Factors affecting fractionation of stable carbon and nitrogen isotopes in birds

Paul E. Allen

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Factors Affecting Fractionation of
Stable Carbon and Nitrogen Isotopes in Birds

by

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12-7-98

Date
Factors Affecting Fractionation of Stable Carbon and Nitrogen Isotopes in Birds

Abstract:

Stable isotope methodology has been used to investigate dietary patterns of animals when diets consist of isotopically distinct food sources. However most studies assume that fractionation between animal tissues and each dietary nutrient is the same. By feeding Cedar Waxwings (Bombycilla cedrorum) treatment diets containing isotopically distinct plant- and insect-derived protein, I examined the effect of different relative proportions of plant- and insect-derived nitrogen on diet-blood plasma nitrogen fractionation. Extensive mass loss by all subjects complicated the comparison of fractionation for the treatments, but fractionation estimates could be adjusted for mass loss. Adjusted fractionation values ranged from 4.0-5.9% for diets containing from 0-84% insect-derived protein. The largest adjusted fractionation occurred at low levels (26%) of insect protein in treatment diets and was 48% greater than fractionation of the diet containing no insect protein. Adjusted fractionation for medium levels (54%) of insect protein was slightly less than that of the insect-free diet. When the diet contained high levels (84%) of insect protein, fractionation did not differ from the insect-free diet. A critical result from this study was the demonstration of a positive relationship between mass loss and fractionation.

A second study examined whether age or maturity of growing birds affects fractionation, an important consideration for using stable isotope methodology to study the diets of growing birds. Ten pairs of zebra finches were allowed to breed and their offspring were collected when either they were half-grown, or the day they fledged. Fractionation between liver tissue and food from crop samples was analyzed for the two juvenile age groups as well as the adults. Results indicate that nitrogen fractionation in the liver is not constant throughout avian development. Thus stable isotope methodology cannot be used to accurately trace nitrogen sources in growing birds without controlling for age or maturity. For carbon, no differences in fractionation were found among age groups, but large variances in the stable carbon isotope signatures of samples may have obscured potential differences.
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Chapter 1

Introduction

Cedar Waxwings are unusual among temperate passerines because they rely mainly on fruit and other plant parts for their protein requirements, yet also eat protein-rich insects, especially as nestlings (Judd 1901, Lea 1942, Putnam 1949, Witmer 1996). A large proportion of the diet of newly hatched Cedar Waxwings chicks is comprised of insects but, by the time they fledge, chicks are eating a substantial amount of fruit (Lea 1942, Putnam 1949). Many important ecological and physiological questions arise from considering the nestling diet of this unusual temperate species. For example, how does feeding fruit to young affect the cost of reproduction for adults, since fruit is generally easy to find when its available? Fruit generally does not ripen until late in the temperate breeding season so how does the utilization of fruit affect the timing of breeding and juvenile survival? What physiological characteristics of the young allow them to thrive on fruit while most other temperate passerines use insects for their primary nestling diet?

Before pondering these and other questions arising from the frugivory of Cedar Waxwing nestlings, it is important to consider more fundamental questions: How much plant and insect protein do waxwing young really eat? How does plant and insect protein utilization differ between nestling waxwings and other temperate passerine nestlings? Stable isotope methodology is a potential tool for answering these fundamental questions and the research described here was designed to answer some essential questions about stable isotope methodology as it applies to birds.

Stable isotopes of carbon and nitrogen have proven useful for determining the dietary patterns of animals when their diets consist of isotopically distinct food types (Petelle et al. 1979, Boutton et al. 1983, Ambrose and DeNiro 1986, Hobson 1987, Hobson 1990, Mizutani et al.
1990, Hobson and Sealy 1991, Alisauskas and Hobson 1993, Angerbjörn et al. 1994, Alexander et al. 1996). These analyses rely on the observation that the stable isotope composition of animal tissues can often be predictably related to the stable isotope composition of the nutrients used to synthesize the tissues (DeNiro and Epstein 1978, 1981). Though the relationship between consumer tissues and nutrients is direct, the stable isotope ratios of carbon and nitrogen, $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$, in consumer tissues is often systematically changed or fractionated with respect to the isotope composition of the nutrients supplied to the tissues (DeNiro and Epstein 1977, Rau et al. 1983, Fry and Sherr 1989, Minagawa and Wada 1984). This fractionation is specific to the nutrient-tissue combination examined (Tieszen et al. 1983, Krueger and Sullivan 1984, Ambrose and Norr 1993, Tieszen and Fagre 1993, Hobson 1995). The $^{15}\text{N}/^{14}\text{N}$ ratio of tissues generally increases as trophic level increases (DeNiro and Epstein 1981, Minagawa and Wada 1984) because the metabolic processes of consumers typically show a very slight but systematic affinity of $^{15}\text{N}$ over $^{14}\text{N}$ during nutrient utilization (Macko et al. 1986). This difference in nitrogen isotope composition between trophic levels is one of the sources of distinction between food types that allow stable isotope methodology to be applied to studies of animal food habits. The diet of Cedar Waxwings is ideal for study using stable isotopes since the fruit and insects comprising it are from different trophic levels and, therefore, should have distinct nitrogen isotope signatures.

To a certain extent, stable isotope methods of studying dietary habits are complementary to traditional methods like analysis of gut contents, use of emetics, fecal analysis, and direct observation (Rosenberg and Cooper 1990). While traditional methods ideally provide information about total nutrient intake, stable isotope techniques track nutrients actually assimilated and used for synthesis of particular tissues (Gannes et al. 1997). Using traditional methods, a single sample typically provides diet information for a time scale of a few minutes or hours. Stable isotope methods can provide long-term information about dietary intake because
tissues integrate the isotope signatures of nutrients used to synthesize them. Since turnover rates differ among tissues, a relatively large range of historic information is potentially available in a single animal. Bone collagen has a turnover rate on the order of years (Tieszen et al. 1983, Boutton et al. 1984, Hobson and Clark 1992a), while blood plasma turns over in a matter of days (Hobson and Clark 1993). In addition, some metabolically-inert products like feathers (Mizutani et al. 1990, 1992), eggshells (von Schirnding et al. 1982, Schaffner and Swart 1991, Hobson 1995), and baleen (Schell et al. 1989) have the potential to provide snapshots of the isotope composition of the nutrients assimilated when they were synthesized without the continuous integration that occurs in metabolically-active tissues. While stable isotope methods will never provide the detailed taxonomic information about prey species that traditional methods sometimes can, there is great potential with isotope methods for non-destructive sampling using blood, feathers, or eggshells.

