The Journal of Experimental Biology 215, 3354-3361

doi:10.1242/jeb.068528

RESEARCH ARTICLE

A test of alternative models for increased tissue nitrogen isotope ratios during fasting in hibernating arctic ground squirrels

Trixie N. Lee[1,2,*], C. Loren Buck[3], Brian M. Barnes[2] and Diane M. O'Brien[2]

[1]Department of Biology and Wildlife, University of Alaska Fairbanks, PO Box 756100, Fairbanks, AK 99775, USA, [2]Institute of Arctic Biology, University of Alaska Fairbanks, PO Box 757000, Fairbanks, AK 99775, USA and [3]Department of Biological Sciences, University of Alaska Anchorage, 3101 Science Circle, Anchorage, AK 99508, USA

*Author for correspondence (trixie.lee@alaska.edu)

SUMMARY

We describe two models explaining the increase in tissue nitrogen isotope ratios ($\delta^{15}N$) that occurs during fasting in animals. The catabolic model posits that protein breakdown selectively removes the lighter isotope of nitrogen (^{14}N) from catabolized tissues, causing an increase in the proportion of heavy nitrogen isotope (^{15}N). The anabolic model posits that protein synthesis during fasting results in elevated $\delta^{15}N$ values, as the unreplaced loss of ^{14}N to urea results in a higher proportion of ^{15}N in plasma amino acids used for protein synthesis. We effected a range of lean mass loss in arctic ground squirrels (*Urocitellus parryii*) fasting during hibernation and then collected organ and muscle tissues for analysis of $\delta^{15}N$ values. The catabolic model predicts increased $\delta^{15}N$ values in both liver and muscle, as these tissues undergo significant catabolism during hibernation. The anabolic model predicts no change in muscle, but an increase in $\delta^{15}N$ values in liver, which has high levels of protein synthesis during euthermic phases of hibernation. We found a significant increase in liver $\delta^{15}N$ values and no change in muscle $\delta^{15}N$ values with lean mass loss, which supports the anabolic model. Heart, small intestine and brown adipose tissue also showed an increase in $\delta^{15}N$ values, indicating protein synthesis in these organ tissues during hibernation. Urine was 3.8‰ lighter than plasma, and both urine and plasma increased in $\delta^{15}N$ values with lean mass loss. This study helps clarify the mechanisms causing $\delta^{15}N$ change during nutritional stress, thus increasing its utility for physiological research and reconciling previously contradictory results.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/215/19/3354/DC1

Key words: stable nitrogen isotope, fasting, hibernation, arctic ground squirrel, anabolic model.

Received 21 November 2011; Accepted 11 June 2012

INTRODUCTION

The ecological and physiological strategies by which animals cope with adaptive fasting or starvation stress are subjects of enduring interest in physiological ecology (Mrosovsky and Sherry, 1980; Castellini and Rea, 1992; McCue, 2010). Some animals, including hibernating mammals, long distance migrants, and birds that incubate eggs continuously, undergo prolonged fasting as part of their annual cycle. These animals anticipate fasting by building up large fat stores to support metabolic costs, but they may also catabolize significant amounts of lean mass during the fast (Hobson et al., 1993; Buck and Barnes, 1999). Other animals exposed to unpredicted decreases in food availability may have insufficient fat stores and rely on catabolism of lean mass for energy to an even greater extent. Loss of lean mass during either fasting or starvation can impact survival or subsequent reproductive success of animals, but the importance and consequences of the use of lean tissue are difficult to investigate without repeated captures and analyses of body condition.

An increase in the nitrogen stable isotope ratio ($\delta^{15}N$) of tissues may serve as an indicator of lean mass loss during nutrient deprivation. Here, we focus on fasting and, given that the physiological responses to anticipated fasting and unanticipated starvation are similar, we use the term fasting to encompass these and other forms of nutrient deprivation, including protein deficiency. Several studies have shown increases in $\delta^{15}N$ values in tissues during fasting or nutritional stress (Hobson et al., 1993; Scrimgeour et al., 1995; Fuller et al., 2005; Boag et al., 2006; Alamaru et al., 2009). However, a growing number of studies have either failed to observe an increase (Castillo and Hatch, 2007; Kempster et al., 2007; McCue and Pollock, 2008; McFarlane Tranquilla et al., 2010; Mayor et al., 2011) or only detected it in specific tissues (Doucett et al., 1999; Gloutney et al., 1999; Cherel et al., 2005; Guelinckx et al., 2007). Reconciling these apparently contradictory results will require a more complete understanding of the mechanisms underlying changes in $\delta^{15}N$ values in fasting animals (Gannes et al., 1997; Martínez del Rio et al., 2009).

Animal nitrogenous waste has a low $\delta^{15}N$ value relative to body nitrogen (Steele and Daniel, 1978; Minagawa and Wada, 1984; Sponheimer et al., 2003); therefore, the whole-animal $\delta^{15}N$ value is expected to increase when urinary loss is not replaced by dietary nitrogen (Gannes et al., 1997). However, it is less clear how individual tissues are affected by the fasting body's net loss of light nitrogen. Previous studies have suggested two mechanisms to explain increases in tissue $\delta^{15}N$ values during fasting. The first mechanism we term the 'catabolic' model, in which a disproportionate loss of ^{14}N-containing amino acids during protein breakdown causes an increase in the residual $\delta^{15}N$ value of any tissue undergoing catabolism (Gannes et al., 1997; Gloutney et al., 1999; Martínez del Rio and Wolf, 2005; Gaye-Siessegger et al., 2007; Lohuis et al., 2007; McCue, 2008; McFarlane Tranquilla et

al., 2010). This model derives from the results of Hobson et al. in which fasting geese lost significant mass from both liver and muscle (58% and 44%, respectively), and both of these tissues increased in $\delta^{15}N$ values (Hobson et al., 1993). However, several authors have advocated an alternative 'anabolic' model in which protein synthesis, not breakdown, leads to increases in tissue $\delta^{15}N$ values (Scrimgeour et al., 1995; Focken, 2001; Fuller et al., 2005; Boag et al., 2006; Wolf et al., 2009; Habran et al., 2010). In this model, loss of lighter nitrogen from the free amino acid pool as a result of excretion causes the $\delta^{15}N$ value of residual amino acids to increase when not replaced by exogenous sources (Sick et al., 1997). These 'heavy' amino acids are incorporated into tissues during protein synthesis when they are being rebuilt, causing increased tissue $\delta^{15}N$ values. Here, the terms anabolic and catabolic refer to which of the two concurrent metabolic processes is the proximate cause of increasing tissue $\delta^{15}N$ values and do not imply a net metabolic state of the tissue. Understanding which mechanism is at work is crucial to accurately interpreting $\delta^{15}N$ values in investigations of animal physiology during nutritional deprivation. For example, if the catabolic model is correct, $\delta^{15}N$ values would increase in tissues in proportion to their use as catabolic protein stores. However, if the anabolic model is correct, increasing $\delta^{15}N$ values would identify tissues maintained by protein synthesis.

