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Abstract
I conducted a field experiment to examine the use of body fat as an energy reserve by juvenile female Belding's ground squirrels, Spermophilus beldingi, *prior to hibernation. Squirrels were divided into two groups: the squirrels in one group had their foraging times restricted by being placed in a cage for part of each day for four consecutive days (deprived group), and the squirrels in the other group foraged ad lib. (ad lib. group). The deprived group foraged in its normal habitat morning and evening but had access only to water and lettuce while captive. Total body electrical conductivity (TOBEC), an in vivo method of estimating fat-free mass, was used to follow changes in body composition. The deprived group lost mass, while the ad lib. group gained mass. Total body electrical conductivity measurements indicated that the deprived group lost fat-free mass and not fat, whereas mass gained by the ad lib. group was mostly fat. This is consistent with an emphasis on fat anabolism prior to hibernation. I also show that the TOBEC method is affected by variation in stomach contents, and I evaluate conditions under which an intraspecific calibration equation is needed.*

Introduction

Energy reserves are thought to have an important influence on animal decision making (see references in Walsberg [1988]; Roby [1991]). While energy stored as food caches is easy to measure, the endogenous energy reserves (usually lipids) carried by most animals are difficult to measure in vivo. Endogenous reserves can be approximated as indices of body condition by using a combination of body mass and body length, but such indices may be misleading because different energy substrates have different caloric densities. The lipid in adipose tissue is primarily an energy reserve (Pond

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1978), although muscle tissue is also a labile energy store (see, e.g., Davidson, Evans, and Uttley 1986; Harlow and Buskirk 1991). The energy densities of hydrated lean body tissue and fat tissue differ by nearly an order of magnitude. For example, 10 g of hydrated lean mass (approximately 75% water, 20.6% protein; 4.3 kcal/g protein) contains 37.1 kJ, and 10 g of adipose (93% lipid; 9.4 kcal/g lipid) contains 366.4 kJ (Bintz 1985). Estimates of body composition that distinguish adipose from lean mass may provide a more accurate estimate of energy reserves than could a mass index.

This article will present the results of a field experiment on the effect of food restriction on body composition in ground squirrels (*Spermophilus beldingi*). Total body electrical conductivity (TOBEC) was used to obtain in vivo estimates of fat-free mass independent of fat and total body mass. Variables that affect the TOBEC estimates of fat-free mass, as well as conditions for which a specific calibration equation for TOBEC is needed, will be discussed.

Material and Methods

Study Animal

A field experiment was conducted from July 1 to August 3, 1990, on a population of Belding's ground squirrels living at the Sierra Nevada Aquatic Research Laboratory at 2,160-m elevation in eastern California (for further details, see Bachman [1993]). Belding's ground squirrels do not store food. Hence, energy requirements for approximately 8 mo of hibernation are met solely by the energy contained in body tissues (Morton, Maxwell, and Wade 1974). This study focused on juvenile female squirrels, which typically emerge from their natal burrows by the end of May and begin hibernating in late August. A juvenile squirrel is depositing measurable quantities of fat by the time its total mass reaches 130 g (Morton et al. 1974). At the start of the experiment, the lightest individual weighed 178 g.

Experimental Protocol

Five squirrels were partially deprived of natural foraging opportunities (deprived group), while five others foraged ad lib. (ad lib. group), as described below. Each deprived squirrel was paired with an ad lib. squirrel. Both individuals were sampled simultaneously to minimize differences due to factors other than food deprivation. Pairs were matched for body mass, body length, and home range location in the study area. Water was available to all squirrels in their habitat, and all were accustomed to being trapped with Tomahawk live traps. Body mass was measured to the nearest 0.1 g with an American Scientific Products top-loading balance. Body length was measured from nose to base of tail to the nearest 0.5 cm. Total body electrical conductivity was measured with an EM-SCAN SA2 (AgMed, Springfield, Ill.).