One obstacle preventing general use of stable isotopes in dietary studies is that fractionation is not well understood. This thesis focuses on two questions about fractionation that need to be answered before using stable isotope methodology on Cedar Waxwing nestlings in the field. Chapter 2 describes a study designed to determine whether fractionation depends on the relative proportion of plant- and insect-derived protein present in the diet. Chapter 3 describes research to determine whether fractionation is constant throughout avian development. Chapter 4 discusses the implications of this research for using stable isotope methodology on birds.
Chapter 2

Fractionation of Plant- and Insect-derived $^{15}$N

by Cedar Waxwings (*Bombycilla cedrorum*)

Introduction

Before applying stable isotope methodology to study the diet and protein utilization of Cedar Waxwing nestlings, it is important to know whether fractionation changes depending on the relative proportion of plant- and insect-derived protein in their diet. Fractionation is known to be specific to nutrient-tissue combinations (Tieszen et al. 1983, Krueger and Sullivan 1984, Ambrose and Norr 1993, Tieszen and Fagre 1993, Hobson 1995) but little work has been performed to establish whether fractionation changes as the relative proportion of two sources of the same nutrient changes (Tieszen et al. 1993). Waxwing nestlings would encounter changing sources of protein, from insects to fruit, as they develop.

In diet studies employing stable isotope methodology to determine the relative importance of two food types in the diet, the isotope signature of consumer tissue ($\delta_{T,p}$) can be modeled as a linear combination of the stable isotope signatures of the two foods:

$$\delta_{T,p} = p \delta_A + (1 - p) \delta_B + \Delta_T.$$  

Here, $\delta_A$ and $\delta_B$ are the stable isotope signatures of two food types, A and B, and $\Delta_T$ is the fractionation associated with the tissue of interest. The proportion of food A in the diet is $p$ and the proportion of food B is $(1 - p)$. A significant drawback of this model is that fractionation is assumed to be constant for a specific tissue, although it is well-established that fractionation is specific to nutrient-tissue combinations. This model also fails to account for differential assimilation efficiency of diet components and for differential allocation of nutrients to specific tissues, once assimilated (Gannes et al. 1997).
Recasting the model slightly and allowing fractionation to vary explicitly addresses these problems:

\[
\delta_{T, \Psi} = \Psi \delta_A + (1 - \Psi) \delta_B + f_A(\Psi, T, A, B).
\]

Here, the proportion of the tissue synthesized from nutrients derived from food A is \(\Psi\), while food B provides the remaining nutrients \((1 - \Psi)\) for synthesis. Fractionation, \(f_A\), is explicitly written as a function of \(\Psi\) as well as the tissue \((T)\) and foods \((A \& B)\) involved. This model specifically avoids using \(p\), the proportion of a particular food in the diet. Stable isotope methodology cannot reveal more than general trends in \(p\) since the relationship between diet and the nutrients used to make a tissue is obscured by different assimilation efficiencies of diet components and different allocation strategies.

The present study was designed to describe the fractionation of nitrogen in the tissues of Cedar Waxwings consuming protein from plant and insect sources in various relative amounts. If significant changes in fractionation occur as the types and the relative proportion of protein in the diet change, then efforts to use stable isotope methodology to examine fruit and insect consumption and utilization of protein by nestling Cedar Waxwings will be complicated. Nitrogen fractionation would have to be calibrated for different relative amounts of various types of protein.

Methods

I caught 13 wild Cedar Waxwings using mist nets in late September 1996 at Missoula, Montana. Four birds had the plumage characteristics of hatch-year (HY) juveniles, while the remaining were after-hatch-year (AHY) adults. The sex of the birds was not determined. The waxwings were randomly distributed between two indoor walk-in flight cages (1 m x 2 m x 2 m) in the same room at the laboratory animal facilities of The University of Montana (Animal Use Protocol BSTM-96-B). Throughout their captivity, the light-dark cycle was changed weekly to...
correspond to the natural day-night cycle, but the temperature was held constant at 20-21°C (68-70°F). Food and water was provided ad libitum.

All food was formulated using the semi-synthetic, banana-based diet of Denslow et al. (1987) as the foundation. During their first week in captivity, the birds were habituated to the banana diet using a formulation of it to which thawed frozen blueberries had been added. After the habituation period, all birds were fed the standard banana diet during a pretreatment period of 86 d. The pretreatment diet was intended to normalize the stable isotope signature of blood plasma to a standard baseline for all the birds. Mass of birds was recorded at least biweekly during pretreatment. After a period of mass gain at the beginning of captivity, the birds' masses were stable for the duration of the pretreatment period.

With the exception of trace amounts of animal-derived protein in the vitamin and mineral supplement used, the standard banana diet contained only plant-derived protein. The majority came from soy protein isolate and wheat germ components, though the bananas themselves contributed some protein (Table 2.1). The three treatment diets were based on the standard banana diet with insect-derived protein substituting for part or all of the wheat and soy proteins (Tables 2.1 & A1.1). The different trophic levels of plants and insects in the treatment diets assured some isotopic distinction with respect to nitrogen. The carbon isotope signatures of the diets were not considered.

The insect protein component was comprised of equal amounts (by weight) of mealworm larvae (Tenebrio molitor) and crickets (Acheta domestica). Insects were ground to a paste with a food processor before use. The treatment diets were formulated to have the same protein and calorie content per gram of diet (wet weight) as the standard formulation (Tables A1.2 & A1.3). In the low insect diet, 26% of the protein was derived from insects while the medium and high insect diets contained 54% and 84% insect protein, respectively. Each time a batch of food was prepared, a sample was frozen and later freeze-dried. After freeze-drying, the food samples were
oven-dried and ground to talcum powder consistency with a shatterbox (model 8500 with an 8505 alumina ceramic grinding container, SPEX Certiprep, Methuchen, NJ).

Table 2.1. Sources of protein in standard banana and treatment diets. For complete list of ingredients and amounts, see Table A1.1.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Banana (%)</th>
<th>Soy (%)</th>
<th>Wheat Germ (%)</th>
<th>Cricket (%)</th>
<th>Mealworm (%)</th>
<th>Total Insect Protein (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>20</td>
<td>56</td>
<td>24</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>High Insect</td>
<td>17</td>
<td>-</td>
<td>-</td>
<td>44</td>
<td>40</td>
<td>84</td>
</tr>
<tr>
<td>Med. Insect</td>
<td>18</td>
<td>28</td>
<td>-</td>
<td>29</td>
<td>26</td>
<td>54</td>
</tr>
<tr>
<td>Low Insect</td>
<td>18</td>
<td>40</td>
<td>16</td>
<td>14</td>
<td>12</td>
<td>26</td>
</tr>
</tbody>
</table>

Two weeks before the treatment diets began, 72 d into the pretreatment period, three AHY birds were randomly assigned to each of three treatment groups, high, medium, or low insect. One HY bird was randomly assigned to each treatment group and the fourth HY bird was arbitrarily assigned to the medium group. Each group was housed in a separate walk-in flight cage in the same room. After 86 days of pretreatment, on day 0 of the experiment, the standard banana diet was replaced with the treatment diets. The treatment diets were available for 25 days, after which they were replaced by the standard banana diet.

Since dried blood plasma was the material used for stable isotope analyses, whole blood samples drawn from the birds had to be relatively large. Frequent sampling at the beginning of the treatments was necessary to track accurately the expected exponential change in the plasma stable isotope signature. I took samples at intervals of no less than three days to avoid endangering the health of the birds. Samples during treatment were drawn on days 2, 5, 11, and 25. A single sample drawn from each bird four days before the start of the experiment (day -4) served as a pretreatment baseline. For modeling, I treated the samples from day -4 as representing blood composition on day 0, just before treatment. I was unable to collect usable
samples from three birds on day -4 and from another bird on day 2 because of difficulties drawing the blood. Blood samples consisted of 300 µl of blood drawn from the jugular vein of each subject with a heparinized syringe. Samples were immediately refrigerated. Whole blood was centrifuged (2-5 min, 10,000 RPM) within 0-4 d (usually within 1 d) after sampling, and plasma and cellular components were separated. After separation, plasma was immediately frozen and later freeze-dried.