Protein catabolism and anabolism are generally thought to be balanced during protein turnover at steady states in feeding animals (Waterlow, 2006). During fasting, however, catabolism and anabolism become unbalanced to an extent that differs among tissues. In muscle, for example, catabolism greatly exceeds anabolism during fasting, and protein synthesis effectively ceases (Cherel et al., 1991; Waterlow, 2006). In contrast, protein synthesis in the liver continues at levels just below normal or increases with catabolism, imposing a demand for amino acids (Garlick et al., 1975; Cherel et al., 1991; Waterlow, 2006). These differences in protein turnover in muscle and liver during fasting lead to different predictions of isotopic change by the catabolic and anabolic models in these tissues. Specifically, if catabolism is the proximate mechanism for increasing $\delta^{15}N$ values in tissue during fasting, we would expect to see increased $\delta^{15}N$ values in both muscle and liver that were proportional to lean mass loss. In contrast, if anabolism is the mechanism for increasing $\delta^{15}N$ values in tissue during fasting, we would expect to see it in the liver only.

Arctic ground squirrels (*Urocitellus parryii*, Richardson 1825) present a useful model of natural fasting to distinguish between the anabolic and catabolic models. These small mammals (~800 g) hibernate for 7–8 months every year without eating or drinking, but they often excrete nitrogen as urea during periodic arousals from torpor to high body temperature. Free-living arctic ground squirrels lose a significant fraction of lean mass when hibernating at low ambient temperatures (Buck and Barnes, 1999). Although arctic ground squirrels readily tolerate body temperatures below 0°C during torpor under natural conditions (Buck et al., 2008), they must increase metabolism to prevent freezing as ambient temperatures decrease further (Buck and Barnes, 2000). Thermogenesis at sub-zero ambient temperatures appears to rely on a shift in metabolic fuels from strictly fat to increased use of carbohydrates generated from gluconeogenesis, the substrates of which are thought to be derived in part from protein breakdown (Buck and Barnes, 2000). The variability in lean mass loss under conditions to which arctic ground squirrels are naturally and periodically exposed generates a range of catabolism that should be sufficient to differentiate between the catabolic and anabolic models.

We evaluated the validity of the anabolic and catabolic models by comparing their differing predictions of change in $\delta^{15}N$ values in liver and muscle with measured changes in hibernating arctic ground squirrels. To generate variability in lean mass loss, we exposed squirrels to two temperature treatments that induced differential metabolic rates and fuel use for different durations of hibernation (Buck and Barnes, 2000). We then collected samples of liver, muscle, urine, plasma and other tissues. We used the patterns of $\delta^{15}N$ change with increasing lean mass loss in muscle and liver to distinguish whether catabolic or anabolic processes are responsible for tissue isotopic change during fasting. Based on these outcomes, we used the supported model to interpret changes in $\delta^{15}N$ values in other tissues of interest during hibernation (heart, small intestine and brown adipose tissue).

MATERIALS AND METHODS

Animals

This study took place in the autumn and winter of 2007–2008 and used 40 arctic ground squirrels (*U. parryii*, family Sciuridae) from a captive colony at the University of Alaska Fairbanks. Both male and female, non-growing squirrels were used. Squirrels were either captured near the University of Alaska Fairbanks' Toolik Field Station (68°38′N, 149°36′W) in the Arctic or born in captivity. The most recently captured squirrels used in this study had been in captivity for 4 months prior to the start of the experiment. Squirrels in captivity were maintained on a standard diet of Mazuri Rodent Chow ($\delta^{15}N$=2.7±1.2‰, $\delta^{13}C$=–20.9±0.8‰). Females with pups were provided with Purina Cat Chow ($\delta^{15}N$=2.9±0.6‰, $\delta^{13}C$=–16.2±0.3‰) as a supplement to support the increased protein demands of lactation from 2 weeks after birth (Vaughan et al., 2006). This supplement was removed from females at weaning and from juveniles 8 weeks prior to the beginning of the experiment. Animals were maintained on a 19.5 h:4.5 h light–dark cycle at an ambient temperature of 18–22°C until 13 August 2007 when they were moved to 8 h:16 h light–dark cycle and +2°C. As animals began to hibernate, they were sampled as described below and moved to a quiet chamber without food to continue hibernation. We placed wood shavings on the backs of torpid animals and noted an arousal interval when shavings had been disturbed (Pengelley and Fisher, 1961). All animal use procedures were approved by the University of Alaska Fairbanks Institutional Animal Care and Use Committee, protocol 06-40.

Study design

As animals began to hibernate, we randomly assigned them to treatment groups. Control animals (N=5) were sampled after 3–8 days of hibernation at +2°C. Experimental animals were assigned to two temperature treatments (+2 or –10°C) and three hibernation durations at each temperature (45, 68 or 90 days) for a total of six treatment groups (N=5 per group, N=30 total). Because squirrels in captivity under constant conditions may shorten their annual cycle, leading to decreased hibernation duration (Pengelley et al., 1976), we selected relatively short durations and assigned additional squirrels to each temperature treatment. One of these extra animals was allowed to hibernate for 115 days at –10°C and four squirrels at +2°C were sampled after naturally terminating hibernation after 82, 139, 177 and 232 days. Thus, the total sample size was N=40.

Estimation of lean mass through isotope dilution

We used isotope dilution to estimate lean mass using a calibration equation based on chemical analyses of body composition (Lee et al., 2011). We estimated initial lean mass of all squirrels except the control group on the second day of hibernation and final lean mass

of all animals prior to euthanasia (see below). In all cases (except the four squirrels that naturally terminated hibernation), we induced the squirrel to arouse to high body temperature by handling. Once the animal had achieved high body temperature, it was anesthetized by gas anesthesia (isoflurane, 3–5%) and a claw was clipped to collect a background sample of blood into heparinized capillary tubes that were then either flame-sealed for cool storage (deuterium analyses) or clay-sealed and frozen at –20°C (carbon and nitrogen analyses). A 3% deuterium injection was administered intraperitoneally according to the equation: injection volume (ml)=mass (g)×0.000867, and the animal was allowed to recover from anesthesia. After 61.4±2.4 min (mean ± s.d.), the animal was anesthetized again and a sample of deuterium-enriched blood was taken by claw clip. Experimental squirrels were then placed in the chamber at their assigned ambient temperature where they returned to torpor within 1–3 days.

Tissue sample collection

Animals were moved to a warm room (18–22°C) and induced to arouse. We monitored rectal temperature during arousal *via* a wax-tipped thermocouple inserted approximately 2.5 cm into the rectum; 9 h after the rectal temperature reached 30°C, we anesthetized the animal and repeated isotope dilution as above to estimate lean mass. After 1 h, the animal was anesthetized again and the second, enriched, isotope sample was taken by cardiac puncture, during which we also collected blood (2 ml) into heparinized Vacutainers for carbon and nitrogen isotope analysis of plasma and red blood cells. The animal was then euthanized while still under anesthesia by an overdose of sodium pentobarbital injected into the heart. Tissues (heart; liver; small intestine; brown adipose tissue; subcutaneous and abdominal white adipose tissue; and gastrocnemius, quadriceps, abdominal and scapular skeletal muscles) were removed and frozen at –20°C for isotope analysis. Urine samples were collected directly from the bladder with a syringe when possible (N=33) and frozen in cryovials at –20°C. Squirrels that terminated hibernation naturally were anesthetized and sampled as above on their third day at high body temperature.

