

# Protein Use and Muscle-Fiber Changes in Free-Ranging, Hibernating Black Bears

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## ABSTRACT

Studies of the metabolic and physiological changes that bears undergo during hibernation have, for the most part, supported the paradigm that bears use only fatty tissues as a metabolic substrate during hibernation. This study was performed to document the extent of protein loss and alteration of muscle-fiber characteristics of selected muscles in black bears during winter dormancy. Muscle biopsies were removed from the gastrocnemius and biceps femoris from seven free-ranging female black bears on the Uncompahgre Plateau in west-central Colorado. Six of the seven bears produced cubs during the hibernating season. Muscle samples were collected from the left hind limb shortly after bears entered their dens (fall), and additional samples were collected from the right hind limb just prior to bears leaving their dens (spring). Protein concentration, fast- and slow-twitch muscle-fiber ratios and muscle-fiber cross-sectional areas, and citrate synthase activity were measured in the laboratory. While protein concentration decreased in both muscles during the hibernation period, it was lower than predicted for lactating females. In addition, muscle-fiber number and cross-sectional area were unchanged in these muscles, suggesting only limited muscle atrophy. In support of these observations, there was a moderate but significant increase in the proportion of fast-twitch fibers only in the biceps femoris, with a concomitant decrease in citrate synthase activity, but no alteration of the fiber ratio in the gastrocnemius during hibernation. These findings suggest that hibernating bears, particu-

larly lactating females, do use some protein, in concert with fat catabolism, as a metabolic substrate and as a source of water. However, the extent of this protein use is moderate and is associated with limited alteration of muscle structure, characteristic of disuse atrophy.

## Introduction

There continue to be contrasting opinions regarding winter dormancy in the American black bear (*Ursus americanus*). The use of the word "hibernator" to describe bears in general has been a topic of scientific debate for decades. Unlike hibernators that experience rhythmic alterations of deep torpor and arousal, bears remain in a relatively shallow torpor for 5–7 mo. During this time, bears experience no regular periods of arousal and maintain near-normal body temperatures of 31°–35°C (Nelson et al. 1983). Female black bears give birth to cubs while occupying their dens, usually in January, and will nurse their young until they emerge in the spring (Nelson 1973; Nelson et al. 1983). While many aspects of the physiological processes associated with hibernation are generally understood, two are less clear and are the focus of this study.

### *Protein and Fat Use during Hibernation*

Bears are relatively large mammals and while hibernating use some 17,000 kJ of energy per day, yet do not eat, drink, urinate, or defecate for the duration of winter dormancy (Nelson 1973; Nelson et al. 1983). This required energy is believed to be provided almost exclusively by use of fat reserves, with little or no net muscle protein loss (Nelson et al. 1973, 1975; Lundberg et al. 1976). It has often been suggested that none of the common end products of protein catabolism accumulate during black bear hibernation. Total serum protein, urea, uric acid, total amino acids, and ammonia do not increase during hibernation, indicating an absence of net protein loss (Nelson et al. 1973, 1983). Ahlquist et al. (1976) and Lundberg et al. (1976) obtained similar results using captive black bears and believe that this absence of accumulated catabolic end products is due in part to an increase in the effectiveness of protein synthesis, wherein, rather than being eliminated, much of the nitrogen from protein breakdown reenters protein anabolic pathways. Therefore, even though previous studies suggest little net protein loss in overwintering bears, protein turnover, that

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is, the cyclic degradation and synthesis of protein, may still be high. For example, Lundberg et al. (1976) found that winter protein turnover increases three- to fivefold over summer levels without a measurable net protein loss.

Even though it is generally thought that there is a marked reduction in protein catabolism during hibernation by small mammals (Bintz et al. 1979; Yacoe 1983; Krilowicz 1985), a certain amount of protein use is required for all fasting or overwintering animals (Le Maho et al. 1981; Yacoe 1983). A basal level of protein catabolism may be needed to sustain a continuous use of fat by providing a source of citric acid cycle intermediates from amino acid deamination (Bintz et al. 1979; Yacoe 1983), to provide a source of water during periods of limited food and water intake, and to provide water, as well as a nitrogen source, for milk production during lactation (Bintz et al. 1979).

