlings reached 10 d we measured the length of the right seventh primary feather using a flexible plastic ruler accurate to 0.1 mm. On the day the nestlings fledged, we measured the right and left tarsi and right and left seventh primary feathers. Measurements were taken daily between 3:00 pm and 6:00 pm, 15 min following a feeding. We pulled the left and right seventh primary feathers post mortum and measured them to the nearest 0.5 mm. Only feathers with the complete tip intact were included (39 or 72%). The right and left legs were completely removed from the birds and the length of the tarsi measured.

We scored the amount of subcutaneous fat in the furcular deposit in the nestlings after their death using an qualitative scale ranging from zero to five: zero for a nestling with no fat in the deposit, one for streaks of fat, two for the cavity completely lined with fat, three for the cavity filled with fat flush to the rest of the chest, four for fat beyond the furcular cavity, and five for a gross bulging fat deposit (Wingfield et al. 2003).

The left breast muscle was carefully and fully removed, and then weighed and lyophilized (hereafter called freeze-dried) and weighed again. The keel, now completely exposed, was measured using calipers.

2.2.4 Stable Isotope Analyses

Three samples from each of the five different batches of diet were randomly selected. The diet samples were freeze-dried and ground to a fine powder using a Crescent Wig-L-Bug. In order to extract the lipids, we combined each sample with 10 mL of 20:10:8 methanol:chloroform:water solution and filtered it through a fritted glass filter three times, rinsed it with 20 mL of methanol, followed by air drying, and weighing into tin capsules (C: 0.49 mg, N: 1.50 mg). In all cases, we analysed each sample in duplicate.

The first outermost left tail feather was pulled after death and sealed in a dry plastic bag until stable isotope analysis (following Smith et al. 2003). To clean the feathers of organic contaminants, we soaked them for 24 h in a 50:50 methanol:chloroform solution, then rinsed them twice in the same solution prior to air-drying. We cut the feathers (barbs and rachis) into small (~1 mm) sections using stainless steel scissors, and weighed them (0.50 mg).

Two heparinized capillary tubes with whole blood from the severed jugular vein were collected, as were the liver and right breast muscle. After collection, the samples were frozen for stable isotope analysis. The frozen whole blood, liver, and muscle were freeze-dried and ground to a fine powder. We extracted the lipids from half of each sample of liver and muscle. We performed the lipid extraction by rinsing the sample in a 50:50 methanol:chloroform solution three times and air-drying. We did not extract lipids from the blood because the lipid content in blood is very small (Bearhop et al. 2000). We then weighed the samples (0.50 mg).

We performed the stable isotope analyses in the Laboratory for Stable Isotope Science at *the* University *of* Western Ontario. Nitrogen and carbon stable isotope ratios

were determined on N_2 and CO_2 gases produced by combusting the samples in an elemental analyzer coupled to a continuous flow mass spectrometer (Thermo Finnigan Delta^{plus} XL ® Mass Spectrometer, Costech ® Elemental Analyser). The stable isotope composition was expressed as the normalized ratio of the sample divided by the standard in parts per thousand according to the following formula:

$$\delta X = [(R_{sample}/R_{standard})-1] \times 1000 \qquad \text{[Eq'n 2.1]}$$

where X is the ratio of ^{15}N:^{14}N or ^{13}C:^{12}C, the international standard for carbon is Vienna Peedee Belemnite (VPDM), the standard for nitrogen is atmospheric nitrogen, and R_{sample} and $R_{standard}$ are the ratios of heavy and light isotopes in the sample and standard, respectively (Gannes et al. 1998). The data were normalized to international standards (VPDB, Air) using laboratory standards – ANU Sucrose, NBS 22, IAEA N1 and IAEA N2 inserted at intervals throughout the run. The accuracy of the measurements (determined using nicotinamide) was within 0.02‰ for both carbon and nitrogen; the measurement precision for nitrogen was ± 0.08‰ and for carbon was ± 0.07‰. The measurement precisions for the unknown tissue samples were: blood (N ± 0.02‰, C ± 0.02‰), feather (N ± 0.07‰, C ± 0.07‰), liver with lipids (N ± 0.02‰, C ± 0.02‰), liver without lipids (N ± 0.02‰, C ± 0.01‰), muscle with lipids (N ± 0.03‰, C ± 0.01‰), and muscle without lipids (N ± 0.03‰, C ± 0.02‰).

Because each batch of diet contained different eggs, we calculated the discrimination between the tissue and diet for both carbon and nitrogen according to the following formula:

$$\Delta X_{tissue\text{-}diet} = \delta X_{tissue} - \delta X_{diet} \qquad \text{[Eq'n 2.2]}$$

which made the birds fed different diets directly comparable, despite having slightly different isotopic signatures. The diet, on average, had a $\delta^{15}N$ value of 2.98 ± 0.16 ‰, and a $\delta^{13}C$ value of –17.41 ± 0.22 ‰.

2.2.5 Blood Analyses

Immediately before a bird was euthanized, we drew blood from the ulnar vein into one heparinized capillary tube; the blood was then used to measure the concentration of glucose (AccuSoft Advantage ® blood glucose monitor). After decapitation, blood was collected into a capillary tube in order to measure the hematocrit. All blood was collected within three minutes of death. The blood was spun down in a refrigerated centrifuge at 2000 rpm for 10 min, within 12 h. We calculated the hematocrit as the height of the packed blood red blood cells divided by the total height of the blood column; we then extracted and froze the plasma for the measurement of corticosterone. Between and 5 and 21 μL of plasma were extracted for corticosterone measurement via radioimmunoassays following extraction in dichloromethane in the laboratory of Dr. John Wingfield at the University of Washington (Wingfield et al. 1992).

2.2.6 Fluctuating Asymmetry

Fluctuating Asymmetry analyses were done on the tarsi and the seventh primary feathers. The right and left legs were removed from the birds, and the tarsi bones separated and defleshed. Of the 54 birds, 13 (24%) had at least one leg damaged while frozen and were not included in the analyses. The length of the tarsus was measured five times by the same person with the three median measurements recorded. We measured the total

length of the seventh primary feathers twice using a plastic ruler. Only complete feathers with undamaged tips were included. All measurements were done blind to the birds' identity.

FA was calculated as the difference of the unsigned length (R-L). Following Palmer and Strobeck (2003), we calculated the significance of the between sides variation relative to measurement error using repeatability measure $[ME5]_{rA}$.

$$[ME5]_{rA} = \frac{MS_{interaction} - MS_{error}}{MS_{interaction} + (df_{interaction} \times MS_{error})} \qquad \text{[Eq'n 2.3]}$$

For the tarsi, FA was significantly larger than the measurement error ($p < 0.001$, $R^2 = 0.999$) with a repeatability of 0.840. The tarsi did not show directional asymmetry (two-tailed pairwise t-test: $t_{40} = 1.81$, $p = 0.078$) or antisymmetry as the distribution was unimodal and normally distributed (Wilk's Shapiro test: $W = 0.97$, $p = 0.391$). FA in the seventh primary feathers was significantly larger than the measurement error ($p < 0.001$, $R^2 = 0.999$) with repeatability of 0.847. The length of the feathers, even after being transformed, still displayed antisymmetry using Shapiro Wilk's test ($W = 0.93$, $p = 0.027$) but not when using Kolmogorov-Smirnov ($d = 0.14$, $p > 0.2$). The feathers showed no directional asymmetry (two-tailed pairwise t-test: $t_{37} = 0.72$, $p = 0.476$) (following Eeva et al. 2003).

2.2.7 Fault Bars

Fault bar analyses were carried out on the second and third outer left tail feathers and the sixth and seventh right and left primary wing feathers. Each feather was pulled from the bird after death, and analysed under a dissecting microscope to count the number of fault

bars, which appear as translucent bands across the feather. We then measured the length of the feather vane, and calculated the average number of fault bars per centimetre of vane. All measuring and counting were done blind to the birds' identity.

2.2.8 Statistical Analyses

We compared the effect of nutritional stress between the *ad libitum* and food-stressed birds using mixed-model nested ANOVAs with treatment and sex as the independent variables and nestling brood as the nested random variable (STATISTICA 6.0, StatSoft Inc., 2001). The data were checked for normality and homogeneity of variances and were square root transformed when necessary. In all cases, the tests were two-tailed and α was set at 0.05. All means are presented ± SEM.

The growth curves for body mass were calculated using the inverse equation (y = -x/age + constant). The logistic equation was not used because the masses for the first three days of life were not available. We calculated a condition index (a ratio of mass to morphological traits, Hochachka and Smith 1991) as mass/tarsus length^3 (following Saino et al. 2003). The distribution of hematocrit had three outliers (greater that two standard deviations away from the treatment mean) that were removed from the data set (two *ad libitum* females and one food-stressed female). Glucose concentrations were analyzed using a 2-way ANCOVA with body mass a covariate because glucose concentrations vary with body mass, with larger body mass being correlated with higher glucose levels (Ben-David et al. 1999; Christensen et al. 2000). The order in which the nestlings were bled was included as a covariate in the analysis of corticosterone.

Stable isotope analyses were conducted on the two largest nestlings from each brood. Nestlings in each brood varied in size and by selecting the two largest, we avoided adding extra variation caused by differences in body condition. The statistical analyses for $\Delta^{15}N$ of liver and muscle were performed on samples that had not been lipid-extracted, while analyses of $\Delta^{13}C$ in liver and muscle were done on samples that had been lipid-extracted (see Chapter 3). In the analysis of the effect of nutritional stress on $\Delta^{13}C$ values, hatch date was included as a covariate for the analyses of blood, feather, and muscle.

2.2.9 Ethical Considerations

The University of Western Ontario Animal Care Committee approved all methods in this experiment, which followed the principles and guidelines of the Canadian Council of Animal Care (protocol number 2004-037-04).

2.3 Results

2.3.1 Growth During the Experiment

The growth and development of the sparrow nestlings was significantly affected by the amount of food provided. Nestling growth was rapid and continuous until the age of fledging (10 ± 0.1 d) when it slowed until the end of the experiment. Birds fed *ad libitum* fledged one day earlier than the birds under food stress ($F_{1,37} = 47.29$, $p < 0.001$; *ad libitum* 9.6 ± 0.1 d, N = 28; food-stressed 10.5 ± 0.1 d, N = 26). Despite fledging earlier, birds fed *ad libitum* still weighed 15% more than the food-stressed birds on fledging day (17.2 g ± 0.3, N = 28 and 15.0 g ± 0.3, N = 26 respectively) ($F_{1,37} = 44.43$, $p < 0.001$).

While there was no effect of sex on fledging mass ($p > 0.10$), there was an interaction, where the mass of males was more affected by food-restriction than was the mass of the females ($F_{1,37} = 5.80$, $p = 0.021$). For the duration of the experiment the food-stressed birds weighed approximately 85% of birds fed *ad libitum* (Figure 2.1). The growth rate of the birds (measured as the absolute value of the slope of the inverse equation) was greater in birds fed *ad libitum* than the food-stressed birds ($F_{1,37} = 46.06$, $p < 0.001$) (Table 2.1), with an effect of sex where males had greater growth rates than females ($F_{1,37} = 4.15$, $p = 0.049$), and no interaction between food stress and sex ($p > 0.10$). The birds fed *ad libitum* had a predicted final mass 15% greater than that of the food-restricted birds ($F_{1,37} = 100.70$, $p < 0.001$). Males had a larger predicted final mass than females ($F_{1,37} = 10.13$, $p = 0.003$) (Table 2.1), and a significant interaction where the asymptotic mass of males was more affected by food restriction than the final predicted mass of females ($F_{1,37} = 7.04$, $p = 0.012$).