Two mg of each food or plasma sample were placed in individual tin analysis capsules (5x9 mm, Costech Analytical Technologies, Inc., Valencia, CA). These samples were analyzed using mass spectrometry by the Institute of Ecology Analytical Chemical Laboratory at the University of Georgia (Athens, GA). The stable nitrogen isotope composition or signature of a sample is measured as the ratio of the two nitrogen isotopes, $^{15}N/^{14}N$. It is expressed as the deviation from a standard isotope ratio in parts per thousand (‰):

$$\delta^{15}N = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 10^3$$

Here, $R_{\text{sample}}$ is the measured sample ratio and $R_{\text{standard}}$ is the isotopic ratio of atmospheric air. In addition to giving the stable isotope composition of a sample, mass spectrometry also provides the total nitrogen content of the sample.

For each bird with a pretreatment plasma sample, I estimated diet-plasma fractionation for the pretreatment diet by subtracting the mean $\delta^{15}N$ value of the pretreatment diet samples from the $\delta^{15}N$ value of the plasma sample. For each bird, I calculated fractionation estimates for the treatment diets by fitting an exponential model to the $\delta^{15}N$ value of plasma samples taken during the treatment period. At time $t$, I modeled plasma $^{15}N$ as

$$\delta^{15}N(t) = \delta^{15}N(\infty) + [\delta^{15}N(0) - \delta^{15}N(\infty)] e^{-\lambda t}$$

The estimated pretreatment plasma $^{15}N$ signature is $\delta^{15}N(0)$. $\delta^{15}N(\infty)$ represents the tissue $^{15}N$ signature when the plasma has completely turned over while in the presence of the treatment diet. Since $\delta^{15}N(\infty) = \delta^{15}N_{\text{diet}} + \Delta_{\text{diet-plasma}}$, the model simplifies to

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\[ \delta^{15}N(t) = \delta^{15}N_{\text{diet}} + \Delta_{\text{diet-plasma}} + [\delta^{15}N(0) - (\delta^{15}N_{\text{diet}} + \Delta_{\text{diet-plasma}})] e^{-rt} \]

where \( r \) is the turnover rate constant of the plasma and \( \Delta_{\text{diet-plasma}} \) is the fractionation between the treatment diet and the plasma. Using the \( \delta^{15}N \) values of blood plasma samples taken before treatments began \((t = 0, \text{if available})\) and during treatment \((t = 2, 5, 11, 25)\), I estimated \( \Delta_{\text{diet-plasma}}, \delta^{15}N(0), \) and \( r \) for each bird with non-linear regression using Levenberg-Marquardt estimation.

I recorded the mass of each bird when blood samples were drawn. The relative mass of each bird on day 25 was calculated as mass on day 25 divided by mass on day 0. For each bird, relative mass loss over the treatment period was calculated as 1.0 minus relative mass on day 25. I analyzed mass loss with a repeated measures ANOVA where the daily masses of individual birds were treated as the "within" factor. In that analysis, I tested for variation among birds as a function of treatment and examined the day \( \times \) treatment interaction.

The complex effect of mass loss on fractionation was analyzed with an ANCOVA by nesting relative mass loss as a covariate within treatment. I used the resulting treatment-specific parameter estimates for mass loss effects to calculate a residual or adjusted fractionation estimate by subtracting predicted mass loss effects from fractionation. Additionally, fractionation estimates for the pretreatment period did not differ among groups (ANOVA, \( n = 2 \) and 7, \( F = 0.748, P = 0.508 \)), so all fractionation estimates from that period were considered together as a fourth "treatment" group. These fractionation estimates from the pretreatment group had no mass loss effect since there was no mass loss during pretreatment; they could be compared with the adjusted (residual) fractionation estimates of the three original treatment groups. When fractionation among the four groups was compared, a factor representing the effect of individuals was added to the ANOVA analysis since each individual contributed two fractionation estimates, one from the pretreatment period and one from the treatment period.
All statistical analyses and curve fits were performed using SPSS (SPSS 1995). No effects of age (i.e., HY versus AHY) were detected in any analyses, so age was left out of all analyses and data were combined.

Results

Food $\delta^{15}N$.— Relative to the standard banana diet, the $\delta^{15}N$ of treatment diets increased as the amount of insect protein included in the treatment diet formulations increased (Fig. 2.1). The $\delta^{15}N$ of each diet differed (ANOVA, $n = 3$ and 20, $F = 152.336, P < 0.001$; all Tukey's HSD post-hoc comparisons $P < 0.001$). No difference in the total nitrogen content of the diets could be detected (ANOVA, $n = 3$ and 20, $F = 0.462, P = 0.712$). Mean ($\pm$ SE) nitrogen concentration of the diets was 2.76% ($\pm$ 0.078), 2.63% ($\pm$ 0.039), 2.67% ($\pm$ 0.103), and 2.68% ($\pm$ 0.116) for the standard banana, low insect, medium insect, and high insect diets, respectively.

Mass Loss. — The treatments resulted in severe nutritional stress as indicated by mass loss by all birds during the experimental period (Table 2.2; Fig. 2.2). However, mass loss was detected only between day 11 and day 25. A repeated measures analysis indicated that body mass did not systematically differ among treatment groups throughout the treatment period ($df = 2$ and 10, $F = 0.1538, P = 0.262$). A significant day $\times$ treatment interaction ($df = 8$ and 40, $F = 2.374, P = 0.034$) resulted from a significant treatment parameter estimate (estimate $\pm$ SE = $-5.745 \pm 2.247$) for the low insect treatment on day 11. Other than this case where the predicted mass of the low treatment group on day 11 was significantly smaller than that of the other two groups, none of the treatment parameter estimates for mass were significantly different from zero. Within individuals, mass differed significantly among days ($df = 4$ and 40, $F = 153.361, P < 0.001$). Parameter estimates for the effects of day confirm the visual impression (Fig. 2.2) that the masses from day 25 differed significantly from the masses on other days. The estimated intercept ($\pm$ SE) for masses on day 0, 2, 5, 11, and 25 were 43.2 ($\pm$ 1.10), 42.8 ($\pm$ 1.38), 43.4 ($\pm$ 1.53), 44.2 ($\pm$ 1.50), and 27.8 ($\pm$ 1.30), respectively.
The proximate reason for the mass loss observed in the birds was a significantly lower consumption of the treatment diets during the latter part of the treatment period relative to consumption levels for the pretreatment diet. Although I made no quantitative measurements, the amount of food required to satisfy the daily *ad libitum* consumption during the latter part of the treatment period was much less than that required during pretreatment. The birds ate less of the treatment diets than they did of the pretreatment diet. One day after the treatments ended, two of the weakest birds died and another was euthanized. One of the dead birds had been in the high insect treatment while the other two were from the medium insect treatment.