Some researchers believe that the resulting metabolic water produced by fat catabolism can maintain a positive water balance in bears (Nelson et al. 1983). However, Bintz et al. (1979) calculated that catabolism of fat along with protein in a ratio of 2 : 1 would be necessary to maintain a water balance for hibernating ground squirrels. Under normal circumstances, the release of water from protein is potentially negated by its loss in urine to detoxify urea. However, bears, like some ground squirrels, recycle urea during winter hibernation (Nelson et al. 1973, 1975), which allows them to retain much of the bound water released as a result of protein breakdown. The problem of maintaining a metabolic water balance is exacerbated for hibernating female bears during gestation and lactation because of the extra requirements of both energy and water needed for milk and fetus production. The need for this additional water and nitrogen necessary for cub growth further taxes protein reserves (skeletal muscle and nonmuscle) of the lactating female. These physiological challenges would seem to suggest that a net loss of protein during hibernation, especially by lactating females, is probably inevitable.

#### *Muscle Atrophy and Fiber-Type Alteration*

Another phenomenon typically associated with protein degradation during long periods of inactivity is the atrophy of skeletal muscles. However, one of the most consistent observations made by biologists in the field is the apparent lack of impaired locomotor ability by bears aroused during hibernation (T. D. I. Beck, personal observation, over 140 denning bears). It remains unclear how bears remain inactive, within a confined space, for 4–6 mo without any apparent impairment of muscle function. Many muscle disuse models have been described, developed primarily from studies of either rodent hibernators (see, e.g., Wickler et al. 1987, 1991; Musacchia et al. 1989; Steffen et al. 1991) or animals exposed to some type of acute limb immobilization (Musacchia et al. 1983; Desplanches et al. 1987). How-

ever, no study has yet addressed muscle-fiber integrity during the unique circumstances experienced by black bears that remain inactive for extended periods of time.

There have been two traditional models for studying muscle disuse atrophy in mammals: plaster-cast immobilization and hind limb suspension. These conditions of disuse cause muscle atrophy characterized by a decrease in muscle-fiber protein, cell size and number, and altered ratio of muscle-fiber types, resulting in an impaired muscle function (Musacchia et al. 1989). However, the profile of muscle atrophy that results from these models appears to be inconsistent, as evidenced by the broad range of findings. In addition, these models have limitations in that they are performed under unnatural conditions and they do not allow for repeated sampling of the same individuals over time. Recent studies using hibernating small mammals have partially circumvented these concerns and have identified some unique properties in what appears to be a more natural muscle disuse model. But there are two major limitations to small mammal hibernators as a model to investigate effects of chronic immobility. First, because of their small size, the same individual cannot be repeatedly sampled, and different groups of animals must be killed and compared during stages of hibernation. Second, small hibernators arouse with violent muscle shivering approximately every 15–20 d, which may provide sufficient exercise to retard changes in muscle-fiber-type ratios and even enhance citrate synthase (CS) activity levels. Study of the black bear allows researchers to circumvent these limitations; it is large enough to resample during the winter, and it does not undergo periodic arousal with violent shivering.

Despite these advantages, relatively little work has been done on overwintering muscle physiology for this species. Koebel et al. (1991) found that protein content as well as concentrations of glycogen, triglycerides, and other principal energy-supplying substrates of muscles are unchanged in black bears during hibernation, and that CS activity, an indicator of muscle oxidative capacity, is also unchanged during the denning period. However, their study was conducted on three nonlactating bears held in captivity and does not necessarily reflect the dietary, thermal, and activity profiles of naturally foraging and denning bears.

The specific questions addressed in this study were: (1) How much (if any) protein is catabolized from specific muscle areas by lactating and nonlactating females? (2) Are muscle-fiber type and cross-sectional area maintained during hibernation, resulting in a retention of muscle integrity for spring emergence? and (3) Does CS activity during the denning period become altered in a pattern similar to that of small mammal hibernators or other disuse atrophy models?