2.3.2 Physical Size and Condition

Our morphological measures indicated that birds given *ad libitum* food were in better condition than were those under food-restriction at 23 d post-hatch. Body mass, condition index, fat score, seventh primary length, keel length, and wet and dry breast muscle mass were all significantly larger in the birds fed *ad libitum* than in the food-stressed birds ($p < 0.05$), with treatment having no effect on tarsus length ($p > 0.10$, Table 2.2). Body mass, primary length, and keel length were significantly larger in males than females ($p < 0.05$), while condition index, fat score, tarsus length and muscle mass showed no relationship with sex ($p > 0.10$). Body mass, primary length, keel length, and

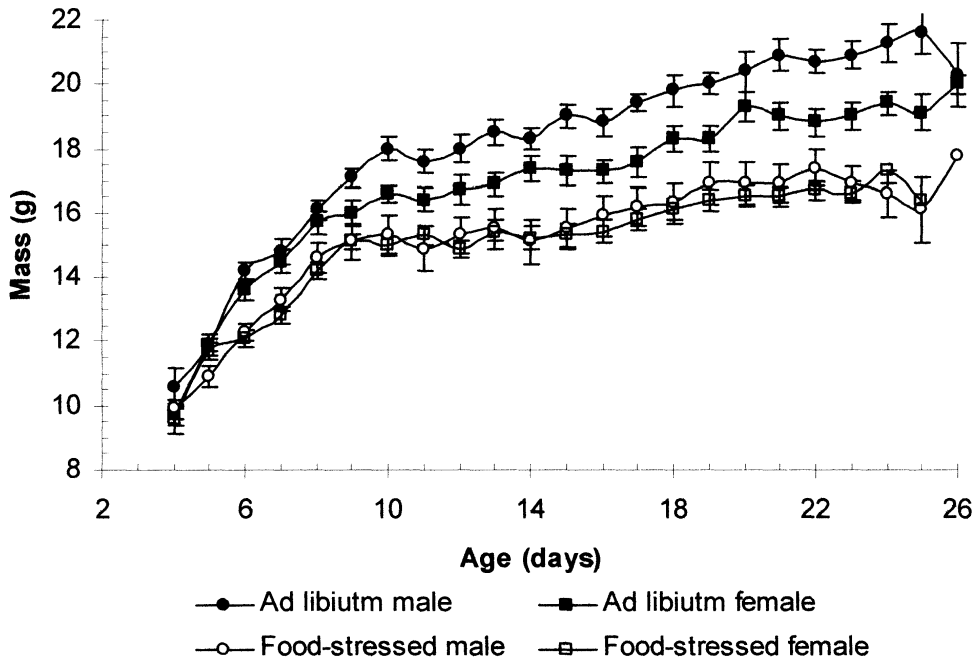

Figure 2.1. Increase in body mass during the course of the experiment.

Table 2.1. Parameters from the inverse equation. Predicted final mass was the asymptotic weight and the growth rate represents the slope of the equation.

	Predicted Final Mass (g)	Growth Rate
Ad libitum male N = 14	22.7 ± 0.4	51.6 ± 2.0
Ad libitum female N = 14	20.7 ± 0.5	44.1 ± 2.9
Stressed male N = 11	18.2 ± 0.7	32.5 ± 3.7
Stressed female N = 15	17.8 ± 0.4	29.9 ± 3.1

Table 2.2. Morphological measurements at the end of the treatment.

Trait	*Ad libitum* male	*Ad libitum* female	Stressed male	Stressed female	Treatment effect	Sex effect	Interaction
Mass (g)	20.6 ± 0.4 N = 14	18.8 ± 0.4 N = 14	16.9 ± 0.5 N = 11	16.2 ± 0.3 N = 15	$F_{1,37} = 78.51$ $p < 0.001$	$F_{1,37} = 10.28$ $p = 0.003$	$F_{1,37} = 5.423$ $p = 0.025$
Condition Index	2.06 ± 0.06 N = 14	2.01 ± 0.06 N = 14	1.73 ± 0.06 N = 11	1.68 ± 0.04 N = 15	$F_{1,37} = 38.63$ $p < 0.001$	$F_{1,37} = 0.39$ $p = 0.536$	$F_{1,37} = 0.41$ $p = 0.526$
Fat Score	1.93 ± 0.22 N = 14	2.11 ± 0.22 N = 14	0.95 ± 0.14 N = 11	1.17 ± 0.12 N = 15	$F_{1,37} = 29.61$ $p < 0.001$	$F_{1,37} = 1.83$ $p = 0.184$	$F_{1,37} = 0.04$ $p = 0.842$
Tarsus (mm)	21.6 ± 0.2 N = 14	21.1 ± 0.2 N = 14	21.3 ± 0.3 N = 11	21.3 ± 0.2 N = 15	$F_{1,37} = 1.01$ $p = 0.321$	$F_{1,37} = 3.14$ $p = 0.084$	$F_{1,37} = 1.34$ $p = 0.254$
Primary (mm)	55.8 ± 0.5 N = 12	52.8 ± 0.5 N = 14	53.5 ± 1.2 N = 11	52.7 ± 0.6 N = 14	$F_{1,34} = 4.84$ $p = 0.035$	$F_{1,34} = 9.70$ $p = 0.004$	$F_{1,34} = 6.00$ $p = 0.020$
Keel (mm)	16.7 ± 0.2 N = 10	15.0 ± 0.1 N = 9	14.8 ± 0.7 N = 6	14.3 ± 0.2 N = 13	$F_{1,24} = 25.2$ $p < 0.001$	$F_{1,24} = 23.53$ $p < 0.001$	$F_{1,24} = 6.54$ $p = 0.017$
Wet Muscle (g)	1.15 ± 0.05 N = 10	0.98 ± 0.05 N = 9	0.74 ± 0.14 N = 6	0.81 ± 0.03 N = 13	$F_{1,24} = 41.03$ $p < 0.001$	$F_{1,24} = 1.96$ $p = 0.174$	$F_{1,24} = 6.54$ $p = 0.017$
Dry Muscle (g)	0.32 ± 0.01 N = 10	0.27 ± 0.01 N = 9	0.21 ± 0.04 N = 6	0.23 ± 0.01 N = 13	$F_{1,24} = 40.04$ $p < 0.001$	$F_{1,24} = 0.23$ $p = 0.636$	$F_{1,24} = 6.63$ $p = 0.017$

muscle mass all had significant interactions between food stress and sex ($p < 0.05$). In all cases, males were larger than females but only when given ad libitum food. Under food-restriction, males and females were of comparable size. Thus males suffered more than females when food was in short supply. There was no interaction for condition index, fat score, or tarsus length ($p > 0.10$).

2.3.3 Stable Isotope Analyses

The $\Delta^{13}C$ values were more responsive to nutritional stress than the $\Delta^{15}N$ values. Only muscle $\Delta^{15}N$ was affected by food stress, but in a direction opposite to what was expected; food-stressed birds had less positive $\Delta^{15}N$ values than the birds fed *ad libitum* ($F_{1,11} = 7.60$, $p = 0.019$, Table 2.3). The $\Delta^{15}N$ values of blood, feathers, and liver did not vary with food treatment ($p > 0.10$, Table 2.3), and there was no effect of sex nor an interaction between food stress and sex ($p > 0.10$, Table 2.3). While the $\Delta^{15}N$ values of the muscle may have been statistically different between the two treatment groups, this difference likely has little biological significance. The effect (though statistically significant) was only 0.12 ‰ while the precision of the measurement is ± 0.08 ‰.

The food-restricted birds had significantly more negative and less positive $\Delta^{13}C$ values than the birds fed *ad libitum* (Table 2.4); blood was enriched 0.25 ‰, feather was enriched 0.16 ‰, muscle was enriched 0.18 ‰, and liver was enriched 0.14 ‰. Only blood showed a significant effect of sex where the males had less negative $\Delta^{13}C_{blood\text{-}diet}$ values than females (Table 2.4). There was also a significant interaction between sex and stress for blood, with the change in $\Delta^{13}C_{blood\text{-}diet}$ values being larger for males than females when reared under food stress (Table 2.4). The raw δ values are in Appendix 2.

2.3.4 Blood Analyses

The blood analyses of condition were affected by the nutritional state of the bird. Food-stressed birds had significantly higher concentrations of corticosterone than did birds fed *ad libitum* (28.7 ± 4.3 ng/mL and 15.2 ± 2.1 ng/mL) (2-way ANCOVA, treatment: $F_{1,25}$ = 6.93, p = 0.014; covariate (bleeding order): $F_{1,25}$ = 16.17, p < 0.001). The concentrations of glucose were significantly greater in the food-stressed birds (24.4 mmol/L ± 0.87, N = 26) than in the birds fed *ad libitum* (20.7 mmol/L ± 0.95, N = 23) (2-way ANCOVA, treatment: $F_{1,32}$ = 6.39, p = 0.016; covariate (mass): $F_{1,32}$ = 5.43, p = 0.026). There were no significant effects of sex nor an interaction between stress and sex (p > 0.10). Hematocrit was significantly greater in the birds fed *ad libitum* (0.63 ± 0.01, N = 20) than in the food-stressed birds (0.59 ± 0.01, N = 24) ($F_{1,28}$ = 4.38, p = 0.046), with no effect of sex or an interaction between stress and sex (p > 0.10).

2.3.5 Indicators of Developmental Stress

Developmental stress, measured through FA and fault bars, was unaffected by our imposed nutritional stress. Food-restricted birds did not have significantly greater FA than did the birds fed *ad libitum* in the tarsi (0.21 ± 0.04 N = 16 and 0.15 ± 0.02 N = 27, respectively) ($F_{1,26}$ = 1.41, p = 0.246) or in the seventh primary feathers (0.07 ± 0.01 N = 21 and 0.04 ± 0.02 N = 16, respectively) ($F_{1,21}$ = 2.53, p = 0.127), with no effects of sex nor an interaction between stress and sex (p > 0.10). When the degree of FA was compared between the tarsi and the feathers for each individual, the feathers were more asymmetric than were the bones (0.013 ± 0.003 and 0.007 ± 0.001 respectively) (paired t-test: t_{28} = 2.32, p = 0.028). Nutritional stress had no effect on the number of fault bars in

either the tail ($F_{1,37} = 0.04$, $p = 0.842$) or wing ($F_{1,37} = 1.27$, $p = 0.267$). The birds fed *ad libitum* had 0.80 ± 0.12 (N = 28) and 0.12 ± 0.03 (N = 28) fault bars/centimetre in the tail and wing, respectively, while the food-stressed birds had 0.85 ± 0.12 (N = 26) and 0.06 ± 0.03 (N = 26) fault bars/centimetre in the tail and wing, respectively. The number of fault bars in the tail and wings was not affected by sex nor by an interaction between sex and food stress ($p > 0.10$).