Table 2.2. Mass loss between treatment days 0 and 25.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Mass loss (g)</th>
<th>Relative mass loss (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High insect</td>
<td>4</td>
<td>15.8 (2.57)</td>
<td>36.5 (5.36)</td>
</tr>
<tr>
<td>Med. insect</td>
<td>5</td>
<td>15.4 (4.03)</td>
<td>35.6 (8.42)</td>
</tr>
<tr>
<td>Low insect</td>
<td>4</td>
<td>13.8 (1.73)</td>
<td>33.7 (3.75)</td>
</tr>
</tbody>
</table>

Table 2.3. Estimates of pretreatment tissue $^{15}$N values ($\delta^{15}$N(0)), diet-plasma fractionation ($\Delta_{diet-plasma}$), and turnover rate parameter ($r$) produced by curve fitting. Fractionation estimates are not adjusted for mass loss.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>$\delta^{15}$N(0) (%)</th>
<th>$\Delta_{diet-plasma}$ (%)</th>
<th>$r$</th>
</tr>
</thead>
<tbody>
<tr>
<td>High insect</td>
<td>4</td>
<td>5.70 (0.061)</td>
<td>4.18 (0.276)</td>
<td>-0.118 (0.030)</td>
</tr>
<tr>
<td>Med. insect</td>
<td>5</td>
<td>5.74 (0.190)</td>
<td>5.40 (0.505)</td>
<td>-0.070 (0.019)</td>
</tr>
<tr>
<td>Low insect</td>
<td>4</td>
<td>5.58 (0.203)</td>
<td>4.75 (0.220)</td>
<td>-0.111 (0.058)</td>
</tr>
</tbody>
</table>

$^{15}$N Fractionation and Turnover.— Visually, the curves fit to the plasma $\delta^{15}$N values of each bird segregate into treatment groups (Fig. 2.3). The curves were compared by analyzing the fractionation and turnover values (Table 2.3) estimated by the fitting process.
Mass loss by all subjects during the treatments had a complex effect on fractionation. Depending on the treatment, fractionation either increased or decreased as relative mass loss increased (ANCOVA, df = 5 and 7; F = 17.200, P = 0.001; treatment effect, df = 2 and 7, F = 2.124, P = 0.190; effect of relative mass loss nested within treatment, df = 3 and 7, F = 6.707, P = 0.018). The coefficients (± SE) for the effects of relative mass loss on fractionation for the low, medium, and high treatments were -3.402 (± 3.484), 5.887 (± 1.345), and 0.245 (± 2.439), respectively. Only the coefficient for the medium treatment differed significantly from zero (P = 0.003). These coefficients were used to calculate adjusted (residual) fractionation estimates.

Fractionation estimates, including adjusted estimates from the treatments, differed by treatment/group (Fig. 2.4; ANOVA, df = 15 and 7, F = 36.251, P < 0.001; treatment/group effect, df = 3 and 7, F = 70.104, P < 0.001; individual effect, df = 12 and 7, F = 0.921, P = 0.571). Fractionation for each group was different from the others except for the high insect treatment and pretreatment group (all Tukey’s HSD comparisons P ≤ 0.001, except Phigh-pretreatment = 0.660).

The estimate of r, the plasma turnover rate constant produced for each bird by the curve fitting procedure, was not affected by treatment or mass loss (Fig. 2.5; ANCOVA, df = 3 and 9, F = 1.393, P = 0.307; treatment effect, df = 2 and 9, F = 2.083, P = 0.181; relative mass loss effect, df = 1 and 9, F = 0.006, P = 0.939). The treatment x relative mass loss interaction was not significant so it was left out of the model.
Figure 2.1. Box plot of \( \delta^{15}N \) for food samples from standard (pretreatment) and treatment diets. The line inside each box represents the median for the group while the box itself shows the limits of the middle half of the data. The whiskers above and below the box reach to the nearest data point not beyond a span of 1.5 times the inter-quartile range. Data points outside the range of the whiskers are plotted as open circles.
Figure 2.2. Body mass declined for all birds in all treatments. Lines for each treatment are LOWESS (locally weighted scatterplot smoothing; Cleveland 1981) curves to indicate group trends.
Figure 2.3. Curves of the form \( \delta^{15}N(t) = \delta^{15}N_{\text{diet}} + \Delta_{\text{diet-plasma}} + \left[ \delta^{15}N(0) - (\delta^{15}N_{\text{diet}} + \Delta_{\text{diet-plasma}}) \right] e^{-rt} \) fit to the plasma \( \delta^{15}N \) values for each bird. Individual plasma \( \delta^{15}N \) values are jittered horizontally for clarity. For each bird, the curve estimation procedure provided estimates of \( r, \Delta_{\text{diet-plasma}}, \) and \( \delta^{15}N(0) \) (Table 2.3). The dashed horizontal line shows the mean plasma \( \delta^{15}N \) (5.66\%) before treatments began.
Figure 2.4. Box plot of $^{15}$N fractionation for the pretreatment period and adjusted (residual) $^{15}$N fractionation estimates for the treatments. Adjusted fractionation estimates are fractionation values with mass loss effects removed (see text). See Fig. 2.1 for conventions of box plots.
Figure 2.5. Box plot of half-life estimates for plasma nitrogen of individuals in the treatment groups. Half-life is calculated as $\ln(0.5)/r$ where $r$ is the turnover rate parameter estimated by the curve fitting procedure. Larger half-life values indicate lower turnover rates. See Fig. 2.1 for conventions of box plots.
Discussion

The extensive mass loss observed in all subjects during the experimental treatments did not begin until halfway through the treatment period. The proximate reason for the mass loss was a decrease in the quantity of treatment food consumed compared to pretreatment consumption. Ultimate explanations for the mass loss are mainly speculative, but the most plausible reasons are related to food palatability and digestion. The birds may have found the treatment diets unpalatable due to altered taste or texture compared to the standard diet. Cedar Waxwings are very sensitive to slight differences in the nutrient content of foods (Martínez del Rio et al. 1989), so it would not be surprising if they were as sensitive about the taste or texture of their food. Waxwings would not normally encounter either crickets or mealworms in the wild, so they may have found the taste or texture of these insects unpleasant even though they were ground into a paste and partially camouflaged by the banana mixture.

The mass loss ultimately also may be related to difficulties digesting the treatment diets. Since digesting insects and fruit present different challenges to the digestive system, the mixture of an insect component and fruit or plant component in the same meal may have prevented efficient digestion of either. Although Cedar Waxwings do rely on both fruit and insects annually (Witmer 1996), birds in the wild apparently do not mix fruit and insects in the same meal (pers. comm. Martínez del Rio). Also, it is unlikely that all the protein in the treatment diets was digestible and available for assimilation since the insect protein was partly chitin. Chitin digestion depends on chitinolytic activity which is absent in birds that do not feed on insects but high in birds that wholly or partly depend on insects or crustaceans (Jeuniaux and Cornelius 1978, Jackson et al. 1992).