Stable isotope analysis

Stable isotope samples were analyzed at the Alaska Stable Isotope Facility at the University of Alaska Fairbanks. For simultaneous carbon and nitrogen analysis, plasma (5.5 µl) and red blood cells (1.8 µl) were pipetted into tin capsules, which were then dried, weighed and crimp-sealed for analysis (0.1–0.3 mg dry mass). Organ tissue, muscle tissue and food samples were dried, powdered, homogenized and weighed out to 0.2–0.3 mg into tin capsules; brown adipose tissue was analyzed in dried fragments of 0.2–0.3 mg. Because white adipose tissue contains little nitrogen, these tissues were prepared in dried fragments of 0.4–1.2 mg for carbon analysis and 4–8 mg for nitrogen analysis during separate analyses. For urine sample preparation, 10 µl of 37% HCl was added to a small, muffled glass microfiber filter prior to the addition of 10 µl of urine to prevent loss of N by ammonification. These samples were dried in an oven (60°C) and placed into tin capsules for analyses. For deuterium (^{2}H) analysis, water was distilled from blood samples in flame-sealed Pasteur pipettes on hot plates and analyzed (Lee et al., 2011). Undistilled plasma from samples that were too small to distil was manually injected into the instrument for analysis. Previous work has shown no difference in lean mass calculated from analysis of distilled blood and undistilled plasma samples (Lee et al., 2011). Several blood samples in flame-sealed Natelson capillary tubes were lost during storage; matching samples from clay-sealed and frozen microhematocrit capillary tubes were analyzed in place of lost samples when available. Matching samples from each type of handling were analyzed to ensure that there was no difference in δ^2H values (t<1.64, P>0.05).

Sample isotope ratios were analyzed at the Alaska Stable Isotope Facility by continuous flow isotope ratio mass spectrometry. The carbon and nitrogen isotope ratios of solid samples were analyzed using a Costech ECS4010 elemental analyzer (Costech Scientific Inc., Valencia, CA, USA) interfaced to a Finnigan Delta^{Plus} XP isotope ratio mass spectrometer (IRMS) *via* the Conflo III interface (Thermo Fisher Scientific, Bremen, Germany). The hydrogen isotope ratio of plasma was analyzed using a ThermoElectron high temperature conversion elemental analyzer interfaced to a Finnigan Delta V Plus IRMS *via* the Conflo III interface (both instruments from Thermo Fisher Scientific). All isotope values are expressed in delta notation as $\delta X=[(R_{sample}–R_{standard})/R_{standard}]\times 1000‰$, where X is the heavy isotope, R is the ratio of heavy to light isotope, and the standards are as follows: N, atmospheric nitrogen (N_{air}); C, Vienna PeeDee belemnite (V-PDB); and H, Vienna standard mean ocean water (V-SMOW). Laboratory reference materials (δ^{15}N=7.0‰, δ^{13}C=–15.8‰) run concurrently with samples had δ^{15}N and δ^{13}C values of 7.0±0.2‰ and –15.8±0.1‰, respectively (N=104). We corrected δ^2H values based on standard calibration.

Statistical analysis

Estimates of lean mass were calculated from δ^2H values according to a species-specific calibration equation (see Lee et al., 2011), and loss could only be calculated on individuals with all four isotope dilution samples intact (N=32 out of 35). Control animals (N=5) were assigned 0 g lean mass loss for analyses. We compared δ^{15}N and δ^{13}C values of different tissues using ANOVA, and we used a t-test to compare all torpor bout lengths between temperature treatments. We used simple linear regression to determine how tissue δ^{15}N and δ^{13}C values varied as a function of lean mass loss (% total lean mass lost during experiment) after removing outliers identified by Mahalonobis distances (δ^{15}N: ≤3 per tissue from 7 of 13 tissues for a total of 10 of 424 measurements; δ^{13}C: ≤2 per tissue from 10 of 13 tissues for a total of 14 of 478 measurements). We performed all statistical analyses with JMP 8.0 (SAS Institute, Cary, NC, USA) using α of 0.05 and report all means ± s.d.

RESULTS

Arctic ground squirrels exposed to the experimental regimes of differing hibernation durations and ambient temperatures of +2 and –10°C exhibited a wide range of loss of total lean mass, from 8 to 49%. By 90 days, squirrels that hibernated at –10°C had lost twice as much lean mass as squirrels hibernating at +2°C (40.3±5.4% *versus* 19.1±9.5%). The extent of lean mass loss increased with hibernation duration at –10°C (0.19% per day, $F_{1,14}$=6.03, P=0.028), but not at +2°C ($F_{1,14}$=0.13, P=0.726; Fig. 1). Squirrels hibernating at +2°C lost on average 22.7±7.1% lean mass regardless of hibernation duration. Torpor bouts in squirrels hibernating at –10°C were significantly shorter (10±3 days, N=15) than those in squirrels hibernating at +2°C (13±3 days, N=15; t=–3.18, P=0.004), leading to more frequent arousals at –10°C. Lean mass loss was positively correlated with the number of times squirrels aroused to high body temperature during hibernation (N=32, R^2=0.524, $F_{1,30}$=32.97, P<0.0001).

Both nitrogen and carbon isotope ratios differed among tissues of animals at the beginning of hibernation (δ^{15}N: $F_{11,46}$=14.37, P<0.0001; δ^{13}C: $F_{12,52}$=13.16, P<0.0001; Table 1), consistent with other studies and predictions based on protein composition and lipid

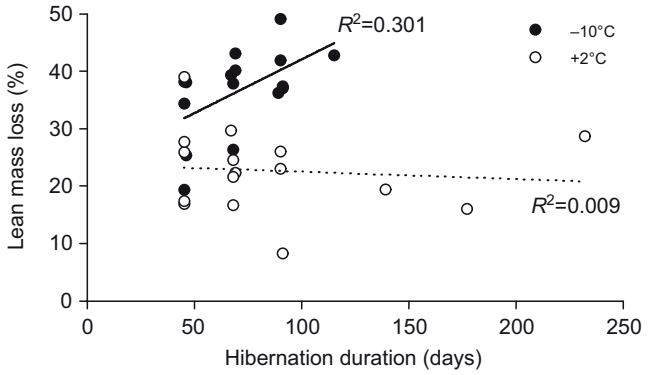

Fig. 1. Lean mass loss as a function of hibernation duration in arctic ground squirrels. The relationship was significant for animals hibernating at –10°C (N=16), but there was no relationship for animals hibernating at +2°C (N=16).