Table 2.3. $\Delta^{15}N_{(tissue\text{-}diet)}$ values for blood, feather, liver, and muscle.

Tissue	*Ad libitum* male	*Ad libitum* female	Stressed male	Stressed female
Blood	2.03 ± 0.08 N = 9	2.02 ± 0.08 N = 5	1.87± 0.08 N = 5	2.02 ± 0.10 N = 9
Feather	2.76 ± 0.08 N = 9	2.68 ± 0.13 N = 5	2.74 ± 0.09 N = 5	2.74 ± 0.09 N = 9
Liver	2.98 ± 0.05 N = 9	3.10 ± 0.08 N = 5	3.13 ± 0.08 N = 4	2.90 ± 0.05 N = 9
Muscle	2.23 ± 0.08 N = 9	2.13 ± 0.06 N = 5	2.14 ± 0.07 N = 4	2.04 ± 0.10 N = 9

Table 2.4. The effects of nutritional stress, sex, and their interaction on $\Delta^{13}C_{tissue\text{-}diet}$ values of blood, feather, muscle, and liver.

Trait	*Ad libitum* male	*Ad libitum* female	Stressed male	Stressed female	Treatment effect	Sex effect	Interaction
Blood	-1.32 ± 0.11 N = 9	-1.46 ± 0.10 N = 5	-1.64 ± 0.29 N = 5	-1.61 ± 0.14 N = 9	$F_{1,11} = 46.86$ $p < 0.001$	$F_{1,11} = 5.72$ $p = 0.036$	$F_{1,11} = 5.16$ $p = 0.044$
Feather	0.44 ± 0.09 N = 9	0.23 ± 0.09 N = 5	0.14 ± 0.26 N = 5	0.23 ± 0.11 N = 9	$F_{1,11} = 9.59$ $p = 0.010$	$F_{1,11} = 0.87$ $p = 0.371$	$F_{1,11} = 0.30$ $p = 0.595$
Liver	-0.52 ± 0.10 N = 9	-0.46 ± 0.10 N = 5	-0.72 ± 0.21 N = 4	-0.66 ± 0.10 N = 9	$F_{1,11} = 8.16$ $p = 0.016$	$F_{1,11} = 0.50$ $p = 0.494$	$F_{1,11} = 0.10$ $p = 0.758$
Muscle	-0.79 ± 0.08 N = 9	-0.92 ± 0.05 N = 5	-0.96 ± 0.29 N = 4	-0.99 ± 0.11 N = 9	$F_{1,10} = 7.96$ $p = 0.018$	$F_{1,10} = 0.03$ $p = 0.866$	$F_{1,10} = 1.69$ $p = 0.223$

2.4 Discussion

The degree of food stress experienced by the Song Sparrows in this study was sufficient to significantly alter their growth and development without increasing morbidity or mortality through starvation. The food-stressed birds had slight developmental delays and fledged one day later than did the nestlings fed *ad libitum* (10.5 and 9.6 d, respectively) and consistently weighed less than did the food-stressed birds (Figure 2.1). The imposed nutritional stress was severe enough to result in birds that were smaller and in poorer condition, with smaller fat deposits, shorter seventh primary feathers, shorter keel lengths, and less pectoral muscle mass. Blood analyses also indicated effects of nutritional stress through increased corticosterone, anemia and glucose levels. Despite these features, we could not detect nutritional stress using stable nitrogen or carbon isotope analysis and the birds did not display any increased developmental instability.

Hobson et al.'s (1993) experiment concerning the effects of nutritional stress on stable isotope composition has been cited over one hundred times. The hypothesis that ^{15}N-enrichment is the result of nutritional stress and increased protein catabolism has been applied to a wide variety of organisms, from stream invertebrates (Jardine et al. 2005), to shrews (Baugh et al. 2004), to fish (Gaye-Siessegger et al. 2004b). In some of these studies, unexpected increases in $\delta^{15}N$ values have been attributed to a decrease in body condition with a concomitant increase in protein catabolism, even though body condition was not evaluated (see Ben-David et al. 1997; Kidd et al. 1999; Kurle and Worthy 2001). In other cases, animals that should have been experiencing increased protein catabolism because of the nutritional stress arising from reproduction, migration, or rapid growth, have not shown enrichment in ^{15}N (see Pfeiler et al. 1998; Hobson and

Schell 1998; Gloutney et al. 1999; Schmidt et al. 1999; Gorokhova and Hansson 1999; Doucett et al. 1999; Ben-David et al. 1999). These authors offer various explanations for why ^{15}N-enrichment was not found. Our results suggest that the ^{15}N-enrichment observed by Hobson et al. (1993) may not be a general pattern applicable across all species (Gannes et al. 1997). Before we can conclude that variations in $\delta^{15}N$ values reflect differences in nutritional stress amongst individuals, more laboratory studies are needed on a variety of species, undergoing different types of nutritional stress and increased protein catabolism.

Our nutritionally stressed juvenile Song Sparrows did not show enrichment in ^{15}N (i.e., more positive $\Delta^{15}N$ values), unlike the juvenile quail (Hobson et al. 1993). This difference may have occurred because our level of nutritional stress was relatively moderate. While the food-stressed nestlings were smaller and had higher corticosterone levels than did those fed *ad libitum*, the birds did not show developmental instability, or increased mortality. By comparison, the nutritionally stressed Japanese Quail were raised under an extreme level of food stress, provided with just enough food to maintain a constant body mass, while birds fed *ad libitum* tripled in the size over the same period. The level of food stress chosen in our experiment was designed to mimic food stress that would occur in the wild, without causing morbidity and mortality. Altricial nestlings require a high volume of food while growing and would have starved if we had employed the same level of food stress as did Hobson et al. (1993). It is possible that extreme nutritional stress is required before ^{15}N enrichment occurs. Altricial young, in fact, may not be capable of reaching this level of nutritional stress for a long enough period to show dramatically increased protein catabolism and concomitant increases in $\delta^{15}N$. If this is

the case, then using $\delta^{15}N$ values in the wild as a measure of nutritional stress may not be useful for species that cannot survive for prolonged periods with little/no food.

The $\Delta^{13}C$ values obtained for the Song Sparrows were also inconsistent with the pattern predicted for nutritional stress. Instead of being enriched in ^{13}C (Hatch et al. 1995; Oelbermann and Scheu 2002), the nutritionally stressed animals were depleted of ^{13}C in all tissues examined relative to those fed *ad libitum*. One possible explanation is that animals grown on low protein diets may use carbon from non-protein components of the diet to synthesize new amino acid carbon backbones (Gannes et al. 1998). Since lipids and carbohydrates are typically more depleted in ^{13}C than are proteins (Peterson and Fry 1987), these new amino acids and proteins would have lower $\delta^{13}C$ values than those derived directly from the diet (which would be more plentiful in the birds fed *ad libitum*) (Webb et al. 1998; Gaye-Siessegger et al. 2004a; Savidge and Blair 2004). The unexpected ^{13}C enrichment may also be derived from the composition of the diet. The fate of the carbon isotopes under different levels of food intake may be more complicated than conventionally assumed. The diet of the birds (see Appendix 1) was composed of both C_3 (wheat) and C_4 (corn) plants, each of which has unique isotopic signatures. Bruckental et al. (1985) fed sheep known proportions of lucerne hay (C_3: -27.9‰) and maize grain (C_4: –10.5‰). Sheep fed at maintenance levels digested both dietary components at amounts equal to the weighed digestibility of each component. When the amount of food consumed was increased, a high level of maize grain in the diet reduced the digestibility of the lucerne hay. In the present experiment, it is possible that the stressed nestlings (equivalent to the maintenance sheep) digested both the C_3 and C_4 components of the diet. The birds fed *ad libitum* (equivalent to the sheep fed at high

levels) digested more of the C_4 component of the diet and, therefore, became more enriched in ^{13}C. More intensive studies are needed to determine the possible impacts of different types of food under different levels of nutrition.

The blood parameters were successful in identifying birds that were exposed to nutritional stress. During food stress, corticosterone levels become chronically high (Heath and Duffy 1998; Clinchy et al. 2004). Corticosterone, the primary stress hormone in birds, is associated with a host of physiological and behavioural effects that act to increase the body's energy supply (Heath and Duffy 1998; Salvante and Williams 2003; Love et al. 2003). Another function of corticosterone is an increase in production of glucose from non-carbohydrate sources (Marra and Holberton 1998). Under stress, glucose is produced from catabolized skeletal muscle, which further depletes the protein reserves (Sockman and Schwabl 2001; Holberton and Wingfield 2003; Salvante and Williams 2003). Hematocrit was lower in the food-stressed birds, indicating anaemia and poorer chances of survival (Dubiec and Cichoñ 2001). These indices are both simple to measure and can be used as a reliable indicator of condition.

Nutritional stress significantly affected all morphological features save one – tarsus length. Tarsus length is simple to measure and is one of the most common measurements done in the field to determine size and physical condition. That said, however, other experiments of nutritionally stressed passerine nestlings have also revealed a lack of effect on tarsus length (see Lepczyk and Karasov 2000; Shulter et al. 2004; Martins 2004). Interestingly, the other skeletal measurement, keel length, was significantly smaller when grown under food stress. Song Sparrow fledglings are not capable of extended flight for approximately a week after fledging and rely instead on

their legs to escape from predators. Animals faced with nutritional deficits may therefore preferentially maintain high growth rates in some skeletal features such as tarsus length (Wright et al. 2002), over other features such as keel length. As well, a smaller keel might indicate that the individual has a physically smaller core body. A smaller body would require fewer resources and less energy to maintain. Tarsus length, therefore, may be under genetic control, while the other morphological features are more sensitive and responsive to the current environment (Reynolds et al. 2003), and make better indicators of condition.

Individuals raised with *ad libitum* food allocate more resources to the mechanisms that maintain developmental symmetry (Yalcin et al. 2001; Aparicio and Bonal 2002; Greico 2003). Despite being physically smaller and in poorer condition, developmental instability, measured through fault bars and FA, was unaffected by the feeding regime. Nutritional stress had no effect on the number of fault bars in either the wing or tail feathers (also see Negro et al. 1995). Feather fault bars can result from a wide variety of stressors including nutrition, dehydration, illness, environmental conditions, and human handling (Bortolotti et al. 2002; Jovani and Tella 2004; Jovani and Blas 2004). Because the nestlings were hand-reared, it is possible that the high fault bar incidence on the feathers was the result of human contact.

We also found no evidence of fluctuating asymmetry in the tarsus or seventh primary feathers, something also reported in other studies (see Hovorka and Robertson 2000; Searcy et al. 2004). The feathers, which are more responsive to the environment than the tarsi bones, were more asymmetric than were the tarsi, but even here we found no effects. Our results suggest that nestlings, even under food stress, appear to allocate

resources preferentially to the mechanisms that maintain developmental stability and reduce the impact of developmental noise. The nestlings may have traded a larger physical size for developmental stability.