The field experiment was conducted as follows. On day 1, a pair of squirrels was weighed and body composition was estimated. On each day of the "food deprivation period," days 2–5, both the ad lib. and deprived individuals were trapped between 0730 and 0900 hours. The ad lib. squirrel was weighed and released. The deprived squirrel was weighed and placed in a cage indoors with access only to water and lettuce until its release (near its burrow) between 1600 and 1700 hours. On days 6–9, the pair foraged unrestricted in their habitat. On day 10, the pair was weighed and body composition was estimated. This schedule allowed the deprived squirrel to forage in its normal habitat in the early morning and evening, with access to its own burrow overnight. Squirrels were typically active from 0700 to 1900 hours. Days 6–10 included an injection of doubly labeled water (Nagy 1983), blood sampling, and behavioral observations used in a study of field metabolism (Bachman 1992).

Measuring Body Composition

Theoretically, the most accurate methods of estimating fat mass in vivo measure fat directly (e.g., by lipid-soluble gas depletion), but current methods are time-consuming (Forbes 1987; Henen 1991). Methods for estimating fat-free mass are faster and are adequate if errors associated with indirectly estimating fat are acceptable (Forbes 1987; Morton, Kirkpatrick, and Smith 1991) or if estimating fat-free mass is the goal.

The ability of the EM-SCAN to estimate fat-free mass is affected by body temperature, movement during TOBEC measurement, subject position and shape in the measurement chamber, and abnormal electrolyte and body water status (see, e.g., Bracco et al. 1983; Klish et al. 1984; Segal et al. 1985; Fiorotto et al. 1987; Boileau 1988; Keim et al. 1988; Walsberg 1988; Castro, Wunder, and Knopf 1990; Roby 1991; Scott, Grant, and Evans 1991). The following precautions were taken to minimize the impact of these variables on this study.

Although endotherm body temperature is generally stable enough to not be a source of intermeasurement variation by TOBEC, it may increase because of stress, activity, or excessive heat load (Walsberg 1988). To minimize radiative heat load, squirrels were trapped only in the morning hours with shaded traps and were brought indoors at capture. The potential for heat production due to stress and activity was minimized by covering captives with a dark cloth. Also, frequent trapping prior to the study habituated the squirrels to humans as well as to sitting in traps. Squirrels in the deprived group appeared to sleep for most of the day when in captivity. The rectal temperatures at the time of TOBEC estimates, measured to the nearest 0.1°C with a BAT-12 (Bailey Instruments), varied from 37.7° to 40.5°C, with a mean of 39.3°C.

To minimize movement by the subject during TOBEC measurement, the squirrels were anesthetized with methoxyflurane until the initial stages of relaxation. Full anesthesia was not desirable, given the frequency with which TOBEC was measured. Movement was limited by wrapping the squirrel in an elastic bandage with velcro closures. The bandage itself had no effect on TOBEC. Conductivity indices varied between wrappings of the same squirrel (conductivity index [C-index] refers to the output of the EM-SCAN SA2) but were similar in a single wrap. Struggling resulted in increased values. The final C-index was an average of at least three different wraps, with three readings per wrap, to give an average of 10.8 (SE = 0.9) readings. The average coefficient of variation for the C-index was 4.0%.

Measuring Total Body Water

Total body water was determined by isotopic dilution on day 6, after the weight manipulation. Squirrels were injected intraperitoneally with 0.75 mL of 99% D_2O and held without food or water for 2 h. Approximately 50 μL of blood was taken from a clipped toenail and put into hematocrit tubes, which were immediately flame sealed and refrigerated until isotope analysis was performed at UCLA (for details of isotope analysis, see Wood et al. [1975]; Nagy [1983]).

Calibrating TOBEC

In July, 1991, 11 squirrels were collected from the study site. Readings of TOBEC were taken according to the procedures described above. Immediately afterward, each squirrel was sacrificed through continued exposure to methoxyflurane anesthesia. Stomach contents were immediately removed and weighed. The conductivity of the gut contents was estimated as the difference between the C-index of the empty EM-SCAN calibration tube (which has a constant, measurable conductivity) and the tube with the weighing boat and contents included. The weighing boat itself had no measurable conductivity. The carcass and stomach contents were then frozen.