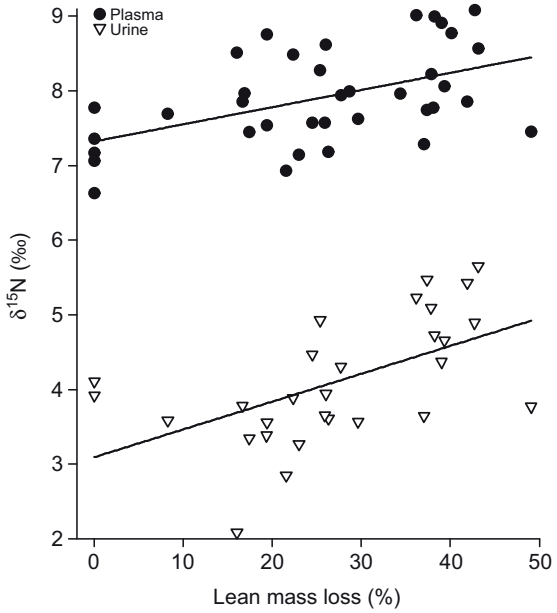

Fig. 2. Plasma and urine $\delta^{15}N$ values significantly increased as lean mass loss increased during hibernation in arctic ground squirrels (see Table 2). Plasma had consistently heavier $\delta^{15}N$ values than urine.

content (DeNiro and Epstein, 1977; Tieszen et al., 1983; Wolf et al., 2009). As squirrels lost lean mass, $\delta^{15}N$ values of plasma and urine increased (Fig. 2, Table 2). Plasma $\delta^{15}N$ values were consistently elevated relative to urine values throughout the range of lean mass loss (mean difference between paired samples: 3.8±0.9‰). As predicted by the anabolic model, $\delta^{15}N$ values in liver tissue increased with increasing lean mass loss while $\delta^{15}N$ values showed no change in the four skeletal muscles sampled. Heart, brown adipose tissue and small intestine also showed significant positive associations between $\delta^{15}N$ values and lean mass loss (all P<0.05, see Table 2, Fig. 3). White adipose tissue (subcutaneous and abdominal) and red blood cell $\delta^{15}N$ values did not change with lean mass loss (Fig. 4, Table 2). Carbon isotope ratios did not change with lean mass loss in any tissue except plasma, in which $\delta^{13}C$ values increased with increasing lean mass loss ($F_{1,33}$=4.70, P=0.038; all other tissues P>0.15).

DISCUSSION

Our results support the anabolic model as the primary mechanism by which nitrogen stable isotope signatures change in animal tissues during fasting. The anabolic model requires that urea synthesis selectively remove light isotopes from the plasma amino acid pool, causing the residual amino acid pool to increase in $\delta^{15}N$ value. Our results in fasting arctic ground squirrels supported this expectation: urine $\delta^{15}N$ values were on average 3.8‰ lighter than those of plasma, and both urine and plasma $\delta^{15}N$ values increased linearly with increasing lean mass loss. More crucially, we observed increasing $\delta^{15}N$ values in the liver, which is expected to continue synthesizing proteins even during extreme fasting, but not in four different skeletal muscle tissues, which are expected to undergo protein breakdown but little, if any, synthesis. The results from skeletal muscle contradict the catabolic model, which predicts that tissues that are broken down during fasting should increase in $\delta^{15}N$ values. Fig. 5 summarizes the key requirements of the anabolic

Table 1. Values of $\delta^{15}N$, $\delta^{13}C$ and C:N from all tissues in arctic ground squirrels sampled at the beginning of hibernation

Tissue	$\delta^{15}N$	$\delta^{13}C$	C:N
Plasma	7.2±0.4	–22.3±0.9	5.5±2.2
Heart	6.9±0.6	–22.5±0.7	4.5±0.2
Liver	7.6±0.5	–22.6±0.3	5.8±0.5
Small intestine	5.6±0.4	–22.2±0.5	4.3±0.2
Brown adipose tissue	4.2±1.0	–24.6±0.3	18.6±4.9
Quadriceps muscle	5.5±0.4	–21.9±0.5	4.3±0.6
Gastrocnemius muscle	5.7±0.3	–21.5±0.5	3.7±0.2
Scapular muscle	5.9±0.5	–22.2±0.5	4.6±0.7
Abdominal muscle	5.6±0.6	–21.6±0.8	3.7±0.3
Red blood cells	4.8±0.2	–21.5±0.3	3.3±0.1
Urine	5.0±1.2	–22.6±2.1	1.4±0.9
Subcutaneous WAT	4.4±0.5*	–24.8±0.3	371.2±111.8*
Abdominal WAT	**	–24.9±0.2	**

Values are means ± s.d. for the control group (N=5).
Subcutaneous and abdominal white adipose tissue (WAT) sample sizes are reduced because some samples had too little nitrogen present for a reliable analysis (*N=3, **N=0).

Table 2. Regression analyses of tissue $\delta^{15}N$ values against lean mass loss (%) in arctic ground squirrels sampled throughout hibernation

Tissue	N	R^2	F	d.f.	P
Plasma	37	0.248	11.53	1,35	**0.002**
Urine	29	0.307	11.97	1,27	**0.002**
Heart	34	0.273	12.03	1,32	**0.002**
Liver	37	0.153	6.34	1,35	**0.017**
Small intestine	36	0.305	14.92	1,34	**<0.001**
Brown adipose tissue	36	0.191	8.00	1,34	**0.008**
Quadriceps muscle	37	0.013	0.45	1,35	0.506
Gastrocnemius muscle	36	0.001	0.04	1,34	0.849
Scapular muscle	36	0.017	0.57	1,34	0.454
Abdominal muscle	35	0.003	0.10	1,33	0.756
Red blood cells	37	<0.001	0.00	1,35	0.957
Subcutaneous WAT	17	0.054	0.85	1,15	0.370
Abdominal WAT	7	0.013	0.06	1,5	0.810

Subcutaneous and abdominal white adipose tissue (WAT) sample sizes are reduced because some samples had too little nitrogen present for a reliable analysis.
Significant results are presented in bold.

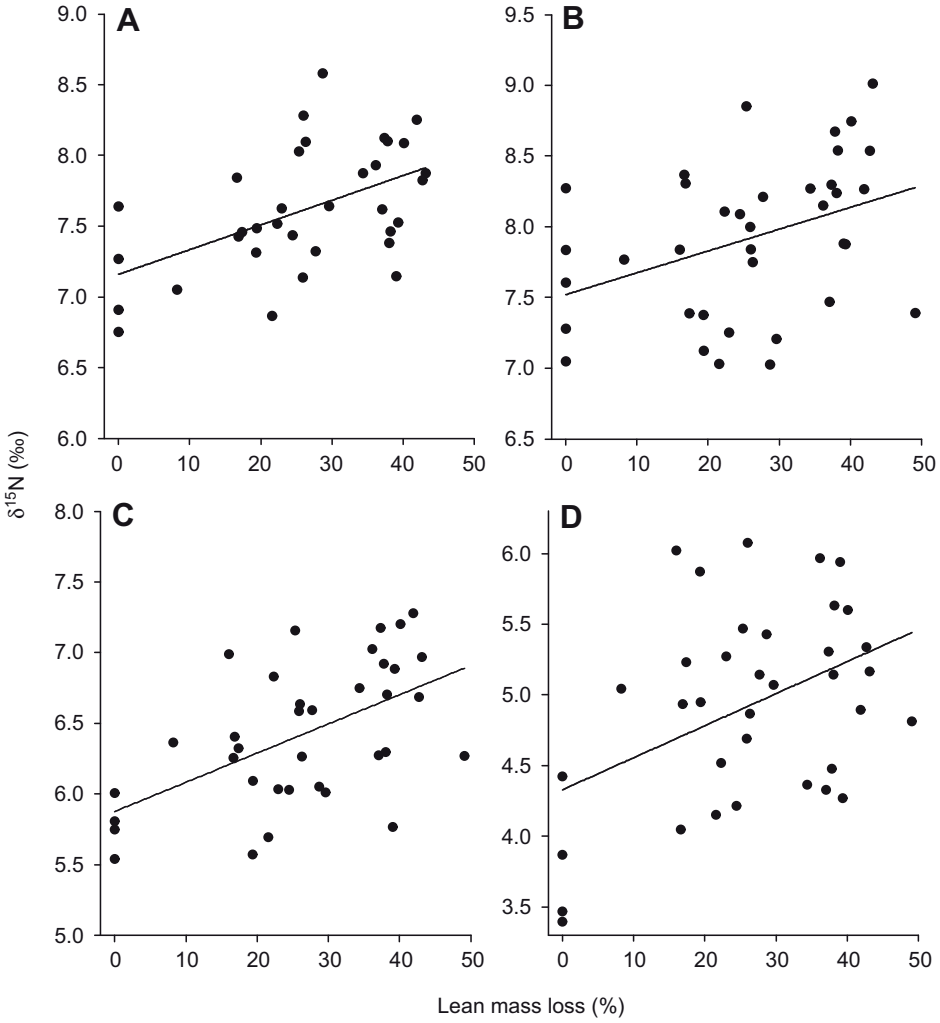

Fig. 3. Organ $\delta^{15}N$ value as a function of lean mass loss in hibernating arctic ground squirrels: (A) heart, (B) liver, (C) small intestine and (D) brown adipose tissue (see Table 2).