The growth and development of male and female nestlings were affected differently by the feeding regimes. Song Sparrows are sexually dimorphic, with males typically larger than females as nestlings and adults (Hochachka and Smith 1991). Males in this experiment had greater body mass, primary length, and keel length than did females. Body mass, primary length, keel length, and muscle mass all had significant interactions between food stress and sex. Males grew larger than females but only when given *ad libitum* food. Thus, under ideal conditions males grow larger than females, but under nutritional restrictions males and females will reach similar sizes. Life-history theory predicts that when conditions are harsh, nestlings of the larger sex are more vulnerable and should experience increased mortality because of their higher food requirements (Torres and Drummond 1997). This is consistent with our results since male Song Sparrows were in poorer condition than were females when food was restricted.

While stable nitrogen and carbon isotope analysis may have potential as a measure of condition, additional laboratory studies are needed to test the assumptions and limitations of this technique more fully. Stable nitrogen and carbon isotope ratios are the result of complex interactions among ecology, physiology, and biochemistry. Without a thorough understanding of how stable isotope compositions vary at each of these levels, our interpretations of these δ-values will never be accurate (Gannes et al. 1997). Our

study shows that $\delta^{15}N$ and $\delta^{13}C$ values do not provide a good indication of body condition in juvenile passerines exposed to moderate levels of food stress.

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Chapter 3: Diet-tissue fractionation and effects of lipid extraction in juvenile Song Sparrows

3.1 Introduction

Stable isotope analysis has developed into a useful tool for ecologists. This technique provides a new way to study some of the most complex areas of ecology such as migratory patterns and geographic origin (Marra et al. 1998), diet reconstruction (Hobson and Clark 1992a; Hobson 1995), trophic ecology (Adams and Sterner 2000), and nutritional status (Hobson et al. 1993, but see Chapter 2). Stable isotope analysis can address these areas because dietary items have unique carbon and nitrogen isotopic compositions, which become incorporated into body tissues (Hobson and Clark 1992a).

In their seminal paper, Gannes et al. (1997) made a call for more laboratory experiments that test the assumptions used in the interpretation of stable isotope studies. For example, it was previously assumed that isotopic changes from diet to tissue were similar across all species (Gannes et al. 1997). It is now clear, however, that these tissue-diet discrimination factors are not only species-specific but tissue-specific as well (Hobson and Bairlein 2003). Because tissue-diet discrimination factors are specific, previous studies that generalized across species or from one tissue to the next may need reinterpretation. Previous studies conducted on small passerines were commonly interpreted using isotopic parameters obtained from larger (e.g., American Crows, *Corvus brachyrhynchos*), and unrelated avian species (e.g., Japanese Quail, *Coturnix japonica* and Ring-billed Gulls, *Larus delawarensis*) (Hobson and Clark 1992a, 1992b). Recent studies on Garden Warblers (*Sylvia borin*) (Hobson and Bairlein 2003) and Yellow-

rumped Warblers (*Dendroica coronata*) (Pearson et al. 2003; Podlesak et al. 2005) indicate that such extrapolation is unreliable. Before using an ecological explanation to stable isotope ratios collected from wild animals, therefore, the species-specific tissue-diet discrimination factors must be known (Hobson and Clark 1992a). Tissue-diet discrimination factors must be determined under controlled conditions on animals given known amounts food that are isotopically homogenous. While this approach may be straightforward, such analysis has been performed for only a few species (Bearhop et al. 2002).

Another complication that arises when trying to generalize results from stable isotope measurements across studies and species is that the method of tissue preparation can vary. Results from stable isotope analyses can be compared only if the tissue were prepared using similar techniques. Sample preparation methods, however, typically differ between researchers and the types of organisms used (Sotiropoulos et al. 2004). For example, lipid extraction is a common preparation technique used in stable isotope ecology. Lipids are depleted of ^{13}C relative to carbohydrates and proteins (DeNiro and Epstein 1978). Tissues rich in lipids, such as the muscle and liver are more depleted of ^{13}C than were lipid-poor tissues such as blood, hair, and feathers. Thus, lipids are commonly removed in lipid-rich tissues to reduce the variation in $\delta^{13}C$ values caused by differences in lipid content (Sotiropoulos et al. 2004). Removing the lipids leaves the carbohydrates and proteins and allows for more specific information to be obtained on metabolic pathways and use of the dietary proteins and carbohydrates (Bearhop et al. 2000; Kelly 2000; Sotiropoulos et al. 2004).

The common techniques used to extract lipids were developed for carbon and nitrogen extraction methods done off-line. The gases (N_2 and CO_2) were produced separately, then analysed using a dual-inlet mass spectrometer. In modern continuous flow instruments, carbon and nitrogen are analysed concurrently from the same sample (Sotiropoulos et al. 2004). Lipid extraction should cause enrichment in ^{13}C because of the removal of lipids, but may also cause enrichment in ^{15}N because some proteins are also lost during lipid extraction (see Sotiropoulos et al. 2004). Controlled laboratory studies are required to assess such impacts before we can apply the isotopic results more broadly (Gannes et al. 1997; Kelly 2000).

Songbirds are studied extensively in the wild, and stable isotope analysis has become popular because of its many potential applications (for example see Rubenstein et al. 2002; Herrera et al. 2003; Norris et al. 2004; Hobson et al. 2004). Very few captive studies have been done, however, to calibrate how stable isotopes differentiate in these small and metabolically active birds (see Hobson and Bairlein 2003; Pearson et al. 2003; Podlesak et al. 2005). In this study, we raised nestling Song Sparrows (*Melospiza melodia*) from post-hatch until independence under controlled laboratory conditions. Between 23 to 26 days post-hatch, the nestlings were euthanized and their blood, feathers, liver, muscle, and excreta were collected. We determined the tissue-diet discrimination factors in these tissues ($\Delta^{15}N$ and $\Delta^{13}C$) and compared them with those reported in the literature for other avian species. We also examined the effects of lipid extraction on the stable nitrogen and carbon isotope compositions of liver and muscle.

3.2 Methods and Materials

3.2.1 Collection and Housing

This study was conducted on animals involved in an experiment examining the effects of nutritional stress on $\delta^{13}C$ and $\delta^{15}N$ values (see Chapter 2). Nestling Song Sparrows were collected from London, Ontario, Canada, during the spring and summer of 2004. Nests were located using adult behavioural cues and they were monitored every 3 to 4 days. We removed the nestlings from the nest between 2 and 7 d post-hatch. We removed 83% of 54 nestlings between the ages of 2 and 5 d post-hatch, with the remainder brought in 6-7 d post-hatch. Nestlings were aged based on our observations of hatch day and feather tract development.

A total of 15 broods of 54 young were collected. Each brood was transferred from its nest into an artificial nest (a plastic bowl lined with paper towels). To identify individuals, we applied non-toxic marker in unique combinations to the legs of nestlings under 6 d post-hatch, and switched to plastic colour leg bands thereafter. We randomly assigned each brood to one of our two treatments ("*ad libitum*" or "stressed", see below). In total, there were 8 broods of 28 young (14 male and 14 female) in the *ad libitum* group, and 7 broods of 26 young (11 male and 15 female) in the food-stressed group. Sexing was done by direct gonad observation during dissection.

Broods were raised in artificial nests until fledging, at which time each brood was housed in a 35 cm x 25 cm x 30 cm cage containing a source of drinking water. At all stages, all birds were maintained in the same room under identical conditions including a photoperiod of 14 light:10 dark and a temperature of 26°C at the nestling phase and 21°C after fledging.

3.2.2 Treatments

From the time of collection, until their euthanization, we fed the nestlings a standard hand-rearing diet of nestling mash (470 mL Mazuri® Small Bird Maintenance (see Appendix 1 for ingredients), 120 mL wheat germ, 30 g soy protein powder, 2 hard-boiled chicken eggs with shells removed, and 950 mL water, blended in a food processor). We mass-produced food five times throughout the experiment. The food in each batch was completely consumed before switching to the next batch. Each batch was stored frozen in small containers and thawed when needed, with samples of the food periodically collected and frozen for stable isotope analysis. We fed the nestlings using 1cc Bectin-Dickinson syringes, which allowed the amount fed to be controlled and measured to the nearest 0.1 mL. All nestlings were fed from 7:00 am until 9:00 pm. Nestlings between the ages of 2 and 13 d were fed every 30 minutes, while those 14 d and older were fed every 45 minutes. The *ad libitum* birds were fed to satiation at every feeding. We calculated the average amount fed to the *ad libitum* birds for each day post-hatch, and fed the birds in the food-stressed treatment 65% of this amount. We chose this level of food-restriction to mimic food stress occurring in the wild without inducing starvation (Searcy et al. 2004). Survival rate was excellent for the birds. Three nestlings (one at two days post-hatch, and two at four days post-hatch) died within 18 hours of being brought in from the field. There were no other fatalities. The birds were euthanized between the ages of 23 d to 26 d, via decapitation with surgical scissors after anaesthetisation with isofluorine. The birds were then frozen.

3.2.3 Stable Isotope Analyses

Three samples from each of the five different batches of diet were randomly selected. The diet samples were freeze-dried and ground to a fine powder using a Crescent Wig-L-Bug. To extract the lipids from the diet, we combined each sample with 10 mL of 20:10:8 methanol:chloroform:water solution and filtered it through a fritted glass filter three times, rinsed it with 20 mL of methanol, followed by air drying, and weighing into tin capsules (nitrogen 1.5 mg; carbon 0.49 mg). In all cases, we analysed each sample in duplicate.

We collected excreta from nestlings between the ages of 4 d and 8 d. Excreta were not collected per individual, but per brood, and preserved in 70% ethanol until stable isotope analyses were performed. Preservation in ethanol does not alter the carbon or nitrogen isotopic composition of the sample (Cherel et al. 2000). We rinsed and centrifuged the excreta in distilled water (following Forero et al. 2002) at 12 000 rpm for 12 minutes. The excreta were then freeze-dried, ground to a fine powder, and weighed (0.85 mg).

The first outermost left tail feather was pulled after death and sealed in a dry plastic bag to await stable isotope analysis (following Smith et al. 2003). To clean the feathers of organic contaminants, we soaked them for 24 h in a 50:50 methanol:chloroform solution, rinsed them twice in the same solution and then air-dried the sample. We cut the feathers (barbs and rachis) into small (~1 mm) sections using stainless steel scissors, and weighed them (0.5 mg).

Two heparinized capillary tubes with whole blood from the severed jugular vein were collected, as were the liver and right breast muscle. After collection, the samples

were frozen while awaiting stable isotope analysis. To prepare for analysis, the frozen whole blood, liver, and muscle were freeze-dried and ground to a fine powder. We extracted the lipids from half of each sample of liver and muscle. We performed the lipid extraction by rinsing the sample in a 50:50 methanol:chlorform solution three times and air-drying. We did not extract lipids from the blood because the lipid content in blood is very small (Bearhop et al. 2000). We then weighed the samples (0.5 mg).