Chemical analysis was conducted according to Folch (Folch, Lees, and Sloan-Stanley 1957; Stein and Smith 1982). Frozen carcasses (including stomach contents) were ground in a Wiley mill. Four 5-g samples were taken from each carcass. Samples were homogenized with 95 mL of chloroform:methanol (2:1). The filtrate was mixed with 20% of its volume of 0.9% aqueous NaCl, and the chloroform and methanol layers were allowed to separate overnight. From each sample, 25 mL of the chloroform layer was evaporated under nitrogen to constant mass, and the amount of fat in the sample was calculated. The average of four samples was used to determine total body fat for each carcass, and fat-free mass was calculated by subtraction. In addition, a 45-g sample from each carcass was lyophilized to constant mass to estimate total body water. According to Behnke (1961), fat-free mass is an in vitro measurement, while lean body mass is an in vivo measurement and includes the essential lipids associated with tissue structure. I will refer to the nonlipid compartment as fat-free mass, which includes water and nonlipids in tissues and gut contents.

Statistical Analysis

A paired t-test was used for most comparisons. All significance levels are presented for two-tailed tests, although one-tailed tests were justified when the foraging deprivation period was predicted to result in a reduction in the aspect of body composition being compared. Means are presented with standard errors in parentheses. For some paired tests, the sample size is four pairs because body composition at the start of the experiment was not available for one ad lib. individual.

Results

EM-SCAN Calibration

The chemically derived estimates of fat-free mass were regressed on the C-indices (see Scott et al. 1991), which resulted in the relationship shown in figure 1. This simple linear regression was not significantly improved by a polynomial model ($r^2 = 0.98$) or by including body length in a multiple regression ($r^2 = 0.96$).

To examine the effects of other variables on the C-index, the C-index was regressed on fat-free mass, and then the residual variation was analyzed.

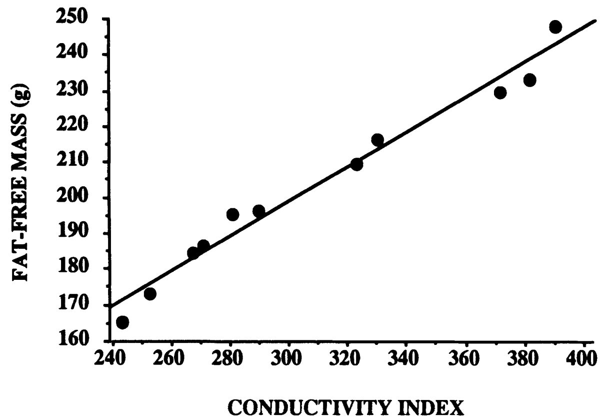

Fig. 1. Linear regression of fat-free mass on C-index. Fat-free mass was determined by carcass analysis on 11 squirrels after TOBEC was measured in vivo. Fat-free mass was 0.401 (C-index) + 73.312 (r^2 = 0.98, P = 0.0001).

The C-index residuals were not significantly related to the variables of total extracted fat (r^2 = 0.011, P = 0.764), body length (r^2 = 0.008, P = 0.790), rectal temperature (r^2 = 0.047, P = 0.790), or the percentage of mass that is fat (r^2 = 0.017, P = 0.706). This should not be interpreted to mean that body length and temperature cannot affect TOBEC, because the range of values for these variables was narrow (see Material and Methods). Body lengths for the 11 squirrels used in the calibration ranged from 19.5 cm to 24 cm (mean = 21.2 cm ± 0.4 cm), and seven values fell between 20.5 cm and 21.5 cm.

Body Composition of the Ad Lib. and Deprived Squirrels

There were no significant differences in total body mass between the ad lib. and deprived groups at any time (table 1). However, the change in total body mass between the three measurement periods shows significant differences between the groups (table 2). The deprived squirrels lost a significant amount of total mass during the deprivation period as compared to the gain by the ad lib. group (−9.4 g vs. +11.3 g), the impact of which lasted through to the end of the experiment. During the 10 d of the experiment, the ad lib. squirrels had gained substantially more mass than had the deprived squirrels (+11.0 g vs. +2.0 g).