model, which are crucial to our understanding of the physiology that causes changes in $\delta^{15}N$ values among tissues during fasting.

The anabolic model posits that proteins synthesized during fasting and lean mass loss should incorporate amino acids with elevated $\delta^{15}N$ values. Fasting during hibernation includes phases of torpor, during which synthesis and degradation are arrested, and periodic arousals to high body temperature, when these processes return to high levels (Zhegunov et al., 1988; Gulevsky et al., 1992; van Breukelen and Martin, 2001; Velickovska et al., 2005). In our study, the net result was a dramatic loss of lean mass. Using the anabolic model, we interpret increased $\delta^{15}N$ values in heart, brown adipose tissue and small intestine to indicate that ground squirrels are actively synthesizing proteins in these tissues during the season of hibernation. Many organs function at full or even enhanced levels during periodic arousals, and some continue to function on a reduced level during torpor; for example, the heart rate is 5–10 beats min^{-1} (Storey, 2010). In small hibernators, periodic rewarming during arousals creates a heavy workload on the heart as it circulates the blood heated by non-shivering thermogenesis in brown adipose tissue (Storey, 2010). Indeed, the relative mass of heart and brown adipose tissue is greater in hibernating ground squirrels than in summer ground squirrels (Wickler et al., 1991). The digestive activity of the gastrointestinal tract, including the small intestine, is significantly reduced in animals that fast during hibernation (Hume et al., 2002), but evidence is emerging that the small intestine may stay active in other roles, such as immune function (Kurtz and Carey, 2007). The idea of small intestine function during hibernation is supported by our results. Finally, plasma $\delta^{13}C$ values increased with lean mass loss, suggesting a shift in mobilized fuels from lipid, which is isotopically light (DeNiro and Epstein, 1977), to protein and

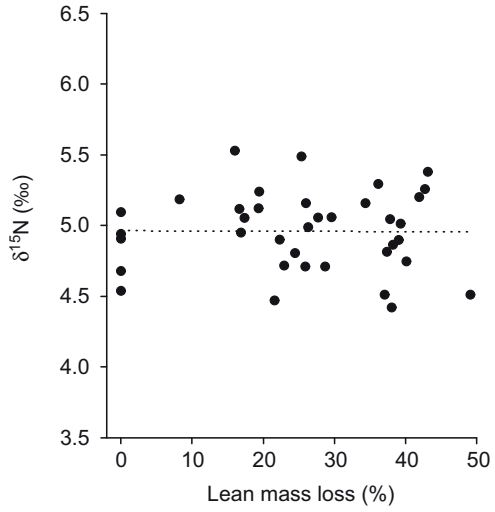

Fig. 4. Red blood cell $\delta^{15}N$ values are not related to lean mass loss and exemplify the result found in skeletal muscles (see Table 2).

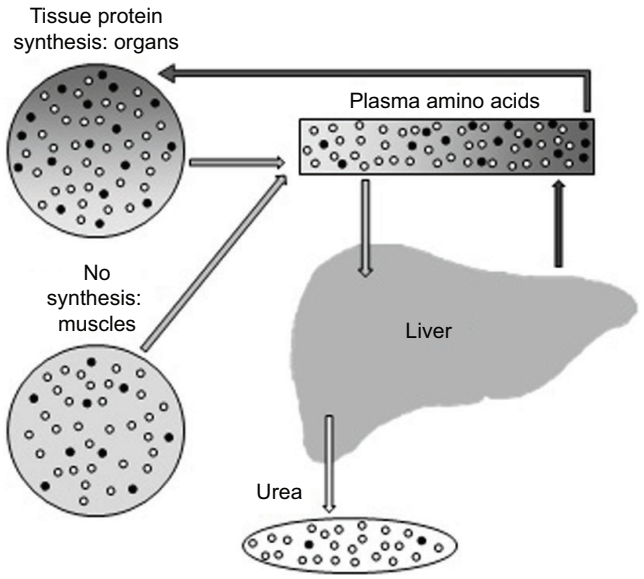

Fig. 5. A conceptual anabolic model of increasing $\delta^{15}N$ values of tissue during fasting. Dark circles and shading indicate heavy isotopes and increases in tissue $\delta^{15}N$ values, respectively; the abundance of heavy isotopes is grossly exaggerated for graphical purposes. Light circles and shading indicate light isotopes and no increase in tissue $\delta^{15}N$ values, respectively. We assume that amino acids from protein catabolism become part of the free amino acid pool in the plasma. Lighter amino acids are degraded to urea in the liver and excreted, while the remaining heavier amino acids are returned to the pool of amino acids in the plasma, increasing the $\delta^{15}N$ values of the plasma. Proteins formed from these heavier amino acids will have higher $\delta^{15}N$ values, as will the organs that are actively synthesizing protein and incorporating the amino acids with increased $\delta^{15}N$ values. The free amino acid pool carried in the plasma should demonstrate increases in $\delta^{15}N$ values proportional to the unreplaced loss of nitrogen from the breakdown of body proteins.

carbohydrate (isotopically heavy) as described previously (Buck and Barnes, 2000).

Discussions of increased tissue $\delta^{15}N$ values during fasting are commonly framed in the context of the catabolic model (Gannes et al., 1997; Gloutney et al., 1999; Kelly, 2000; Doi et al., 2007; Gaye-Siessegger et al., 2007; Lohuis et al., 2007; McCue, 2008; McFarlane Tranquilla et al., 2010). We conducted a review of studies citing Hobson et al. (Hobson et al., 1993), arguably the classic paper demonstrating increased tissue $\delta^{15}N$ values with nutritional stress, and noted whether studies invoked a version of the catabolic or anabolic model when ascribing a mechanism to this effect (Fig. 6). We found that isotope ecologists tend to invoke the catabolic and anabolic models with approximately equal frequency (Fig. 6C). Nutritional stress is not always clearly defined, so we have used the term nutritional status to also include studies in which animals may not have reached physiological levels of stress (Fig. 6A) and