The stable isotope analyses were performed in the Laboratory for Stable Isotope Science at *the* University *of* Western Ontario. Carbon and nitrogen stable isotope ratios were determined on CO_2 and N_2 gases produced by combusting the samples in an elemental analyzer coupled to a continuous flow mass spectrometer (Thermo Finnigan Delta^{plus} XL® Mass Spectrometer, Costech ® Elemental Analyser). The ratio of stable isotopes is expressed as the normalized ratio of the sample divided by the standard in parts per thousand according to the following formula:

$$\delta X = [(R_{sample}/R_{standard})-1] \times 1000 \qquad \text{[Eq'n 3.1]}$$

where X is the ratio of $^{15}N:^{14}N$ or $^{13}C:^{12}C$, the international standard for carbon is Vienna Peedee Belemnite (VPDB) and the standard for nitrogen is atmospheric nitrogen (AIR), and R_{sample} and $R_{standard}$ are the ratios of heavy and light isotopes in the sample and standard (Gannes et al. 1998). The data were calibrated to international standards using laboratory standards – ANU Sucrose, NBS 22, IAEA N1 and IAEA N2 inserted at intervals throughout the analysis. The accuracy of the measurements (determined using nicotinamide) was within 0.02‰ for both carbon and nitrogen; the measurement precision for nitrogen was ± 0.08‰ and for carbon was ± 0.07‰. The measurement precisions for the unknown tissue samples were: blood (N ± 0.02‰, C ± 0.02‰), feather

(N ± 0.07‰, C ± 0.07‰), liver with lipids (N ± 0.02‰, C ± 0.02‰), liver without lipids (N ± 0.02‰, C ± 0.01‰), muscle with lipids (N ± 0.03‰, C ± 0.01‰), muscle without lipids (N ± 0.03‰, C ± 0.02‰), and excreta (N ± 0.06‰, C ± 0.13‰). The raw data is found in Appendix 2.

Because each batch of diet contained different eggs, we calculated the discrimination between the tissue and diet for both carbon and nitrogen according to the following formula:

$$\Delta X_{tissue\text{-}diet} = \delta X_{tissue} - \delta X_{diet} \qquad \text{[Eq'n 3.2]}$$

which made the birds directly comparable, despite having slightly different dietary isotopic compositions. The diet, on average, had a $\delta^{15}N$ value of 2.98‰ ± 0.16, and a $\delta^{13}C$ value of –17.41‰ ± 0.22.

3.2.4 Statistical Analyses

Stable isotope analyses were conducted on the largest nestlings from each brood. The statistical analysis of the $\Delta^{15}N_{tissue\text{-}diet}$ values for the liver and muscle were performed on samples that had not been lipid-extracted. The comparable analyses for $\Delta^{13}C_{tissue\text{-}diet}$ were conducted on samples that had been lipid-extracted. We compared the magnitude of change on $\Delta^{15}N$ and $\Delta^{13}C$ values caused by lipid extraction between the two feeding levels using mixed-model nested ANOVAs with treatment and sex as the independent variables and brood to which nestlings belonged as the nested term. Nutritional stress did not significantly alter the magnitude of change in Δ-values caused by lipid extraction ($p > 0.10$), so we combined the two treatment groups. Using brood means, the effects of lipid extraction on the liver and muscle $\Delta_{tissue\text{-}diet}$ values were compared using repeated

measures ANOVAs, with tissue as the fixed factor, and lipid treatment as the repeated measures term. In all cases, the tests were two-tailed and α was set at 0.05. All means are presented ± SEM, and all calculations were done using STATISTICA 6.0 (Statsoft, 2001).

3.2.5 Ethical Considerations

The University of Western Ontario Animal Care Committee approved all methods in this experiment, which followed the principles and guidelines of the Canadian Council of Animal Care (protocol number 2004-037-04).

3.3 Results

Over the course of the experiment, the birds fed *ad libitum* doubled in size from 10.0 g at 4 d (± 0.60 g) to 18.1 g at 23 d (± 0.40 g). The tissue-diet discrimination of $\Delta^{15}N$ and $\Delta^{13}C$ for the blood, feathers, liver, muscle, and excreta are shown in Figure 3.1. All tissues were enriched in ^{15}N relative to diet, with liver showing the most enrichment, and blood and muscle the least. Excreta were depleted of ^{15}N relative to diet. Only the feathers showed enrichment in ^{13}C, having positive $\Delta^{13}C_{feather\text{-}diet}$ values, while liver, muscle, and blood were progressively depleted of ^{13}C relative to diet. Excreta were the most depleted of ^{13}C relative to diet. These $\Delta_{tissue\text{-}diet}$ values for Song Sparrows were comparable to those from other controlled avian feeding studies (Table 3.1).

The removal of lipids from the liver and pectoral muscle significantly changed both the $\Delta^{15}N$ and $\Delta^{13}C$ tissue-diet values. Lipid extraction caused a significant enrichment in ^{13}C ($F_{1,54} = 5.56 \times 10^6$, $p < 0.001$) and $\Delta^{15}N$ ($F_{1,54} = 2.96 \times 10^6$, $p < 0.001$)

of the liver and muscle (Figure 3.2). There was an interaction between tissue type and effect of lipid extraction for both $\Delta^{13}C$ ($F_{1,54} = 16.0$, $p < 0.001$) and $\Delta^{15}N$ ($F_{1,54} = 43.0$, $p < 0.001$). Muscle became much more enriched in ^{15}N and ^{13}C than did liver when lipids were extracted. All raw δ values are in Appendix 2.

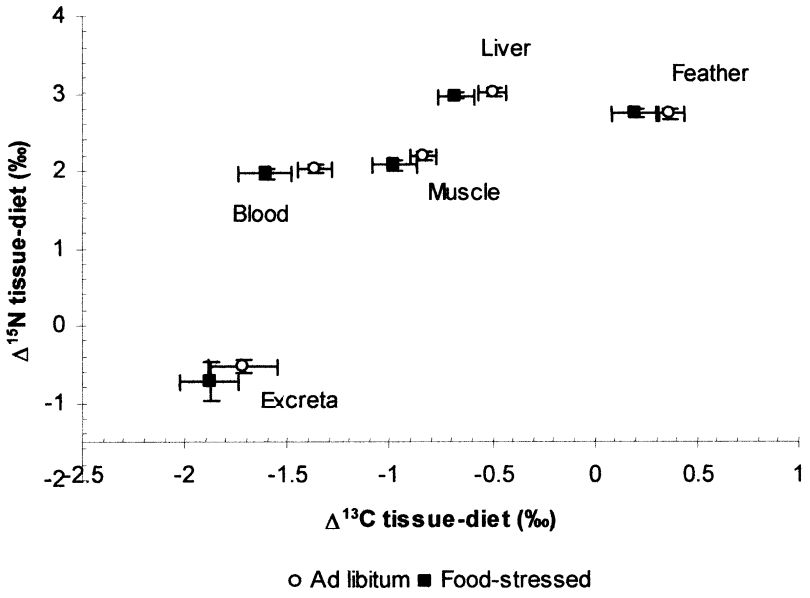

Figure 3.1. The average $\Delta^{13}C$ and $\Delta^{15}N$ (mean ± SE) of the tissues sampled, N = 27.

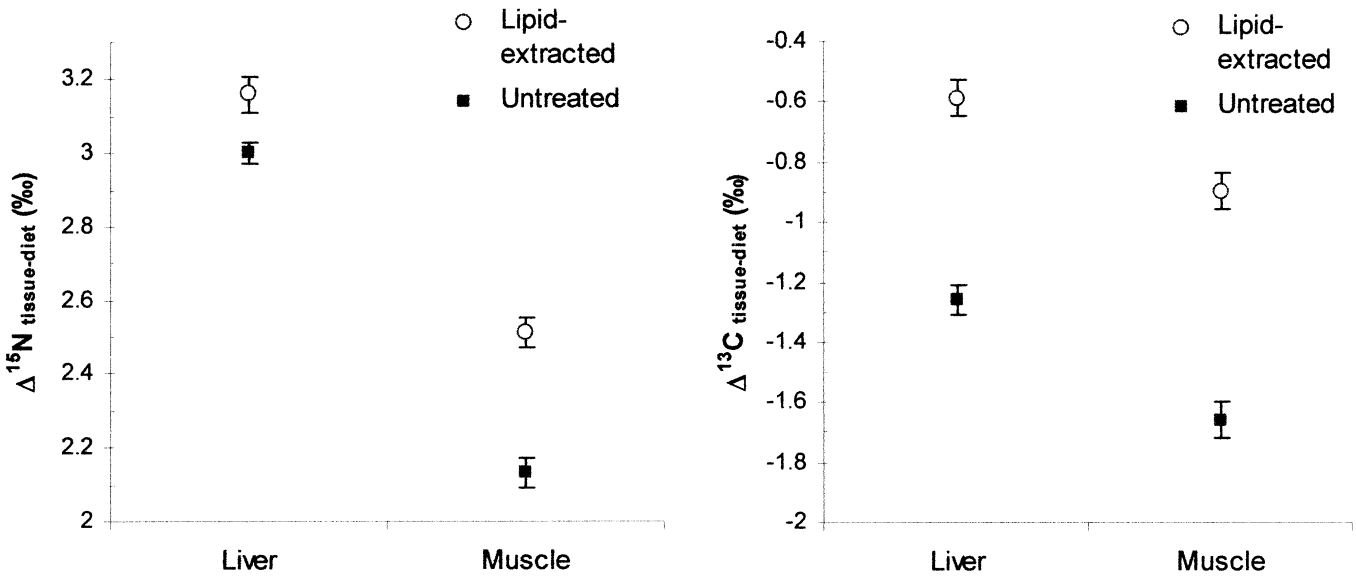

Figure 3.2. Mean ± SEM of the $\Delta^{13}C_{tissue\text{-}diet}$ (left) and $\Delta^{15}N_{tissue\text{-}diet}$ (right) values for lipid-extracted and untreated samples of Song Sparrow liver and pectoral muscle (N= 27).

Table 3.1. Tissue-diet fractionation factors ($\Delta^{15}N$ and $\Delta^{13}C$) for the blood, feathers, liver, and muscle obtained from captive feeding studies. All blood and feather samples were not lipid extracted; only the $\Delta^{13}C$ Song Sparrow liver samples were lipid extracted; all muscle samples were lipid extracted expect for Song Sparrow $\Delta^{15}N$. The diet was not lipid-extracted for the Dunlin, Yellow-rumped Warbler, and Canvasback; lipid-extracted for the Song Sparrow, Japanese Quail, Ring-billed Gull, and Chicken; both were examined in the Garden Warbler, and Great Skua.