Since the gross nutritive value of the treatment diets was very similar to the standard banana mash diet, it seems unlikely that any sort of macronutritional deficiency is to blame for the mass loss. In the standard diet, as in the treatments, bananas supplied most of the
carbohydrates (84-88%) and the total carbohydrate content varied only from 21-24% of dry weight. Because of the relatively high fat content of the crickets and mealworms, the treatments provided more fat (7.6-10.7% of dry weight) than the standard diet (6.4% of dry weight). The crude protein content of the treatment diets (15.9-16.4% of dry weight) was similar to that of the standard diet (15.5% of dry weight). Protein in the bananas alone provided 2.7-2.9% of the protein (dry weight) in the treatment diets. Feeding trials have shown that Cedar Waxwings can maintain body mass on a diet with 2.2% (dry weight) protein and 86% (dry weight) sugar (Witmer 1994). The treatment diets in this study provided similar gross amounts of protein and carbohydrates to that of Witmer's (1994) other feeding trials.

The extensive mass loss potentially complicates attempts to compare fractionation of the plant and insect protein mixtures presented in the treatment diets. The birds almost certainly experienced decreases in lean body mass and it has been shown that tissues of adult animals become enriched in $^{15}$N beyond normal values as lean body mass decreases (Hobson et al. 1993). When daily protein needs are not met, animals recycle protein from their own tissues to a greater extent than usual (Waterlow 1968). Since recycled nitrogen is enriched through the same mechanisms that dietary nitrogen is enriched, the $^{15}$N level in tissues would become enriched (Hobson et al. 1993).

This study did not relate mass loss directly to tissue $^{15}$N levels because the isotope signatures of tissues were in the midst of changing in response to the switch from the pretreatment diet to treatment diets. However, nitrogen fractionation estimates were statistically affected by mass loss, indirectly suggesting that tissue $^{15}$N levels were altered by mass loss. The trends relating mass loss and fractionation were complex. Subjects from the medium and high insect treatments showed a positive trend between mass loss and fractionation. The trend between mass loss and fractionation was negative in the low insect treatment. However, the only significant relationship was that of the medium insect group where, on average, the mass loss of that group increased predicted fractionation by 2.1% (mass loss coefficient for the medium
treatment (5.887‰) multiplied by the mean relative mass loss of the medium insect treatment (0.356)).

Fractionation during the pretreatment period did not differ from fractionation of the high insect treatment after adjustments for mass loss effects. Fractionation of the medium insect treatment adjusted for mass loss was slightly, but significantly, smaller than either pretreatment fractionation or adjusted high insect treatment fractionation. Adjusted fractionation of the low insect treatment was much higher than any other group. However, it is important to reiterate that only the mass loss adjustments for the medium insect treatment was statistically significant. Information about nitrogen balance and protein assimilation efficiency would have been valuable covariates for analyses and helpful in the physiological interpretation of these results.

Mean nitrogen fractionation for the pretreatment diet (4.0‰) and mean adjusted fractionation values for the treatment diets (3.3-5.9‰) were generally higher than the most closely comparable published values. Hobson and Clark (1992b) observed 15N fractionation of 2.2‰ and 3.1‰ for the whole blood of Japanese Quail (Coturnix japonica) and Ring-billed Gull (Larus delawarensis), respectively. There are no other 15N fractionation values published for blood products, but other 15N fractionation values published for vertebrates on a variety of diets range from 2.3-5.8‰ for tissues as diverse as feathers (Mizutani et al. 1992), eggs (Hobson 1995), and several internal organs (DeNiro & Epstein 1981).

In summary, this study shows that fractionation appears to have a complex response to changes in the relative amounts of plant- and insect-derived protein in the diet. The substitution of 26% of the plant protein in the diet by insect protein resulted in an apparent 50% increase in fractionation. Further substitution of insect protein for plant protein resulted in up to a 25% decrease in fractionation compared to baseline levels. These results are potentially confounded by the complex effect that mass loss had on fractionation. This study shows the importance of laboratory calibrations of fractionation when using stable isotope methodology. A further critical result is the demonstration of mass loss effects on nitrogen fractionation.
Chapter 3

Fractionation of $^{13}$C and $^{15}$N in Juvenile Zebra Finches (*Poephila guttata*)

Introduction

Although fractionation has a major impact on the interpretation of stable isotope analyses, the factors affecting fractionation are not completely understood. Before applying stable isotope methodology to study the diet and protein utilization of nestling Cedar Waxwings, it is imperative to know whether fractionation changes during the short, intense period of development in passerines. However, most studies of fractionation have used healthy, adult individuals in which tissues remain at constant size because the rates of tissue synthesis and catabolism are equal. During development, the rate of tissue synthesis exceeds the rate of tissue catabolism, resulting in net growth of the tissue; furthermore, overall rates of synthesis and catabolism are higher during growth (Waterlow et al. 1978). During growth, these changes in the absolute synthetic and catabolic rates or the relative difference in the rates could alter fractionation from what it would be in a healthy individual not experiencing growth.

Previous work has shown that fractionation increased in juvenile quail when tissues that normally would be growing were maintained at an immature size by limited nutrient intake (Hobson et al. 1993). Instead of examining fractionation during growth under conditions of nutritional stress, the present study investigates fractionation during growth in well-nourished individuals. I compared fractionation in half-grown chicks, recent fledglings, and adults.

Since diet has a known affect on fractionation, it was also important that the diets of the adults and chicks be similar. Zebra finches (*Poephila guttata*) are ideal organisms for this research since the adults feed their growing offspring the same food that they eat. By sampling
one group of chicks midway through growth, I determined fractionation in chicks at the point when fastest growth is occurring. I also determined fractionation in offspring after most growth was complete but before all tissues are mature by sampling a second group of young immediately after fledging. By sampling the adult parents of the offspring, estimates of fractionation were obtained from individuals with mature tissues.

Both liver tissue and blood plasma have high turnover rates, recommending their use for this short-term study. The half-life of carbon in the liver and blood plasma of quail is 2.6 and 2.9 d, respectively (Hobson and Clark 1992a), so 95% of carbon in those tissues would turn over in about 23 d. However, the uncertainty of being able to draw large enough blood samples from Zebra Finch chicks to provide adequate dried material for analysis led me to use liver tissue for this research.

**Methods**

Ten pairs of adult Zebra Finches singled out from a large colony were placed in ten cages, 46 cm on each side, for breeding. The pairings were determined by behavioral cues while the birds were in the large colony. The ten pairs were housed in an animal facility at Cornell University (Animal Use Protocol 97-60) with a 14-10 h light-dark cycle at a constant 22° C (72-73° F). A nest box made of a steel can (14 cm tall, 10 cm diameter) was attached externally to each cage in a horizontal orientation. Burlap cloth cut into 10-cm squares and pulled apart into individual strands was provided *ad libitum* as nesting material.

Water and commercial finch food comprised of whole seeds and processed pellets (see Appendix 2) was provided *ad libitum*. Calcium was supplemented by a pinch of crushed, sterilized chicken eggshells on top of the food each day as well as a cuttle bone in each cage. To reduce the influence of individual cuttle bones on the isotope signature of pairs, the cuttle bones were rotated among cages daily. All food used (excluding calcium supplements) was purchased at the beginning of the experiment and mixed to alleviate any differences in the composite isotope...
signature in individual bags of the commercial food. The food (excluding calcium supplements) was sampled every few weeks for stable isotope analysis.