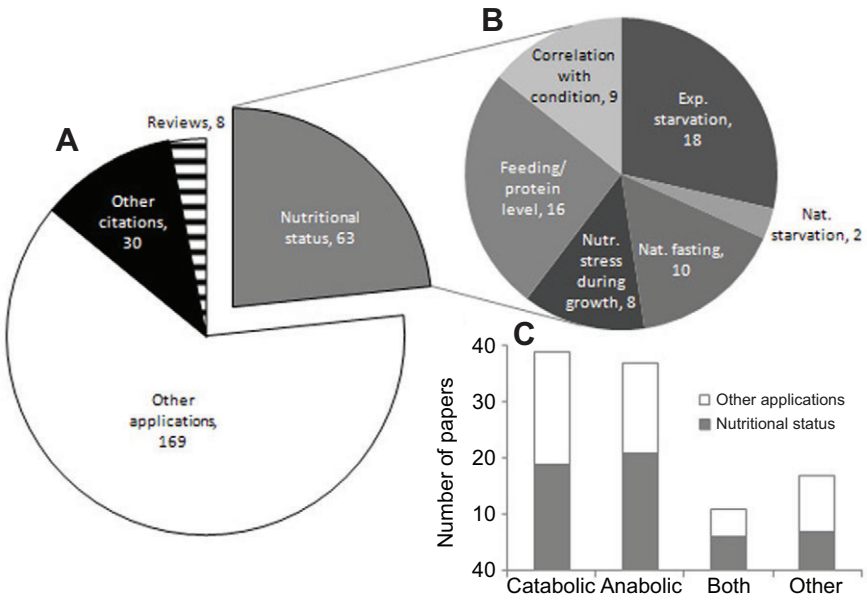

Fig. 6. A review of 270 papers that cited Hobson et al. (Hobson et al., 1993) indexed by ISI Web of Knowledge on 4 August 2011. (A) Papers were categorized as those having data related to changes in $\delta^{15}N$ from physiological mechanisms associated with nutritional status (Nutritional status), those acknowledging that nutritional status could influence $\delta^{15}N$ values during other applications of $\delta^{15}N$ values (Other applications), those citing Hobson et al. (Hobson et al., 1993) in ways not related to nutritional status (Other citations), and reviews and a model that discussed $\delta^{15}N$ values in the context of nutritional status (Reviews). (B) The study types of the data related to nutritional status are also presented in a smaller pie chart. Studies using experimental food deprivation were classified as 'Exp. starvation', whereas natural observations included starvation when food deprivation was not part of the animals' life cycle (Nat. starvation) and fasting (Nat. fasting) when it was. Some studies evaluated the effects of nutritional stress (Nutr. stress) during growth, the effects of different feeding or protein levels on $\delta^{15}N$ values, or tested for correlations between $\delta^{15}N$ values with measures of body condition. Full references for these studies can be found in supplementary material Table S1. (C) Papers in 'Other applications' and 'Nutritional status' were categorized according to the framework of discussion of $\delta^{15}N$ value changes. Studies followed the catabolic model (Catabolic), the anabolic model (Anabolic), both models (Both) or a different mechanism (Other). Several studies (N=118 in Other applications, N=10 in Nutritional status) did not discuss the mechanism.

classified these according to study type (Fig. 6B). Of the studies we reviewed that had original data relevant to nutritional status, about half provided sufficient information for us to evaluate consistency with the anabolic or catabolic model; all of these were consistent with the anabolic model. Many previous results that have been interpreted as contradictory can be reconciled by the anabolic model, especially those studies that failed to find changes in $\delta^{15}N$ values in catabolic tissues (Pfeiler et al., 1998; Doucett et al., 1999; Gloutney et al., 1999; McFarlane Tranquilla et al., 2010). Perhaps the most difficult to reconcile is the finding of Hobson et al. (Hobson et al., 1993) that muscle $\delta^{15}N$ values of geese increased during fasting. However, further work on that population showed no change in muscle $\delta^{15}N$ values during incubation and attributed the previously reported change to physiological changes during egg laying (Gloutney et al., 1999).

Another limitation of the catabolic model is the lack of a physiological mechanism of fractionation involved in catabolism. A systematic isotope effect during catabolism would require isotopic selectivity by the enzymes that mark proteins for breakdown, such as in the ubiquitin proteasome system active in muscle during fasting (Jagoe and Goldberg, 2001). However, there are a number of other factors that influence the selection of proteins for degradation during fasting, including which enzymes are activated (Jagoe and Goldberg, 2001). It is possible that such selection criteria eliminate the possibility of isotopic selectivity or obscure isotope effects. Although the enzymes that hydrolyze dipeptides (dipeptidases) show a preference for ^{14}N (Silfer et al., 1992), digestion of dipeptides is the final step of protein breakdown and should proceed to completion, masking any isotope effect.

Based on our findings and review of the literature, we recommend clarification in the way isotope ecologists understand and use changes in $\delta^{15}N$ values. Strictly speaking, the $\delta^{15}N$ values of an animal's tissues do not change with nutritional stress or even catabolism. Changes in tissue $\delta^{15}N$ values are a consequence of protein synthesis using amino acids derived from catabolism during loss of light nitrogen isotopes to waste. Not all nutritionally stressed animals reuse amino acids from catabolism. For example, growing animals incorporate lighter dietary nitrogen into new tissues in spite of nutritional stress (Kempster et al., 2007; Williams et al., 2007; Sears et al., 2009) and will only show increased $\delta^{15}N$ values if the stress is sufficient to necessitate breaking down and reincorporating endogenous stores (Hobson et al., 1993). In addition, the terms 'catabolic' and 'anabolic', when used as a general description of a whole animal, are not informative regarding changes in $\delta^{15}N$ values that occur on a tissue level. Crucial tissues, such as the liver (Waterlow, 2006), and tissues being formed, such as feathers (Cherel et al., 2005) and hair (Fuller et al., 2005), continue significant levels of protein synthesis while the whole animal may be considered catabolic. Thus, it is crucial to consider metabolic processes at the tissue level before making general conclusions regarding the animal's overall physiological state based on $\delta^{15}N$ values (Ben-David et al., 1999; Gloutney et al., 1999; Gómez-Campos et al., 2011).

In summary, we have shown that $\delta^{15}N$ values increase with lean mass loss in plasma, urine and organ tissues, but do not change in muscle or red blood cells during hibernation in the arctic ground squirrel. In Hobson et al.'s (Hobson et al., 1993) classic work, the authors recognized that metabolism associated with nutritional stress caused tissue $\delta^{15}N$ values to increase and proposed multiple processes that could be involved in this effect, including catabolism, protein mobilization or protein synthesis (which they termed 'redeposition processes'). Our data support the anabolic model, which articulates that the observed increase of $\delta^{15}N$ values during nutritional stress is associated with protein synthesis. We encourage more work on the changes that occur in $\delta^{15}N$ values during fasting, especially studies that measure rates of tissue protein synthesis and catabolism.

ACKNOWLEDGEMENTS

We thank Tim Howe and Norma Haubenstock of the Alaska Stable Isotope Facility for stable isotope analyses. We would also like to thank Franziska Kohl, Jeff Mayfield, Xela Indurkhya, Øivind Tøien, Chris Terzi, and Animal Quarters staff for help with animals and logistics. Sarah Nash, Caroline Van Hemert, Rebecca Young, Maria Bray, Laura Conner and Barbara Taylor provided constructive comments on the manuscript.

FUNDING

This work was supported by a collaborative National Science Foundation International Polar Year Grant [EF-0732755 to B.M.B. and D.M.O., EF-0732763 to C.L.B.]; a University of Alaska Fairbanks Center for Global Change Student Award to T.N.L.; and a United States Army Medical Research and Materiel Command Grant to B.M.B. [05178001].