	Mean tissue-diet discrimination		
	$\Delta^{15}N$ (‰)	$\Delta^{13}C$ (‰)	Source
Blood			
Song Sparrow	2.0	-1.4	This study
Japanese Quail	2.2	1.2	Hobson and Clark 1992a
Ring-billed Gulls	3.1	-0.3	Hobson and Clark 1992a
Canvasback	3.0	1.4	Haramis et al. 2001
Great Skuas	2.8 to 4.2	1.1 to 2.3	Bearhop et al. 2002
Dunlin	2.9	1.3	Evans-Ogden et al. 2004
Garden Warbler	2.4	1.7	Hobson and Bairlein 2003
Yellow-rumped Warbler	1.7 to 2.7	-1.2 to 2.2	Pearson et al. 2003
Feather			
Song Sparrow	2.7	0.4	This study
Japanese Quail	1.6	1.4	Hobson and Clark 1992a
Chicken	1.1	-0.4	Hobson and Clark 1992a
Ring-billed Gulls	3.0	0.2	Hobson and Clark 1992a
Great Skuas	4.6 to 5.0	2.1 to 2.2	Bearhop et al. 2002
Garden Warbler	4.0	2.7	Hobson and Bairlein 2003
Liver			
Song Sparrow	3.0	-0.5	This study
Japanese Quail	2.3	0.2	Hobson and Clark 1992a
Chicken	1.7	0.4	Hobson and Clark 1992a
Ring-billed Gulls	2.7	-0.4	Hobson and Clark 1992a
Dunlin	4.0	1.1	Evans-Odgen et al. 2004
Muscle			
Song Sparrow	2.2	-0.8	This study
Japanese Quail	1.0	1.1	Hobson and Clark 1992a
Chicken	0.2	0.3	Hobson and Clark 1992a
Ring-billed Gulls	1.4	0.3	Hobson and Clark 1992a
Dunlin	3.1	1.9	Evans-Ogden et al. 2004

3.4 Discussion

This study extends the range of species for which tissue-diet nitrogen and carbon isotopic discrimination factors are known. Our study presents such data for blood, feathers, liver, muscle, and excreta in the Song Sparrow, and for the liver and muscle of any passerine species. We found that all Song Sparrow tissues were enriched in ^{15}N relative to diet, while the excreta were depleted in ^{15}N relative to the diet. By comparison, feathers were enriched in ^{13}C relative to diet whereas all other tissues examined were depleted, as was the excreta. The tissue-diet fractionations are within the bounds of similar discriminations established by other avian studies ($\Delta^{15}N$, 0 to 5 ‰; $\Delta^{13}C$ –2 and 3 ‰), but the relative order of enrichment ($\Delta^{15}N$ blood < muscle < feather < liver; $\Delta^{13}C$ blood < muscle < liver < feather) was unique to Song Sparrows (see Table 3.1). We also found that lipid extraction of the liver and muscle caused a significant enrichment in both ^{15}N and ^{13}C.

Discrimination factors are used to calculate trophic position in a food web and to estimate the relative contributions of several food sources to the diet. Good knowledge of the tissue-diet discrimination factors is crucial to these calculations, which can be affected significantly by even small inaccuracies. The weakest link when completing such calculations is understanding how the isotopic composition of a specific tissue differs from the diet from which it was ultimately derived (Cherel et al. 2005). Other factors such as the quality and quantity of food and nutritional status also influence the tissue-diet discrimination factors within a species. Accordingly, the $\Delta_{tissue\text{-}diet}$ values should be determined on a case-by-case basis, both for species and diet (Cherel et al. 2005).

Body tissues isotopically reflect, but are not identical to, the diet from which they were grown (Hobson and Clark 1992a). All body tissues are enriched in ^{15}N relative to the diet because ^{14}N is preferentially incorporated into uric acid (the metabolic waste product) and excreted (Hobson et al. 1993). Carbon typically does not show a strong tissue-diet discrimination, and tissues may be slightly (1-2 ‰) enriched in or depleted of ^{13}C. Within an individual, each tissue has unique $\Delta^{13}C$ and $\Delta^{15}N$ tissue-diet discrimination factors (Gannes et al. 1997). This is caused by: (1) isotopic routing of dietary nutrients to specific tissues, as each dietary component (i.e., proteins, lipids, and carbohydrates) has a unique isotopic signature (Bearhop et al. 2002; Pearson et al. 2003; Hobson and Bairlein 2003; Ayliffe et al. 2004); (2) differing biochemical and metabolic processes during tissue synthesis (Hobson 1995); and (3) the unique biochemical composition of each tissue, as different molecules (including each amino acid) have different isotopic compositions (Bearhop et al. 2000). While it is clear that different biochemical compositions and reactions result in unique tissue-diet discrimination factors, it is not understood how these processes work nor the reasons why certain tissues are more enriched or depleted of ^{15}N or ^{13}C than others. It is crucial, therefore, that tissue-diet discrimination factors be known for the specific tissues of interest; a single value cannot be used to represent the entire animal.

Dietary protein is the body's source of nitrogen. Blood and muscle, tissues rich in protein, are generally considered to most closely reflect the dietary isotopic composition because dietary proteins are preferentially routed to these tissues with little modification (Kelly 2000; Bearhop et al. 2002). Indeed, in our study, blood and muscle showed the least enrichment in ^{15}N (see Table 3.1). The liver, which had the highest ^{15}N enrichment,

is one of the most metabolically active tissues; these reactions may have caused this enrichment. This general pattern appears when all bird species are examined together, but few of them completely fit the pattern as well as did Song Sparrows (see Table 3.1). As expected, excreta were depleted in ^{15}N relative to diet (Figure 3.1), because avian excreta contain uric acid, which is depleted in ^{15}N.

The body procures carbon through proteins, carbohydrates, and lipids, each of which has a unique isotopic composition. The $\Delta^{13}C$ values change very little between the tissues and diet, with enrichments and depletions of 1‰ being most common. Feathers typically show the greatest ^{13}C-enrichment relative to the diet (see Table 3.1). Feathers are 95% protein, and during feather synthesis, dietary amino acids (especially methionine and cysteine - those rich in sulphur and required for keratin synthesis) may be preferentially routed to the feathers (Bearhop et al. 2002). In Song Sparrows, the $\Delta^{13}C_{tissue\text{-}diet}$ discrimination factors for liver, blood, and muscle, were negative. Lipids and carbohydrates are more depleted of ^{13}C than protein. If tissues incorporate these dietary molecules, the tissue would become depleted of ^{13}C. The differences among muscle, liver, and blood may also result from the preferential routing of certain molecules with unique isotopic compositions to certain tissues (Gannes et al. 1998). The excreta were more depleted of ^{13}C than were the tissues (also see Podlesak et al. 2005). This indicates that the birds either preferentially take up ^{13}C, or preferentially excrete ^{12}C, but the exact reason cannot be determined in our experiment.

Both the absolute value of $\Delta_{tissue\text{-}diet}$ and relative order of tissue enrichment in ^{15}N and ^{13}C differed between the Song Sparrows and other species studied (Table 3.1). The $\Delta^{15}N$ values were within the range of values published for other species, while the $\Delta^{13}C$

values tended to reflect lower tissue-diet $\Delta^{13}C$ values relative to those previously reported.

Lipid extraction is a common but not universally employed sample preparation technique used to reduce the variation in $\delta^{13}C$ values caused by differences in lipid content. As the diet is digested, dietary proteins are preferentially incorporated into body tissues, while dietary carbohydrates and lipids are metabolized as fuel, and hence their isotopic signatures do not appear in the body tissues. Protein in the body tissues provides the best estimate of dietary protein. Lipids found in the tissues are variable in amount, and are not always derived directly from dietary lipids (Gannes et al. 1997); consequently, they are commonly removed during sample preparation. Isotopic results for studies that have employed lipid extraction, however, are difficult to compare to studies that have not extracted lipids (Evans-Ogden et al. 2004). In our study, lipid extraction caused a significant ^{13}C-enrichment of tissue because lipids are depleted of ^{13}C (DeNiro and Epstein 1978). The change in $\Delta^{13}C$ caused by lipid extraction in Song Sparrows, however, should not be extrapolated to other birds unless the relative proportion of lipids in the tissue is known. The amount of lipids in the liver and muscle may also show intra-species variation resulting from age, reproductive status, and nutritional status (Sotiropoulos et al. 2004).

The $\Delta^{15}N$ values were also affected by lipid extraction. The ^{15}N-enrichment of the tissue was likely caused by the unintentional leaching of proteins during the lipid extraction process. The two solvents used in the extraction, methanol (polar) and chloroform (non-polar), are not lipid specific. Together they remove lipids by extracting both polar structural fats and non-polar storage fats. The polar structural fats are attached

to proteins, and methanol probably causes the lipid-bound proteins to leach out with the lipids (Sotiropoulos et al. 2004). Sotiropoulos et al. (2004) suggested that a more suitable non-polar solvent should be found to extract storage lipids while minimizing effects on the $\delta^{15}N$ values of the remaining tissue. While this unintentional enrichment was small (0.4‰) it is still a concern because it adds further inaccuracy to estimates of dietary compositions or tissue turnover rates obtained from N-isotope data. Until lipid extraction can be targeted to carbon alone, the $\delta^{13}C$ values should be obtained using lipid-extracted samples and $\delta^{15}N$ values should be obtained using untreated samples.

In summary, stable carbon and nitrogen isotope analysis of tissues has great potential for ecologists, but additional laboratory studies are needed to test the assumptions and limitations of this technique. The discrimination factors ($\Delta^{15}N_{tissue\text{-}diet}$, $\Delta^{13}C_{tissue\text{-}diet}$) determined for juvenile Song Sparrows, for example, were similar, but not identical to previously studied avian species. Lipid extraction can also cause significant enrichment in both ^{15}N and ^{13}C. The unintentional enrichment in ^{15}N will cause errors in calculations of dietary composition, trophic levels, and isotopic turnover rates, and should be avoided.

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Chapter 4: General Discussion and Conclusion

The degree of food stress experienced by the Song Sparrows in this study was sufficient to significantly alter their growth and development without causing starvation-induced mortality. Nutritional stress caused a decrease in condition, and an increase in anemia, and an increase in the concentration of corticosterone and glucose. Despite these features, we could not detect nutritional stress using stable carbon or nitrogen isotope analysis and the birds did not display any increased developmental instability.

Our nutritionally stressed juvenile Song Sparrows did not show ^{15}N-enrichment unlike the juvenile Japanese Quail raised by Hobson et al. (1993). This may have occurred because our level of nutritional stress was relatively moderate, in contrast to Hobson et al.'s (1993) experiment where the food-stressed quail were provided with enough food to maintain a constant body mass, while birds fed *ad libitum* tripled in the size over the same period. If this is the case, then using $\delta^{15}N$ values in the wild as a measure of nutritional stress may not be useful for species such as passerines that cannot survive for prolonged periods under extreme nutritional stress, and will not show large increases in $\delta^{15}N$. It is unknown whether adult passerines would show an enrichment in ^{15}N under nutritional stress.

Instead of being enriched in ^{13}C (Hatch et al. 1995; Oelbermann and Scheu 2002), the nutritionally stressed animals were depleted of ^{13}C. One possible explanation is that animals grown on low protein diets may use carbon from non-protein components of the diet to synthesize new amino acid carbon backbones (Gannes et al. 1998). Since lipids and carbohydrates are more depleted in ^{13}C than are proteins (Peterson and Fry 1987), these new amino acids would have lower $\delta^{13}C$ values than those derived from the diet

(Webb et al. 1998; Gaye-Siessegger et al. 2004; Savidge and Blair 2004). The ^{13}C-enrichment may also be derived from the composition of the diet, which was composed of both C_3 and C_4 plants. The birds fed *ad libitum* may have digested more of the C_4 component of the diet, and therefore became more enriched in ^{13}C (Bruckental et al. 1985). The reactions of carbon isotopes under different levels of food intake are more complicated than is conventionally assumed, and clearly, more intensive studies are needed to determine the possible impacts of different types of food under different levels of nutrition.