The TOBEC estimates of fat-free mass suggest that changes in mass were primarily a consequence of the reduction in the fat-free mass of the deprived

TABLE 1

Body composition at three times during the experiment

	Deprived Mean (±SE)	Ad Lib. Mean (±SE)
Day 1:		
Total mass (g)	216.7	202.9
	(11.9)	(11.3)
Fat-free mass* (g)	207.2	193.3
	(5.2)	(12.5)
Fat-free mass (percentage of total mass)	.96	.95
	(.04)	(.01)
Fat mass* (g)	9.5	10.5
	(8.3)	(2.4)
Day 6:		
Total mass (g)	207.3	214.2
	(9.1)	(13.5)
Fat-free mass (g)	188.6	197.4
	(3.8)	(10.2)
Fat-free mass (percentage of total mass)	.92	.93
	(.03)	(.02)
Fat mass (g)	18.7	16.8
	(8.2)	(5.1)
Day 10:		
Total mass (g)	218.7	213.9
	(10.8)	(12.2)
Fat-free mass (g)	208.9	199.5
	(4.4)	(9.8)
Fat-free mass (percentage of total mass)	.96	.94
	(.03)	(.01)
Fat mass (g)	9.8	14.4
	(7.7)	(3.0)

Note. Body composition was measured at the beginning of the experiment (day 1), after the deprivation period (day 6), and after four days of normal foraging (day 10). All P values for two-tailed, paired t-tests between the deprived and ad lib. groups were not significant.

* $N = 4$, otherwise $N = 5$.

TABLE 2

The change in total mass and body composition of the ad lib. and deprived squirrels over three periods

	Deprived Mean (±SE)	Ad Lib. Mean (±SE)	Paired *t*	*P*
Day 1–6:				
Total mass	−9.4 (4.8)	11.3 (3.4)	−5.288	.006
Fat-free mass*	−18.6 (4.4)	.2 (3.0)	−9.328	.003
Fat mass*	9.2 (2.1)	8.4 (4.5)	−.04	.971
Day 6–10:				
Total mass	11.4 (2.3)	−.3 (2.8)	5.245	.006
Fat-free mass	20.3 (5.9)	2.0 (5.7)	6.107	.004
Fat mass	−8.9 (4.2)	−2.4 (4.1)	−1.412	.231
Day 1–10:				
Total mass	2.0 (3.2)	11.0 (2.6)	−2.946	.042
Fat-free mass*	1.7 (5.2)	7.2 (4.0)	−1.298	.285
Fat mass*	.3 (6.1)	2.5 (2.1)	−1.076	.361

Note. Body composition changes were calculated over the food deprivation period (day 1–6), the subsequent recovery period (day 6–10), and over the whole experiment (day 1–10). All *P* values are for two-tailed, paired *t*-tests between the deprived and ad lib. groups.
* $N = 4$, otherwise $N = 5$.

squirrels (table 2). Throughout the food deprivation period (days 1–6), the deprived squirrels lost an average of 9% of their fat-free mass. This was significantly different from the ad lib. group, although the change in fat mass was not: both groups appeared to gain approximately 8–9 g of fat. The mass recovery observed in the deprived group from day 6 to day 10 may also be attributed to fat-free mass. During this period, the average fat-free mass gain by the deprived group was 20 g, while the ad lib. group gained an average of 2 g. Throughout the whole 10-d experiment, the ad lib. squirrels gained a significant amount of mass relative to the deprived group. The average changes in both fat mass and fat-free mass were greater in the ad lib. group but were not significantly different from those observed in the deprived group.

Potential Sources of Error

Water Balance. Dehydration may lead to an underestimation of fat-free mass by TOBEC (Walsberg 1988; but see Segal et al. 1985). However, dehydration does not explain the decrease in fat-free mass (decrease in TOBEC) in the deprived squirrels. Deuterium dilution estimates of total body water (TBW) on day 6 show no significant difference between the ad lib. and deprived groups (deprived mean = 141.8 mL ± 4.7 mL; ad lib. mean = 145.3 mL ± 9.3 mL; $P = 0.613$). Total body water as a fraction of total mass was not significantly different between the groups (deprived mean = 0.69 mL/g ± 0.02 mL/g; ad lib. mean = 0.68 mL/g ± 0.01 mL/g; $P = 0.723$), nor was TBW as a fraction of estimated fat-free mass significantly different between the two groups (deprived mean = 0.75 mL/g ± 0.02 mL/g; ad lib. mean = 0.74 mL/g ± 0.03 mL/g; $P = 0.389$).