REFERENCES

Alamaru, A., Yam, R., Shemesh, A. and Loya, Y. (2009). Trophic biology of *Stylophora pistillata* larvae: evidence from stable isotope analysis. *Mar. Ecol. Prog. Ser.* **383**, 85-94.

Ben-David, M., McColl, C. J., Boonstra, R. and Karels, T. J. (1999). ^{15}N signatures do not reflect body condition in Arctic ground squirrels. *Can. J. Zool.* **77**, 1373-1378.

Boag, B., Neilson, R. and Scrimgeour, C. M. (2006). The effect of starvation on the planarian *Arthurdendyus triangulatus* (Tricladida: Terricola) as measured by stable isotopes. *Biol. Fertil. Soils* **43**, 267-270.

Buck, C. L. and Barnes, B. M. (1999). Temperatures of hibernacula and changes in body composition of arctic ground squirrels over winter. *J. Mammal.* **80**, 1264-1276.

Buck, C. L. and Barnes, B. M. (2000). Effects of ambient temperature on metabolic rate, respiratory quotient, and torpor in an arctic hibernator. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **279**, R255-R262.

Buck, C. L., Breton, A., Kohl, F., Tøien, Ø. and Barnes, B. M. (2008). Overwinter body temperature patterns in free-living Arctic squirrels (*Spermophilus parryii*). In *Hypometabolism in Animals: Hibernation, Torpor and Cryobiology* (ed. B. G. Lovegrove and A. E. McKechnie), pp. 317-326. Pietermaritzburg, South Africa: University of KwaZulu-Natal.

Castellini, M. A. and Rea, L. D. (1992). The biochemistry of natural fasting at its limits. *Experientia* **48**, 575-582.

Castillo, L. P. and Hatch, K. A. (2007). Fasting increases $\delta^{15}N$-values in the uric acid of *Anolis carolinensis* and *Uta stansburiana* as measured by nondestructive sampling. *Rapid Commun. Mass Spectrom.* **21**, 4125-4128.

Cherel, Y., Attaix, D., Rosolowska-Huszcz, D., Belkhou, R., Robin, J.-P., Arnal, M. and Le Maho, Y. (1991). Whole-body and tissue protein synthesis during brief and prolonged fasting in the rat. *Clin. Sci.* **81**, 611-619.

Cherel, Y., Hobson, K. A., Bailleul, F. R. and Groscolas, R. (2005). Nutrition, physiology, and stable isotopes: new information from fasting and molting penguins. *Ecology* **86**, 2881-2888.

DeNiro, M. J. and Epstein, S. (1977). Mechanism of carbon isotope fractionation associated with lipid synthesis. *Science* **197**, 261-263.

Doi, H., Kikuchi, E., Takagi, S. and Shikano, S. (2007). Changes in carbon and nitrogen stable isotopes of chironomid larvae during growth, starvation and metamorphosis. *Rapid Commun. Mass Spectrom.* **21**, 997-1002.

Doucett, R. R., Booth, R. K., Power, G. and McKinley, R. S. (1999). Effects of the spawning migration on the nutritional status of anadromous Atlantic salmon (*Salmo salar*): insights from stable-isotope analysis. *Can. J. Fish. Aquat. Sci.* **56**, 2172-2180.

Focken, U. (2001). Stable isotopes in animal ecology: the effect of ration size on the trophic shift of C and N isotopes between feed and carcass. *Isotopes Environ. Health Stud.* **37**, 199-211.

Fuller, B. T., Fuller, J. L., Sage, N. E., Harris, D. A., O'Connell, T. C. and Hedges, R. E. M. (2005). Nitrogen balance and $\delta^{15}N$: why you're not what you eat during nutritional stress. *Rapid Commun. Mass Spectrom.* **19**, 2497-2506.

Gannes, L. Z., O'Brien, D. M. and Martínez del Rio, C. (1997). Stable isotopes in animal ecology: assumptions, caveats, and a call for more laboratory experiments. *Ecology* **78**, 1271-1276.

Garlick, P. J., Millward, D. J., James, W. P. T. and Waterlow, J. C. (1975). The effect of protein deprivation and starvation on the rate of protein synthesis in tissues of the rat. *Biochim. Biophys. Acta* **414**, 71-84.

Gaye-Siessegger, J., Focken, U., Abel, H. and Becker, K. (2007). Dietary back-calculation using stable isotopes: can activities of enzymes involved in amino acid metabolism be used to improve estimates of trophic shifts in fish? *Isotopes Environ. Health Stud.* **43**, 129-141.

Gloutney, M. L., Alisauskas, R. T., Hobson, K. A. and Afton, A. D. (1999). Use of supplemental food by breeding Ross's geese and lesser snow geese: evidence for variable anorexia. *Auk* **116**, 97-108.

Gómez-Campos, E., Borrell, A. and Aguilar, A. (2011). Nitrogen and carbon stable isotopes do not reflect nutritional condition in the striped dolphin. *Rapid Commun. Mass Spectrom.* **25**, 1343-1347.

Guelinckx, J., Maes, J., Van Den Driessche, P., Geysen, B., Dehairs, F. and Ollevier, F. (2007). Changes in $\delta^{13}C$ and $\delta^{15}N$ in different tissues of juvenile sand goby *Pomatoschistus minutus*: a laboratory diet-switch experiment. *Mar. Ecol. Prog. Ser.* **341**, 205-215.