We also presented the tissue-diet fractionations factors for blood, feathers, liver, muscle, and excreta in the Song Sparrow, and for the liver and muscle of any passerine species (see Hobson and Bairlein 2003; Pearson et al. 2003; Podlesak et al. 2005). Relative to the diet, all tissues were enriched in ^{15}N, expect the excreta, which were depleted in ^{15}N; all tissues were depleted in ^{13}C, except feather, which was enriched in ^{13}C. While the tissue-diet fractionations are within the bounds of such discriminations established by other avian studies, the magnitude of the discrimination and the relative order of enrichment were unique to Song Sparrows. Discrimination factors should never be estimated using data from other species, and it is crucial that the tissue-diet discrimination factors be known for the specific tissues of interest; a single value cannot be used to represent the entire animal.

Solvent lipid extraction caused a significant increase in both $\Delta^{15}N$ and $\Delta^{13}C$. Lipid extraction caused a significant ^{13}C-enrichment because lipids are depleted of ^{13}C (DeNiro and Epstein 1978). The change in $\Delta^{13}C$ caused by lipid extraction in Song Sparrows, however, should not be extrapolated to other birds unless the relative

proportion of lipids in the tissue is known. The amount of lipid in the liver and muscle may also show intra-species variation resulting from age, reproductive status, and nutritional status (Sotiropoulos et al. 2004). The ^{15}N-enrichment of the tissue was likely caused by the unintentional leaching of proteins during the lipid extraction process because the solvents used are not lipid specific (Sotiropoulos et al. 2004). Sotiropoulos et al. (2004) suggested that a more suitable non-polar solvent should be found to extract storage lipids while minimizing effects on the $\delta^{15}N$ values of the remaining tissue. Until lipid extraction can be targeted to carbon alone, the $\delta^{13}C$ values should be obtained using lipid-extracted samples and $\delta^{15}N$ values should be obtained using untreated samples.

The physiology behind increased protein catabolism causing ^{15}N and ^{13}C enrichment remains largely unknown; before this technique can be used to measure condition in wild populations, far more controlled laboratory studies must be completed. The rate at which the body becomes enriched in ^{15}N and ^{13}C under food stress is unknown. The enrichment may occur continually during food stress, or may only occur once a critical stage in nutritional stress has occurred. It is also unknown how stable isotopes vary in response different hormones. If corticosterone increases during food stress, with a concomitant increase in metabolism, it may cause ^{15}N and ^{13}C enrichment. Lastly, more experiments examining the difference between altricial and precocial young must be done. The fundamental differences between these two maturation patterns may cause differences in the isotopic response to food stress.

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Appendix 1: Ingredients in Mazuri® Small Bird Maintenance

Guaranteed Analysis

Crude protein not less than	14.5 %
Crude fat not less than	5.0 %
Crude fibre not more than	5.0 %
Ash not more than	9.0 %

Ingredients

Ground corn, wheat middlings, dehulled soybean meal, corn gluten meal, glyceryl monostearate, dicalcium phosphate, soybean oil, brewers grains, calcium carbonate, brewers dried yeast, wheat germ, L-lysine, dried egg product, salt, calcium propionate (a preservative), DL-methionine, menadione dimethylpyrimidinol bisulfite (vitamin K), choline chloride, l-ascorbyl-2-polyphosphate (vitamin C), tocopherols (a preservative), pyridoxine hydrochloride, biotin, d-alpha tocopheryl acetate (natural source vitamin E), cholecalciferol (vitamin D_3), vitamin A acetate, folic acid, manganous oxide, zinc oxide, riboflavin, calcium pantothenate, thiamin mononitrate, nicotinic acid, cyanocobalamin (vitamin B_{12}), copper sulfate, zinc sulfate, calcium iodate, cobalt carbonate, sodium selenite.

Approximate Nutrient Composition

Protein, %	**15.4**	**Minerals**	
Arginine, %	0.73	Ash, %	4.5
Cystine, %	0.25	Calcium, %	0.90
Glycine, %	0.59	Phosphorus, %	0.70
Histidine, %	0.39	Phosphorus (non-phytate), %	0.45
Isoleucine, %	0.66	Potassium, %	0.52
Leucine, %	1.66	Magnesium, %	0.16
Lysine, %	0.75	Sodium, %	0.12
Methionine, %	0.48	Chloride, %	0.26
Phenylalanine, %	0.77	Iron, ppm	260
Tyrosine, %	0.50	Zinc, ppm	100
Threonine, %	0.53	Manganese, ppm	89
Tryptophan, %	0.15	Copper, ppm	12
Valine, %	0.74	Cobalt, ppm	0.35
		Iodine, ppm	1.3
Fat, %	**7.0**	Selenium, ppm	0.28

Fiber (Crude), %	**2.8**	**Vitamins**	
Neutral Detergent Fiber, %	14.6	Vitamin K (as menadione), ppm	3.0
Acid Detergent Fiber, %	4.1	Thiamin Hydrochloride, ppm	12
		Riboflavin, ppm	12
Metabolizable Energy, kcal/g	**3.23**	Niacin, ppm	93
		Pantothenic Acid, ppm	18
		Choline Chloride, ppm	1 400
		Folic Acid, ppm	3.9
		Pyridoxine, ppm	10
		Biotin, ppm	0.67
		Vitamin B12, mcg/kg	33
		Vitamin A, IU/gm	12
		Vitamin D3(added), IU/gm	1.8
		Vitamin E, IU/kg	186

Appendix 2: δ values

Blood

ID	$\delta^{15}N$ 1	$\delta^{15}N$ 2	difference	$\delta^{13}C$ 1	$\delta^{13}C$ 2	difference
3	4.74	4.81	0.07	-18.69	-18.66	0.03
5	4.95	4.97	0.02	-18.77	-18.81	0.04
8	4.93	4.91	0.02	-19.46	-19.58	0.12
9	4.83	4.92	0.09	-19.20	-19.27	0.07
10	5.04	4.99	0.05	-19.01	-18.96	0.05
14	4.93	4.97	0.04	-18.69	-18.72	0.03
15	4.67	4.65	0.02	-19.38	-19.35	0.03
16	4.69	4.74	0.05	-18.71	-18.73	0.02
17	4.66	4.68	0.02	-18.87	-18.87	0.00
18	4.64	4.75	0.11	-18.81	-18.87	0.06
21	4.88	4.89	0.01	-19.59	-19.49	0.10
23	4.98	5.01	0.03	-19.02	-19.03	0.01
25	4.79	4.71	0.08	-18.87	-18.95	0.08
26	4.98	4.97	0.01	-18.75	-18.81	0.06
29	5.06	4.98	0.08	-18.47	-18.54	0.07
30	4.65	4.59	0.06	-18.85	-18.91	0.06
33	5.18	5.23	0.05	-18.48	-18.51	0.03
34	5.14	5.10	0.04	-18.56	-18.58	0.02
38	4.95	4.93	0.02	-19.47	-19.50	0.03
40	5.10	5.08	0.02	-19.81	-19.76	0.05
43	5.05	5.03	0.02	-18.55	-18.64	0.09
44	5.19	5.19	0.00	-18.59	-18.54	0.05
49	5.45	5.43	0.02	-18.61	-18.65	0.04
50	5.41	5.32	0.09	-18.70	-18.77	0.07
53	5.53	5.62	0.09	-18.42	-18.41	0.01
54	5.56	5.53	0.03	-18.48	-18.51	0.03
56	5.49	5.57	0.08	-18.39	-18.33	0.06
57	5.51	5.48	0.03	-17.92	-17.98	0.06

Feather

ID	$\delta^{15}N$ 1	$\delta^{15}N$ 2	difference	$\delta^{13}C$ 1	$\delta^{13}C$ 2	difference
3	5.83	5.82	0.01	-16.86	-16.96	0.10
5	5.76	5.70	0.06	-16.96	-17.00	0.04
8	5.92	5.96	0.04	-17.32	-17.23	0.09
9	5.69	5.86	0.17	-17.24	-17.16	0.08
10	5.95	5.87	0.08	-17.01	-17.08	0.07
14	5.45	5.50	0.05	-16.97	-17.02	0.05
15	5.73	5.55	0.18	-17.27	-17.67	0.40
16	5.55	5.99	0.44	-17.09	-17.00	0.09
17	5.19	5.23	0.04	-17.36	-17.48	0.12
18	5.60	5.72	0.12	-17.15	-17.11	0.04
21	6.03	5.85	0.18	-17.15	-17.10	0.05
23	5.81	5.44	0.37	-17.15	-17.30	0.15
25	5.10	5.33	0.23	-17.28	-17.32	0.04
26	5.42	5.25	0.17	-17.36	-17.33	0.03
29	5.85	5.80	0.05	-17.55	-17.03	0.52
30	6.03	5.78	0.25	-16.97	-17.08	0.11
33	5.82	5.85	0.03	-16.92	-16.82	0.10
34	5.96	5.98	0.02	-16.89	-16.85	0.04
38	5.79	5.92	0.13	-17.25	-17.07	0.18
40	5.80	5.75	0.05	-17.72	-17.93	0.21
43	5.62	5.72	0.10	-16.91	-16.90	0.01
44	5.99	5.48	0.51	-16.57	-16.91	0.34
49	5.87	5.89	0.02	-16.72	-16.77	0.05
50	5.88	6.01	0.13	-16.98	-16.85	0.13
53	6.21	6.09	0.12	-16.59	-17.08	0.49
54	6.28	6.39	0.11	-16.81	-16.71	0.10
56	5.64	5.89	0.25	-17.02	-16.75	0.27
57	6.10	6.06	0.04	-16.37	-16.35	0.02

Liver with Lipids

ID	$\delta^{15}N$ 1	$\delta^{15}N$ 2	difference	$\delta^{13}C$ 1	$\delta^{13}C$ 2	difference
3	6.10	6.11	0.01	-18.71	-18.70	0.01
5	6.11	6.09	0.02	-18.73	-18.71	0.02
8	6.03	6.05	0.02	-19.17	-19.16	0.01
9	6.03	6.01	0.02	-19.04	-18.98	0.06
14	5.91	5.91	0.00	-18.90	-18.87	0.03
15	6.12	6.04	0.08	-18.59	-18.62	0.03
16	5.95	5.99	0.04	-18.79	-18.66	0.13
17	5.94	6.05	0.11	-18.55	-18.63	0.08
18	5.92	5.91	0.01	-18.62	-18.66	0.04
21	6.12	6.08	0.04	-18.66	-18.62	0.04
23	5.82	5.81	0.01	-18.70	-18.83	0.13
25	5.73			-18.56		
26	5.83	5.81	0.02	-18.72	-18.81	0.09
29	5.88	5.88	0.00	-18.50	-18.52	0.02
30	5.84	5.86	0.02	-18.56	-18.67	0.11
33	5.97	6.01	0.04	-18.48	-18.53	0.05
34	6.06	6.07	0.01	-18.61	-18.54	0.07
38	6.04	5.85	0.19	-18.79	-18.87	0.08
40	6.24	6.25	0.01	-19.00	-19.01	0.01
43	5.86	5.91	0.05	-18.25	-18.21	0.04
44	6.10	6.17	0.07	-18.09	-18.12	0.03
49	6.15	6.02	0.13	-18.34	-18.34	0.00
50	6.03	6.07	0.04	-18.42	-18.43	0.01
53	6.28	6.22	0.06	-18.34	-18.26	0.08
54	6.33	6.40	0.07	-18.35	-18.38	0.03
56	6.36	6.35	0.01	-18.19	-18.17	0.02
57	6.12	6.15	0.03	-17.99	-18.04	0.05