If some aspect of the deprivation treatment disturbed the electrolyte concentration of the deprived squirrels, TOBEC may not accurately predict TBW, and a systematic difference in the TBW estimates should appear. An equation relating TBW to the C-index was calculated with the calibration data: mL H_2O = 0.27 (C-index) + 64.0 mL ($P \leq 0.0001$, $r^2 = 0.97$). The deuterium estimate of TBW, when subtracted from the estimate given by TOBEC, indicates that the differences in the estimates are not related to either ad lib. or deprived status (mean deprived difference = −0.2 mL ± 4.4 mL; mean ad lib. difference = 2.3 mL ± 5.6 mL; $P = 0.508$) and suggests that electrolyte concentrations did not systematically affect TOBEC readings.

Gut Contents. The ions and water in the squirrel's food had an impact on TOBEC readings. Plant material is high in potassium (Nagy, Shoemaker, and Costa 1976), and Klish et al. (1984) demonstrated that TOBEC is particularly sensitive to potassium concentration. Among the squirrels used to calibrate the EM-SCAN, there was an indication that stomach content mass increases throughout the day (see also Morton 1975). Squirrels caught before

1100 hours had an average of 7.8 g ± 1.5 g of food in their stomach (N = 5), which is equivalent to 1.08 g of fat-free mass. In one squirrel caught at 1730 hours, there was a large mass of food in the stomach, which weighed 43.7 g (16.7% of total mass). This produced a C-index equivalent to 30 g of fat-free mass, which showed that the error introduced by a large quantity of food in a squirrel's stomach could be significant.

However, reduction in stomach and intestinal contents is not likely to account for the observed reduction in fat-free mass in the deprived squirrels. The experimental squirrels were all trapped in the morning, when stomach contents were likely to be no more than 10 g, which is equivalent to 1.1 g of fat-free mass. The intestinal content of a squirrel in the morning is approximately 10 g, which is equivalent to 1.8 g of fat-free mass (9.1 g of a large intestine's contents, not solid feces, has a C-index equivalent to 1.68 g of fat-free mass). If the deprived individuals had completely empty intestines and stomachs, the fat-free mass estimate should be reduced by approximately 3 g. This value, when added to the fat-free mass estimate of the deprived squirrels or subtracted from the fat-free mass of the ad lib. squirrels, did not affect the significance of the test comparing fat-free mass change from day 1 to day 6 of the experiment: the deprived group still lost significantly more fat-free mass ($P = 0.004$).

Discussion

Fat-Free Mass Loss During Deprivation

Studies of body composition changes during total starvation in humans, rats, bats, and ground squirrels show that, initially, labile protein reserves and glycogen are significant sources of energy during a fast. Most of this weight loss is due to the release of water associated with these energy substrates (Brozek and Henschel 1961; Behnke and Wilmore 1974; Schmidt-Nielsen 1983; Yacoe 1983*a*, 1983*b*; Krilowicz 1985). Skeletal muscle provides most of the labile protein, along with contributions from skin, liver, and intestinal epithelia (Bintz and Strand 1983; Forbes 1987; Young, Ramos, and Harris 1988). As a fast continues, the emphasis on lipid catabolism increases and "protein sparing" occurs. The deprived squirrels in my study did not experience total starvation, so it is not clear that this pattern of mass loss would apply. It is possible that during each day measurable amounts of protein were catabolized, as occurs during a fast, and that the nature of the deprivation prevented a noticeable shift to lipid catabolism.