Each breeding pair was checked daily to record progress of nest building, egg laying, and chick rearing. When a clutch became overdue for hatching by approximately one week, the nest and eggs were removed and fresh nesting material was provided in the cage to promote renesting. Most chicks were weighed daily. Offspring were collected and euthanized at one of two times: either when their mass was 6-8 g or after they fledged from the nest box into their cage. Fledglings typically weighed approximately 10 g. Collections were balanced within a nest so that half of the offspring were collected at 6-8 g and half when they had fledged. After all offspring in a cage were collected, the nest box was cleaned and fresh nesting material was provided in the cage to encourage renesting. Adults were allowed to breed for 25 weeks and were euthanized at the end of that period.

All birds were euthanized with CO₂ and immediately frozen. The carcasses were later thawed and the livers dissected out. The livers were dried in an oven and then ground to talcum powder consistency using a mortar and pestle. Any ingested food in the crops of thawed carcasses was removed and frozen. All crop and food samples were oven-dried and ground with a shatterbox (model 8500 with an 8505 alumina ceramic grinding container, SPEX Certiprep, Methuchen, NJ).

Two mg of each food or liver sample were placed in individual tin analysis capsules (5x9 mm, Costech Analytical Technologies, Inc., Valencia, CA). These samples were analyzed using mass spectrometry by the Institute of Ecology Analytical Chemical Laboratory at the University of Georgia (Athens, GA). Isotope composition is expressed as the deviation from a standard isotope ratio in parts per thousand (%o):

\[ \delta X = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 10^3 \]
Here, $X$ is either $^{15}\text{N}$ or $^{13}\text{C}$ and $R_{\text{sample}}$ is the measured sample ratio, $^{15}\text{N}/^{14}\text{N}$ or $^{13}\text{C}/^{12}\text{C}$, respectively. For nitrogen, $R_{\text{standard}}$ is the isotopic ratio of atmospheric nitrogen. For carbon, $R_{\text{standard}}$ is the isotope ratio of the Peedee belemnite marine limestone.

Crop samples were recovered from 17 chicks, three fledglings, and one adult. The $\delta^{15}\text{N}$ of crop samples did not differ between fledglings and chicks ($\bar{x}_{\text{fledglings}}$ (SD) = 4.22 \% (0.160), $\bar{x}_{\text{chicks}}$ (SD) = 4.37 \% (0.414), $t_{3.17} = -0.610, P = 0.549$) so they were combined in further analyses. The $\delta^{15}\text{N}$ of crop samples from offspring differed significantly from that of whole food ($\bar{x}_{\text{crop}}$ (SD) = 4.35 \% (0.387), $\bar{x}_{\text{whole food}}$ (SD) = 5.32 \% (0.228), $t_{20.9} = -6.970, P < 0.001$). The $\delta^{15}\text{N}$ from the single adult crop sample was 5.17 \%. The $\delta^{13}\text{C}$ of crop samples did not differ between fledglings and chicks ($\bar{x}_{\text{fledglings}}$ (SD) = -13.06 \% (0.562), $\bar{x}_{\text{chicks}}$ (SD) = -14.74 \% (1.992), $t_{3.17} = 1.422, P = 0.172$) so they were combined in further analyses. The $\delta^{13}\text{C}$ of crop samples from offspring differed significantly from that of the whole food ($\bar{x}_{\text{crop}}$ (SD) = -14.49 \% (1.938), $\bar{x}_{\text{whole food}}$ (SD) = -17.49 \% (0.719), $t_{20.9} = 6.071, P < 0.001$). The $\delta^{13}\text{C}$ from the single adult crop sample was -12.56 \%. Since the stable nitrogen and carbon isotope signatures of whole food were clearly different from that of the recovered crop samples, I did not consider whole food as representative of the diets of any age group. The whole-food isotope signatures were not used for further analyses.

Fractionation is the difference between the isotope signatures of the tissue of interest and the nutrients used to build or maintain that tissue. In this study, the isotope signatures of the tissue were measured directly from liver samples from each individual. I assumed that the isotope signatures of crop samples recovered from offspring were accurate representations of the nutrients used to build and maintain their liver tissue. For each offspring for which a crop sample was available, I calculated fractionation as the difference between the isotope composition of the offspring's liver and the isotope composition of the crop sample from that individual. For each
offspring without a crop sample available, I calculated fractionation as the difference between the composition of the offspring’s liver and the mean composition of all offspring crop samples.

Calculating fractionation for adults requires an assumption about the adult diet, since only one adult crop sample was recovered. Fractionation in adults could be calculated assuming either that the single crop sample recovered from an adult is representative of the diets of all the adults or that the crop samples recovered from the offspring are representative of the adult diet. A third alternative, assuming that the adult diet was similar to the whole food provided, does not appear to be a valid alternative given the difference between the isotope signature of whole food and that of the crop samples. It is clear the adults showed preferences when choosing food for their chicks, and it is likely that they also had preferences when feeding themselves. For analyses, I calculated two different fractionation values for adults. The first fractionation value for each adult was the difference between the isotope composition of the adult’s liver and isotope composition of the single adult crop sample. The second adult fractionation calculation used the mean isotope composition of the offspring crop samples instead of the adult crop sample.

Results

Eight zebra finch breeding pairs produced 50 offspring in 20 broods; two pairs never produced young. Of the 50 offspring, 16 were not included in these analyses—one newly-hatched chick was euthanized after an injury, 13 died from inadequate parental care, and two underweight chicks euthanized at the conclusion of this experiment were too small to include in the 6-8 g group. Of the 34 offspring analyzed, four were euthanized at the end of the experiment but were included because they were near fledging or their mass was nearly 6 g. Another 6.4 g chick was euthanized after an injury and included with the 6-8 g group. Liver samples were analyzed from 14 fledglings, 20 chicks, and 20 adults.

Nitrogen.—Liver δ¹⁵N differed among fledglings, chicks, and adults (Fig. 3.1; ANOVA, df 11 and 42, adj. \( R^2 = 0.687, F = 11.561, P < 0.001 \); age group effect: df = 2 and 42, F = 36.073,
$P = 0.021$; all Tukey's HSD comparisons $P \leq 0.001$). In addition, the cage from which the individuals came had a significant effect on liver $\delta^{15}N$ (df = 9 and 42, $F = 2.525$, $P = 0.021$). When the adult diet is represented by the adult crop sample, adult nitrogen fractionation did not differ from either chicks or fledglings (Fig. 3.2; ANOVA, df = 2 and 51, adj. $R^2 = 0.100$, $F = 3.950$, $P = 0.025$; $P$-values of Tukey's HSD comparisons between the adults and each other group were $\geq 0.155$). However, fractionation differed between chicks and fledglings (Tukey's HSD, $P = 0.024$). No cage effect was included in this analysis because the model was not significant (ANOVA, df = 11 and 42, $F = 1.358$, $P = 0.229$) when the cage effect was included and no effect of cage was detected in that model (cage effect: df = 9 and 42, $F = 0.811$, $P = 0.609$).