Gulevsky, A. K., Zagnoiko, V. I. and Mishneva, L. G. (1992). The intensity of protein synthesis in organs of heterothermal animals on hibernation. *Cryo Lett.* **13**, 99-108.
Habran, S., Debier, C., Crocker, D. E., Houser, D. S., Lepoint, G., Bouquegneau, J.-M. and Das, K. (2010). Assessment of gestation, lactation and fasting on stable isotope ratios in northern elephant seals (*Mirounga angustirostris*). *Mar. Mamm. Sci.* **26**, 880-895.
Hobson, K. A., Alisauskas, R. T. and Clark, R. G. (1993). Stable-nitrogen isotope enrichment in avian tissues due to fasting and nutritional stress: implications for isotopic analyses of diet. *Condor* **95**, 388-394.
Hume, I., Beiglböck, C., Ruf, T., Frey-Roos, F., Bruns, U. and Arnold, W. (2002). Seasonal changes in morphology and function of the gastrointestinal tract of free-living alpine marmots (*Marmota marmota*). *J. Comp. Physiol. B* **172**, 197-207.
Jagoe, R. T. and Goldberg, A. L. (2001). What do we really know about the ubiquitin-proteasome pathway in muscle atrophy? *Curr. Opin. Clin. Nutr. Metab. Care* **4**, 183-190.
Kelly, J. F. (2000). Stable isotopes of carbon and nitrogen in the study of avian and mammalian trophic ecology. *Can. J. Zool.* **78**, 1-27.
Kempster, B., Zanette, L., Longstaffe, F. J., MacDougall-Shackleton, S. A., Wingfield, J. C. and Clinchy, M. (2007). Do stable isotopes reflect nutritional stress? Results from a laboratory experiment on song sparrows. *Oecologia* **151**, 365-371.
Kurtz, C. C. and Carey, H. V. (2007). Seasonal changes in the intestinal immune system of hibernating ground squirrels. *Dev. Comp. Immunol.* **31**, 415-428.
Lee, T. N., Fridinger, R. W., Barnes, B. M., Buck, C. L. and O'Brien, D. M. (2011). Estimating lean mass over a wide range of body composition: a calibration of deuterium dilution in the arctic ground squirrel. *Rapid Commun. Mass Spectrom.* **25**, 3491-3496.
Lohuis, T. D., Harlow, H. J. and Beck, T. D. I. (2007). Hibernating black bears (*Ursus americanus*) experience skeletal muscle protein balance during winter anorexia. *Comp. Biochem. Physiol.* **147B**, 20-28.
Martínez del Rio, C. and Wolf, B. O. (2005). Mass-balance models for animal isotopic ecology. In *Physiological and Ecological Adaptations to Feeding in Vertebrates* (ed. J. M. Starck and T. Wang), pp. 141-174. Enfield, NH: Science Publishers.
Martínez del Rio, C. M., Wolf, N., Carleton, S. A. and Gannes, L. Z. (2009). Isotopic ecology ten years after a call for more laboratory experiments. *Biol. Rev. Camb. Philos. Soc.* **84**, 91-111.
Mayor, D. J., Cook, K., Thornton, B., Walsham, P., Witte, U. F. M., Zuur, A. F. and Anderson, T. R. (2011). Absorption efficiencies and basal turnover of C, N and fatty acids in a marine Calanoid copepod. *Funct. Ecol.* **25**, 509-518.
McCue, M. D. (2008). Endogenous and environmental factors influence the dietary fractionation of ^{13}C and ^{15}N in hissing cockroaches *Gromphadorhina portentosa*. *Physiol. Biochem. Zool.* **81**, 14-24.
McCue, M. D. (2010). Starvation physiology: reviewing the different strategies animals use to survive a common challenge. *Comp. Biochem. Physiol.* **156A**, 1-18.
McCue, M. D. and Pollock, E. D. (2008). Stable isotopes may provide evidence for starvation in reptiles. *Rapid Commun. Mass Spectrom.* **22**, 2307-2314.
McFarlane Tranquilla, L. M., Hedd, A., Burke, C., Montevecchi, W. A., Regular, P. M., Robertson, G. J., Stapleton, L. A., Wilhelm, S. I., Fifield, D. A. and Buren, A. D. (2010). High Arctic sea ice conditions influence marine birds wintering in Low Arctic regions. *Estuar. Coast. Shelf Sci.* **89**, 97-106.
Minagawa, M. and Wada, E. (1984). Stepwise enrichment of ^{15}N along food chains: further evidence and the relation between $\delta^{15}N$ and animal age. *Geochim. Cosmochim. Acta* **48**, 1135-1140.
Mrosovsky, N. and Sherry, D. F. (1980). Animal anorexias. *Science* **207**, 837-842.
Pengelley, E. T. and Fisher, K. C. (1961). Rhythmical arousal from hibernation in the golden-mantled ground squirrel, *Citellus lateralis tescorum. Can. J. Zool.* **39**, 105-120.
Pengelley, E. T., Asmundson, S. J., Barnes, B. M. and Aloia, R. C. (1976). Relationship of light intensity and photoperiod to circannual rhythmicity in the hibernating ground squirrel, *Citellus lateralis. Comp. Biochem. Physiol.* **53A**, 273-277.
Pfeiler, E., Lindley, V. A. and Elser, J. J. (1998). Elemental (C, N and P) analysis of metamorphosing bonefish (*Albula* sp.) leptocephali: relationship to catabolism of endogenous organic compounds, tissue remodeling, and feeding ecology. *Mar. Biol.* **132**, 21-28.
Scrimgeour, C. M., Gordon, S. C., Handley, L. L. and Woodford, J. A. T. (1995). Trophic levels and anomalous $\delta^{15}N$ of insects on raspberry (*Rubus idaeus* L.). *Isotopes Environ. Health Stud.* **31**, 107-115.
Sears, J., Hatch, S. A. and O'Brien, D. M. (2009). Disentangling effects of growth and nutritional status on seabird stable isotope ratios. *Oecologia* **159**, 41-48.
Sick, H., Roos, N., Saggau, E., Haas, K., Meyn, V., Walch, B. and Trugo, N. (1997). Amino acid utilization and isotope discrimination of amino nitrogen in nitrogen metabolism of rat liver *in vivo*. *Z. Ernahrungswiss.* **36**, 340-346.
Silfer, J. A., Engel, M. H. and Macko, S. A. (1992). Kinetic fractionation of stable carbon and nitrogen isotopes during peptide bond hydrolysis: experimental evidence and geochemical implications. *Chem. Geol.* **101**, 211-221.
Sponheimer, M., Robinson, T. F., Roeder, B. L., Passey, B. H., Ayliffe, L. K., Cerling, T. E., Dearing, M. D. and Ehleringer, J. R. (2003). An experimental study of nitrogen flux in llamas: is ^{14}N preferentially excreted? *J. Archaeol. Sci.* **30**, 1649-1655.
Steele, K. W. and Daniel, R. M. (1978). Fractionation of nitrogen isotopes by animals: a further complication to the use of variations in the natural abundance of ^{15}N for tracer studies. *J. Agric. Sci.* **90**, 7-9.
Storey, K. B. (2010). Out cold: biochemical regulation of mammalian hibernation – a mini-review. *Gerontology* **56**, 220-230.
Tieszen, L. L., Boutton, T. W., Tesdahl, K. G. and Slade, N. A. (1983). Fractionation and turnover of stable carbon isotopes in animal tissues: implications for $\delta^{13}C$ analysis of diet. *Oecologia* **57**, 32-37.
van Breukelen, F. and Martin, S. L. (2001). Translational initiation is uncoupled from elongation at 18 degrees C during mammalian hibernation. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **281**, R1374-R1379.
Vaughan, D. K., Gruber, A. R., Michalski, M. L., Seidling, J. and Schlink, S. (2006). Capture, care, and captive breeding of 13-lined ground squirrels, *Spermophilus tridecemlineatus*. *Lab Anim. (NY)* **35**, 33-40.
Velickovska, V., Lloyd, B. P., Qureshi, S. and van Breukelen, F. (2005). Proteolysis is depressed during torpor in hibernators at the level of the 20S core protease. *J. Comp. Physiol. B* **175**, 329-335.
Waterlow, J. C. (2006). *Protein Turnover*. Oxfordshire, UK: CAB International.
Wickler, S. J., Hoyt, D. F. and van Breukelen, F. (1991). Disuse atrophy in the hibernating golden-mantled ground squirrel, *Spermophilus lateralis. Am. J. Physiol.* **261**, R1214-R1217.
Williams, C. T., Buck, C. L., Sears, J. and Kitaysky, A. S. (2007). Effects of nutritional restriction on nitrogen and carbon stable isotopes in growing seabirds. *Oecologia* **153**, 11-18.
Wolf, N., Carleton, S. A. and Martínez del Rio, C. (2009). Ten years of experimental animal isotopic ecology. *Funct. Ecol.* **23**, 17-26.
Zhegunov, G. F., Mikulinsky, Y. E. and Kudokotseva, E. V. (1988). Hyperactivation of protein synthesis in tissues of hibernating animals on arousal. *Cryo Lett.* **9**, 236-245.