Protein catabolism may also predominate in other circumstances. The use of protein as an energy source may continue beyond the presumed obligatory loss at the beginning of a fast. This pattern of lean mass loss results in "fat sparing" and may explain the loss of fat-free mass in the deprived squirrels. In a 28-d deprivation study, Manning and Bronson (1990) showed that, under conditions of increased energy demand, a food-limited mouse (*Mus musculus*) will use its lean mass and protect its fat. Humans and rats on low protein–high energy diets have been shown to gain fat while losing lean mass (Forbes 1987). Belding's ground squirrels rely primarily on fat as an energy source during hibernation. Although protein catabolism during hibernation is important (see, e.g., Galster and Morrison 1975; Yacoe 1983*a*, 1983*b*; Krilowicz 1985), protein surplus may be more expendable if fat levels are low. Finally, Harlow and Buskirk (1991) show that lean mass catabolism is emphasized in a fasting "lean-bodied" species (American marten, *Martes americana*), while a prairie dog (*Cynomys leucurus*), with nearly one third of its mass as fat, will use its fat when subjected to a fast in late fall. The juvenile female squirrels in my study were tested at least 1 mo prior to hibernation and were still relatively lean (table 1). Just prior to hibernation, a juvenile squirrel may have 44% of its total mass as fat (Morton et al. 1974). A pilot study indicated that late in the active season the protocol used to reduce foraging time could initiate hibernation, so the timing of this experiment was constrained to midseason.

The loss of lean mass during reduced energy intake has been referred to as the "physiologic cost of weight reduction" (Behnke and Wilmore 1974). The extent to which a moderate reduction in lean mass correlates with reduced function in muscles or organs is unknown (Young et al. 1988). Behavioral observations on the days following the deprivation period indicate that the squirrels were not enfeebled (Bachman 1992). Lean mass catabolism can also contribute to the maintenance of water balance. However, because of the availability of freestanding water and lettuce, it is unlikely that water balance was related to the reduction of fat-free mass in my study (Bintz, Bintz, and Riedesel 1971; Bintz and Strand 1983).

Stress associated with the confinement of the deprivation period may have resulted in an elevation of glucocorticoids, which have been shown to stimulate muscle catabolism (Linder 1985). The precautions taken to minimize stress were deemed successful to the extent that I did not observe overt signs of stress and it was not difficult to retrap deprived individuals on subsequent days. Captives did not alarm call or continuously try to escape. Steroid levels were not measured so the possibility remains that a stress response led to increased protein catabolism.

The Use of TOBEC

A number of precautions must be taken when estimating fat-free mass with TOBEC. In this study, systematic errors in fat-free mass estimates associated with positioning, movement, dehydration, temperature, and gut fill either were controlled during measurements or were not evident.

My results suggest that, when using TOBEC to estimate fat-free mass, care should be taken to minimize gut-fill differences between individuals. Many animals may show significant diurnal variation in gut contents, as well as variation in gut contents due to differential gut hypertrophy. The impact of gut contents is of particular relevance to carnivorous animals whose meals are sporadic and may be relatively large. It should be noted that the stomach contents as I measured them may not have the same C-index as they would in vivo because of differences in the general size, shape, and temperature of the conductive mass. The impact of different diets and of potential changes in intake could be explored by feeding trials.

The choice of an equation relating C-index to fat-free mass may greatly affect the estimates of fat-free mass. Walsberg (1988) presented an equation relating C-index to fat-free mass with data from 10 species of rodents in six genera (not including *Spermophilus*). For my squirrels, fat-free mass estimates from Walsberg's (1988) interspecific equation are approximately 24% lower than those obtained with the data from this study with either a linear equation (fig. 1) or an equation similar to Walsberg's (fat-free mass [g] $= 14.632 \times [\text{C-index}]^{1/2} - 59.057$; $r^2 = 0.98$, $P = 0.0001$). Scott et al. (1991) also showed that fat-free mass is not accurately predicted by an interspecific equation for birds and suggested that fat-free mass estimates require species-specific calibration equations. The focus of this article is the change in the fat-free mass over time, which is not affected by choice of equation. Paired *t*-tests for comparing the change in fat-free mass during the food deprivation period in the deprived and ad lib. squirrels were significantly different when the estimate from a calibration equation in the form of a simple linear regression (table 2) was used and when an equation including the square root of the C-index was used (deprived mean = -17.2 g $\pm$ 9.0 g; ad lib. mean = 0.2 g $\pm$ 5.5 g; $P = 0.002$). Accurate estimates of fat-free mass do require a specific calibration equation, and, because the slopes of the lines vary, the absolute values for the change in fat-free mass also depend on the equation used. However, when ranking individuals by fat-free mass content or by changes in fat-free mass, the exact calibration equation is less important. In fact, the raw C-indices themselves are adequate because calibrations are monotonic transformations of the indices.

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