When the adult diet is represented by the offspring crop samples, fractionation differs among all age groups (Fig. 3.2; ANOVA, df = 11 and 42, adj. $R^2 = 0.480$, $F = 5.448$, $P < 0.001$; age group effect: df = 2 and 42, $F = 19.458$, $P < 0.001$; $P$-values of Tukey's HSD comparisons between each pair of groups were $\leq 0.028$). Cage had no effect on fractionation with this model (df = 9 and 42, $F = 0.811$, $P = 0.609$).

**Carbon.**—Liver $\delta^{13}C$ did not differ among fledglings, chicks, and adults but was affected by cage (Fig. 3.3; ANOVA, df = 11 and 42, adj. $R^2 = 0.438$, $F = 4.750$, $P < 0.001$; cage effect: df = 9 and 42, $F = 5.030$, $P < 0.001$; age group effect: df = 2 and 42, $F = 1.318$, $P = 0.279$). When the adult diet is represented by the adult crop sample, cage significantly affects carbon fractionation but age group does not (Fig. 3.4; ANOVA, df = 11 and 42, adj. $R^2 = 0.255$, $F = 2.650$, $P = 0.010$; cage effect: df = 9 and 42, $F = 2.656$, $P = 0.016$; age group effect: df = 2 and 42, $F = 2.752$, $P = 0.075$). When the adult diet is represented by the offspring crop samples, the results are similar to the previous analysis. Cage significantly affects carbon fractionation but age group does not (Fig. 3.4; ANOVA, df = 11 and 42, adj. $R^2 = 0.227$, $F = 2.412$, $P = 0.020$; cage effect: df = 9 and 42, $F = 2.656$, $P = 0.016$; age group effect: df = 2 and 42, $F = 0.648$, $P = 0.528$).
Figure 3.1. Box plot of $\delta^{15}N$ of liver and crop samples. The line inside each box represents the median for the group while the box itself shows the limits of the middle half of the data. The whiskers above and below the box reach to the nearest data point not beyond a span of 1.5 times the inter-quartile range. Data points outside the range of the whiskers are plotted as open circles. The value for the single adult crop sample is shown as a line.
Figure 3.2. Box plot of food-liver $^{15}$N fractionation for chicks, fledglings, and adults.

Fractionation for adults is calculated using two alternatives: 1) the adult diet is represented by the single adult crop sample recovered, and 2) the adult diet is represented by the offspring crop samples. See Fig. 3.1 for box plot conventions.
Figure 3.3. Box plot of $\delta^{13}$C of liver and crop samples. See Fig. 3.1 for box plot conventions.
Figure 3.4. Box plot of food-liver $^{13}$C fractionation for chicks, fledglings, and adults.

Fractionation for adults is calculated using two alternatives: 1) the adult diet is represented by the single adult crop sample recovered, and 2) the adult diet is represented by the offspring crop samples. See Fig. 3.1 for box plot conventions.
Discussion

Nitrogen fractionation between food and liver tissue was slightly, but significantly, reduced in fledglings compared to half-grown chicks. While this study was not designed to investigate the basis any differences found, the change in fractionation may be related to maturity of the liver tissue of these two age groups. When dissecting the offspring, I noted clear physical differences between the livers of the two groups. While the livers of fledglings were solid tissue and remained intact when removed from the carcass, those of chicks were less deeply colored and lost all solid form once the liver membrane was cut. Whether these structural differences correspond to differences in tissue function or maturity and result in the observed differences in nitrogen fractionation between chicks and fledglings is unknown.

Nitrogen fractionation results for adults are difficult to interpret because of the uncertainty of the exact adult diet and corresponding uncertainty of which nitrogen fractionation calculations are most valid. If the single adult crop sample represented the adult diet well, then nitrogen fractionation in adults is intermediate to that of chicks and fledglings and not different from either. If the offspring crop samples represented the adult diet well, then nitrogen fractionation in adults is significantly greater than in either chicks or fledglings. Future studies need to explicitly determine adult diets instead of relying on crop samples for adult diet characterization.

Carbon fractionation did not differ among any of the age groups, regardless of which alternative was used to calculate adult fractionation (Fig. 3.4). The lack of any differences is not surprising given the huge variability of the $\delta^{13}C$ and carbon fractionation calculations. Homogeneity of the whole food samples, resulting in a small variance for $\delta^{13}C$, is probably concealing large variability in the $\delta^{13}C$ of individual seed and pellet types. Combined with individual food preferences by adults, this would explain the large range of $\delta^{13}C$ observed for
liver samples and crop contents (Fig. 3.3). The significant cage effects observed for the carbon analyses lend support to the presence of individual preferences among the adults.

Hobson and Clark (1992b) found food-liver carbon fractionation of 0.4±0.2, 0.2±0.6, and -0.4±1.0 for chicken (Gallus gallus), quail (Coturnix japonica), and gulls (Larus delawarensis), respectively, at the end of their growth stage. The carbon fractionation values for zebra finches of all ages from this study formed a larger range of values but included those from Hobson and Clark (1992b). Mizuntani et al. (1991) found that an adult cormorant (Phalacrocorax carbo) had a carbon fractionation of 1.3%, a value on the high end of the range found for zebra finches.

In summary, this study shows that nitrogen fractionation, at least for the liver, is not constant throughout avian development, and this pattern may be related to changes in liver tissue maturity. Thus, stable isotope methodology cannot be used to accurately trace nitrogen sources in growing birds without controlling for age or maturity. For carbon in the liver, no changes in fractionation were detected in the age groups I sampled. Tentatively, stable isotope methodology may be applied for tracking carbon sources in birds of all ages and maturity, but further studies are required.
Chapter 4

Conclusion

These two studies are critical to understanding factors that might affect fractionation when using stable isotope methodology to examine protein intake and utilization by nestling Cedar Waxwings. I found that the response of nitrogen fractionation to different relative amounts of plant- and insect-derived protein in adult Cedar Waxwing diets is apparently complex. Before using stable isotope methodology on nestling Cedar Waxwings or other birds with distinct protein sources, it will be essential to calibrate nitrogen fractionation with diets comprised of various mixtures of the protein types available. If the results from the Zebra Finch study can be generalized, then nitrogen fractionation is also affected by developmental stage so the age or maturity of birds sampled must be controlled for when applying stable isotope methodology. Again, calibration of fractionation for subjects of different ages or maturity will be helpful.

This research also documents that nitrogen fractionation varies with mass loss. Previous work has shown that the stable nitrogen isotope signature of tissues increases under conditions of nutritional stress (Hobson et al. 1993) but changes in fractionation had never before been linked directly to mass loss in a longitudinal study.