Liver without Lipids

ID	$\delta^{15}N$ 1	$\delta^{15}N$ 2	difference	$\delta^{13}C$ 1	$\delta^{13}C$ 2	difference
3	6.19	6.20	0.01	-17.86	-17.75	0.11
5	6.21	6.25	0.04	-18.02	-18.05	0.03
8	6.30	6.30	0.00	-18.22	-18.24	0.02
9	6.19	6.18	0.01	-18.23	-18.19	0.04
14	6.02	6.00	0.02	-18.20	-18.25	0.05
15	6.22	6.29	0.07	-17.75	-17.75	0.00
16	6.17	6.18	0.01	-17.90	-17.90	0.00
17	6.06	6.09	0.03	-17.96	-17.95	0.01
18	6.09	6.05	0.04	-18.04	-18.01	0.03
21	6.32	6.33	0.01	-17.78	-17.74	0.04
23	6.01	6.05	0.04	-18.13	-18.12	0.01
25	5.95	5.89	0.06	-17.92	-17.96	0.04
26	5.99	6.05	0.06	-18.23	-18.24	0.01
29	6.01	6.02	0.01	-17.85	-17.84	0.01
30	5.97	5.99	0.02	-17.93	-17.94	0.01
33	6.14	6.09	0.05	-18.09	-18.05	0.04
34	6.19	6.29	0.10	-17.91	-17.93	0.02
38	6.08	6.11	0.03	-18.37	-18.35	0.02
40	6.41	6.35	0.06	-18.34	-18.34	0.00
43	6.04	6.09	0.05	-17.75	-17.75	0.00
44	6.16	6.23	0.07	-17.69	-17.67	0.02
49	6.29	6.35	0.06	-17.67	-17.70	0.03
50	6.23	6.28	0.05	-17.79	-17.78	0.01
53	6.46	6.45	0.01	-17.26	-17.30	0.04
54	6.41	6.47	0.06	-17.60	-17.58	0.02
56	6.48	6.49	0.01	-17.53	-17.75	0.22
57	6.31	6.36	0.05	-17.54	-17.56	0.02

Muscle with Lipids

ID	$\delta^{15}N$ 1	$\delta^{15}N$ 2	difference	$\delta^{13}C$ 1	$\delta^{13}C$ 2	difference
3	5.08	4.99	0.09	-18.96	-18.97	0.01
5	5.21	5.28	0.07	-18.93	-18.93	0.00
8	4.84	4.92	0.08	-19.09	-19.13	0.04
9	4.92	4.98	0.06	-19.23	-19.18	0.05
14	5.02	4.97	0.05	-19.02	-19.00	0.02
15	4.78	4.86	0.08	-19.38	-19.31	0.07
16	4.79	4.83	0.04	-19.24	-19.25	0.01
17	4.84	4.81	0.03	-18.89	-18.90	0.01
18	5.10	5.16	0.06	-18.90	-18.89	0.01
21	4.94	4.95	0.01	-19.48	-19.48	0.00
23	4.84	4.94	0.10	-19.19	-19.20	0.01
25	4.95	4.93	0.02	-19.12	-19.16	0.04
26	5.17	5.15	0.02	-19.05	-19.05	0.00
29	5.01	4.91	0.10	-18.76	-18.75	0.01
30	5.02	5.07	0.05	-19.04	-19.02	0.02
33	5.40	5.51	0.11	-19.03	-18.96	0.07
34	5.43	5.46	0.03	-18.88	-18.87	0.01
38	5.05	5.06	0.01	-19.15	-19.38	0.23
40	5.03	5.05	0.02	-19.47	-19.44	0.03
43	5.27	5.22	0.05	-18.79	-18.75	0.04
44	5.26	5.26	0.00	-18.72	-18.73	0.01
49	5.42	5.52	0.10	-18.78	-18.79	0.01
50	5.37	5.52	0.15	-18.82	-18.77	0.05
53	5.52	5.49	0.03	-18.68	-18.72	0.04
54	5.62	5.51	0.11	-18.90	-18.91	0.01
56	5.50	5.65	0.15	-18.78	-18.78	0.00
57	5.79	5.86	0.07	-18.37	-18.38	0.01

Muscle without Lipids

ID	$\delta^{15}N$ 1	$\delta^{15}N$ 2	difference	$\delta^{13}C$ 1	$\delta^{13}C$ 2	difference
3	5.38	5.41	0.03	-18.12	-18.19	0.07
5	5.48	5.46	0.02	-18.12	-18.19	0.07
8	5.25	5.20	0.05	-18.45	-18.56	0.11
9	5.31	5.31	0.00	-18.61	-18.54	0.07
14	5.40	5.54	0.14	-18.26	-18.22	0.04
15	5.33	5.26	0.07	-18.45	-18.44	0.01
16	5.25	5.27	0.02	-18.28	-18.38	0.10
17	5.26	5.17	0.09	-18.21	-18.19	0.02
18	5.50	5.44	0.06	-18.15	-18.10	0.05
21	5.39	5.45	0.06	-18.53	-18.51	0.02
23	5.30	5.22	0.08	-18.50	-18.51	0.01
25	5.36	5.37	0.01	-18.33	-18.37	0.04
26	5.60	5.59	0.01	-18.31	-18.33	0.02
29	5.34	5.27	0.07	-18.09	-18.17	0.08
30	5.48	5.41	0.07	-18.17	-18.07	0.10
33	5.77	5.68	0.09	-18.28	-18.16	0.12
34	5.77	5.85	0.08	-18.17	-18.16	0.01
38	5.42	5.52	0.10	-18.58	-18.56	0.02
40	5.49	5.58	0.09	-18.85	-18.84	0.01
43	5.50	5.48	0.02	-18.06	-18.07	0.01
44	5.60	5.56	0.04	-18.03	-17.98	0.05
49	5.77	5.83	0.06	-18.11	-18.10	0.01
50	5.83	5.87	0.04	-18.10	-18.10	0.00
53	5.82	5.94	0.12	-17.90	-17.90	0.00
54	6.07	5.96	0.11	-18.02	-17.98	0.04
56	5.82	5.87	0.05	-18.06	-18.03	0.03
57	6.17	6.12	0.05	-17.61	-17.58	0.03

Feces

Brood	$\delta^{15}N$ 1	$\delta^{15}N$ 2	difference	$\delta^{13}C$ 1	$\delta^{13}C$ 2	difference
1	2.44	2.54	0.10	-19.96	-20.10	0.14
1	2.97	2.99	0.02	-19.71	-19.73	0.02
1	2.79	2.75	0.04	-19.43	-20.23	0.80
2	2.69			-19.33		
2	2.53			-19.62		
2	2.12	1.70	0.42	-19.93	-20.24	0.31
2	1.64	1.27	0.37	-19.77	-19.73	0.04
2	1.75	1.67	0.08	-20.01	-20.08	0.07
2	2.60	2.62	0.02	-19.19	-19.14	0.05
3	2.00	2.11	0.11	-20.14	-20.19	0.05
3	3.77	3.61	0.16	-19.13	-19.29	0.16
3	3.62	3.60	0.02	-19.57	-20.06	0.49
7	2.22	2.17	0.05	-19.68	-19.21	0.47
7	2.37	2.27	0.10	-20.40	-20.35	0.05
7	2.15	2.20	0.05	-20.06	-19.88	0.18
7	2.45	2.54	0.09	-19.17	-19.48	0.31
11	2.19	2.48	0.29	-19.33	-19.31	0.02
11	2.50	2.27	0.23	-19.39	-19.34	0.05
13	2.99	3.00	0.01	-19.46	-19.48	0.02
15	2.35	2.34	0.01	-19.46	-19.50	0.04
Alex	2.07	2.29	0.22	-19.14	-19.65	0.51
Alex	2.88	2.83	0.05	-19.71	-19.14	0.57
Alex	2.72	2.78	0.06	-17.88	-16.92	0.96
Alex	2.84	2.70	0.14	-17.15	-17.35	0.20
Alex	2.64	2.38	0.26	-17.71	-18.35	0.64
Alex	2.31	2.39	0.08	-17.76	-18.21	0.45

Diet

Diet #	$\delta^{15}N$ 1	$\delta^{15}N$ 2	difference	$\delta^{13}C$ 1	$\delta^{13}C$ 2	difference
1	3.25	3.39	0.14	-18.30	-18.10	0.20
1	3.31	3.32	0.01	-17.98	-17.70	0.28
1	3.17	3.18	0.01	-17.56	-17.63	0.07
2	2.39	2.35	0.04	-18.12	-17.65	0.47
2	2.40	2.46	0.06	-17.95	-17.85	0.10
3	2.89	2.93	0.04	-16.87	-18.09	1.22
4	2.89	2.92	0.03	-16.42	-16.88	0.46
4	3.07	3.06	0.01	-17.04	-16.94	0.10
4	2.96	3.04	0.08	-16.98	-17.02	0.04
5	3.28	3.29	0.01	-17.40	-16.95	0.45
5	3.40	3.30	0.10	-16.26	-16.82	0.56
5	3.30	3.31	0.01	-16.99	-17.14	0.15

Nicotinamide

$\delta^{15}N$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{13}C$
-1.50	-1.57	-32.61	-32.59
-1.46	-1.51	-32.59	-32.6
-1.54	-1.64	-32.59	-32.62
-1.56	-1.39	-32.53	-32.63
-1.69	-1.43	-32.65	-32.64
-1.59	-1.59	-32.74	-32.61
-1.35	-1.40	-32.74	-32.5
-1.48	-1.49	-32.67	-32.58
-1.49	-1.53	-32.68	-32.58
-1.45	-1.45	-32.73	-32.64
-1.61	-1.53	-32.61	-32.67
-1.64	-1.65	-32.61	-32.63
-1.62	-1.39	-32.58	-32.64
-1.71	-1.55	-32.6	-32.65
-1.44	-1.50	-32.52	-32.65
-1.54	-1.50	-32.62	-32.63
-1.53	-1.45	-32.61	-32.64
-1.57	-1.48	-32.61	-32.63
-1.49	-1.51	-32.61	-32.61
-1.52	-1.58	-32.64	-32.62
-1.60	-1.52	-32.48	-32.62
-1.57	-1.55	-32.65	-32.73
-1.67	-1.50	-32.51	-32.74
-1.63	-1.55	-32.48	-32.65
-1.63	-1.61	-32.48	-32.82
-1.61	-1.66	-32.64	-32.75
-1.63	-1.73	-32.62	-32.76
-1.54	-1.56	-32.63	-32.74
-1.55	-1.62	-32.58	-32.7
-1.58	-1.60	-32.65	-32.72
-1.63	-1.61	-32.61	-32.63
-1.72	-1.62	-32.64	-32.46
-1.62	-1.56	-32.67	-32.62
-1.56		-32.63	-32.62
		-32.61	-32.72
		-32.58	-32.59
		-32.58	