Recently, there has been concern that the ease with which stable isotopes can now be measured may lead to animal studies without appropriate laboratory calibration and without regard to physiological processes (Gannes et al. 1997, 1998). The present studies highlight the difficulties that might occur even when performing calibration studies and paying attention to physiology. However, stable isotope methodology still holds great promise for applications in animal ecophysiology.
### Appendix 1

**Nutritional Information for the Cedar Waxwing Diets**

Table A1.1. Nutritional makeup of standard and treatment diets.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Ingredient</th>
<th>Total protein (%)</th>
<th>Total amount (g)</th>
<th>Protein (g)</th>
<th>Carbohydrate (g)</th>
<th>Fat (g)</th>
<th>Energy (kcal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>banana</td>
<td>19.6%</td>
<td>680</td>
<td>7.00</td>
<td>159.32</td>
<td>3.26</td>
<td>625.6</td>
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<tr>
<td></td>
<td>soy protein</td>
<td>56.4%</td>
<td>25</td>
<td>20.17</td>
<td>1.84</td>
<td>0.85</td>
<td>84.5</td>
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<tr>
<td></td>
<td>wheat germ</td>
<td>24.0%</td>
<td>37</td>
<td>8.57</td>
<td>19.17</td>
<td>3.60</td>
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<tr>
<td></td>
<td>vegetable oil</td>
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<td>7</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>61.9</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td></td>
<td><strong>749</strong></td>
<td><strong>35.74</strong></td>
<td><strong>180.33</strong></td>
<td><strong>14.71</strong></td>
<td><strong>905.2</strong></td>
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<tr>
<td>High</td>
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<td>680</td>
<td>7.00</td>
<td>159.32</td>
<td>3.26</td>
<td>625.6</td>
</tr>
<tr>
<td></td>
<td>sugar†</td>
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<td>24</td>
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<td>92.9</td>
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<td>18.65</td>
<td>2.75</td>
<td>5.17</td>
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<td></td>
<td>mealworms</td>
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<td>90</td>
<td>16.79</td>
<td>3.26</td>
<td>12.28</td>
<td>179.1</td>
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<td>7</td>
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<td>0</td>
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<td><strong>189.31</strong></td>
<td><strong>27.71</strong></td>
<td><strong>1080.1</strong></td>
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<td>7.00</td>
<td>159.32</td>
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<td>625.6</td>
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<td></td>
<td>soy protein</td>
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<td>14</td>
<td>11.30</td>
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<tr>
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<td>10.26</td>
<td>1.99</td>
<td>7.50</td>
<td>109.5</td>
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<td></td>
<td>vegetable oil</td>
<td>0.0%</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>61.9</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td></td>
<td><strong>836</strong></td>
<td><strong>39.95</strong></td>
<td><strong>189.00</strong></td>
<td><strong>21.40</strong></td>
<td><strong>1014.7</strong></td>
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<tr>
<td>Low</td>
<td>banana</td>
<td>18.3%</td>
<td>680</td>
<td>7.00</td>
<td>159.32</td>
<td>3.26</td>
<td>625.6</td>
</tr>
<tr>
<td></td>
<td>soy protein</td>
<td>40.1%</td>
<td>19</td>
<td>15.33</td>
<td>1.40</td>
<td>0.64</td>
<td>64.2</td>
</tr>
<tr>
<td></td>
<td>wheat germ</td>
<td>15.8%</td>
<td>26</td>
<td>6.02</td>
<td>13.47</td>
<td>2.53</td>
<td>93.6</td>
</tr>
<tr>
<td></td>
<td>sugar†</td>
<td>0.0%</td>
<td>8</td>
<td>0</td>
<td>7.99</td>
<td>0</td>
<td>31.0</td>
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<tr>
<td></td>
<td>crickets</td>
<td>13.6%</td>
<td>25</td>
<td>5.18</td>
<td>0.77</td>
<td>1.44</td>
<td>33.5</td>
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<tr>
<td></td>
<td>mealworms</td>
<td>12.2%</td>
<td>25</td>
<td>4.66</td>
<td>0.91</td>
<td>3.41</td>
<td>49.8</td>
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<tr>
<td></td>
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<td>0.0%</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>61.9</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td></td>
<td><strong>790</strong></td>
<td><strong>38.20</strong></td>
<td><strong>183.85</strong></td>
<td><strong>18.28</strong></td>
<td><strong>959.5</strong></td>
</tr>
</tbody>
</table>

*Glucose and fructose in 1:1 ratio.

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Table A1.2. Summary of macronutrients for standard and treatment diets. Energy content of all diet formulations was 1.21 kcal · g⁻¹.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Protein (%)</th>
<th>Carbohydrate (%)</th>
<th>Fat (%)</th>
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<tr>
<td>Standard</td>
<td>4.8</td>
<td>24.1</td>
<td>2.0</td>
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<tr>
<td>High insect</td>
<td>4.8</td>
<td>21.2</td>
<td>3.1</td>
</tr>
<tr>
<td>Med. Insect</td>
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<td>22.6</td>
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</tr>
<tr>
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<td>4.8</td>
<td>23.3</td>
<td>2.3</td>
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</tbody>
</table>


<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Protein · g⁻¹</th>
<th>Carbohydrate · g⁻¹</th>
<th>Fat · g⁻¹</th>
<th>Energy (kcal · g⁻¹)</th>
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</thead>
<tbody>
<tr>
<td>banana</td>
<td>0.0103</td>
<td>0.2343</td>
<td>0.0048</td>
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<td>0.0736</td>
<td>0.0339</td>
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<tr>
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<td>0.5180</td>
<td>0.0972</td>
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<td>3.87</td>
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<tr>
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<td>0.0306</td>
<td>0.0574</td>
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<tr>
<td>mealworms</td>
<td>0.1865</td>
<td>0.0362</td>
<td>0.1364</td>
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<td>0</td>
<td>1.0000</td>
<td>8.84</td>
</tr>
</tbody>
</table>
Appendix 2

Nutritional Information for the Zebra Finch Diet

The food used in the zebra finch study was Vita Finch diet by Sunscription (Bowling Green, OH). Ingredients of the Vita Finch diet were white proso millet, golden German millet, red proso millet, flax seed, canary grass seed, niger thistle, rape seed, oat groats, orange extract, dehulled soybean meal, meat and bone meal, fish meal, dehydrated alfalfa meal, brewers dried yeast, calcium phosphate, ground limestone, salt, vitamin A palmitate, D-activated animal sterol (source of vitamin D3), d-alpha tocopheryl acetate (source of vitamin E), monosodium phosphate, menadione sodium bisulfite (source of vitamin K activity), thiamine hydrochloride, niacinamide, cyanocobalamin in mannitol (source of vitamin B12), riboflavin, d-calcium pantothenate, menadione dimethylprimidinol bisulfate, choline bitartrate, pyridoxine hydrochloride, folic acid, biotin, manganese sulfate, ferric sulfate, zinc oxide, calcium iodate, cobalt carbonate, copper oxide, sodium selenite, sucrose, sorbitol, calcium propionate, DL-methionine, L-lysine, lecithin, ascorbic acid (source of vitamin C), natural and artificial flavors.

Nutritional analysis of the Vita Finch diet: crude protein (min.) 12%; crude fat (min.) 3%; crude fiber (max.) 12%; moisture (max.) 14%.
Bibliography


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SPSS. 1995. SPSS Base 7.0 for Windows users guide. SPSS Inc., Chicago, IL.