## **Material and Methods**

### *Study Area*

The study population was located on the northwestern section of the Uncompahgre Plateau in Mesa County, Colorado. The

elevation ranges from 6,000 to 9,000 ft, and the primary canopy cover consists of ponderosa pine (*Pinus ponderosa*), quaking aspen (*Populus tremuloides*), and gambel oak (*Quercus gambelii*), while other canopy and understory species such as blue spruce (*Picea pungens*), serviceberry (*Amelanchier alnifolia*), and sagebrush (*Artemisia* sp.) were somewhat less abundant.

#### Study Population

Eighty-nine bears were ear-tagged, and all bears over 23 kg were fitted with radio-tracking collars transmitting in the 150–152 MHz band. Of these, nine female bears believed to be in a breeding state with a high probability for winter parturition and lactation were selected as the study group. The average fall body mass of the study animals was 104 kg (SE = 24.9).

#### Field Methods

All fieldwork was based out of the Cold Springs Ranger Station in the Uncompahgre National Forest. The fieldwork was divided into two main efforts: the late fall, or early denning period (September–December 1994), and late winter/early spring, or late denning period (March–April 1995). Both aerial and ground radio tracking began in mid-September (aerial reconnaissance was provided by the Colorado Division of Wildlife and the Colorado State Patrol). In both instances, bears were located using a Telonics (Mesa, Ariz.) radio receiver and Yagi-type (Advanced Telemetry Systems, Isanti, Minn.) antennas.

Bears were anesthetized using ketamine hydrochloride/xylazine hydrochloride (200 mg ketamine and 50 mg xylazine mL<sup>-1</sup>) in a jab-stick at a dosage of 8.8 mg ketamine and 2.2 mg xylazine kg<sup>-1</sup> bear weight. After the bear was immobilized, it was extracted from the den by placing it on a heavy plastic tarp while still in the den and dragging the tarp out. The bear was then placed on an inflatable pad and heavy plastic tarp, which provided insulation from the snow and cold. If it was snowing or there was blowing snow, a shelter was constructed over the bear, thereby keeping it dry.

#### Measurements and Surgical Procedures

All physical measurements and surgical procedures were performed on nine female bears during the fall field season and on seven of the same nine bears during the spring field season (bears 6 and 7, which were sampled during the fall season, had left the den sometime during mid- to late March and were not resampled in the spring). Physical measurements and surgical procedures were identical during each season; that is, the surgical biopsies were all removed from the left hind limb during the fall sampling and from the right hind limb during the spring sampling. Six of the seven bears sampled in the spring

had cubs and were therefore considered lactating. Each bear was weighed on a digital load scale (Dyna Link, model MSI-7200), accurate to within 0.1 kg. Snout/vent length was measured to the nearest centimeter.

All handling and surgical procedures were reviewed by Colorado Division of Wildlife and University of Wyoming animal welfare committees. Two small (≈400 mg) muscle-tissue samples were surgically removed from the hind limb of the bear, one from the lateral head of the gastrocnemius and one from the biceps femoris. A sterile field was established and maintained in the areas surrounding each biopsy location.

Each biopsy sample was carefully cut into three equal portions. One of the three pieces of tissue was immediately placed into a small, labeled Ziploc bag and quickly put into a liquid nitrogen thermos, to be used later for assays of enzymatic activity. The second portion of tissue was mounted onto a small cork disc using a tissue-freezing matrix, with the muscle fibers oriented perpendicular to the surface of the cork. This mounted tissue was placed into liquid isopentane cooled by liquid nitrogen and, once frozen, was put into a labeled Ziploc bag and into the liquid nitrogen thermos. The mounted tissue was later used for fiber-type and histological analysis. The final piece of muscle tissue was then put into a labeled Ziploc bag and placed into the liquid nitrogen for later analysis of protein content. All samples were transported frozen to the field station, where they were transferred to a large 35-L liquid nitrogen dewar flask for transport to the laboratory at the University of Wyoming. Immediately following the surgical biopsy, each bear was replaced in its den by sliding it on the plastic tarp to as close to its original position as possible. The den entrance was then covered with vegetation (and snow, if snow was present upon our arrival at the den).

This entire procedure was repeated on seven of the nine bears during the late denning season (March–April). At that time, we reweighed each bear and surgically removed two additional muscle-tissue samples from the hind limb opposite the one sampled in the fall. As during the fall sampling, all bears were replaced in their dens as close to the original position as possible before leaving the den site.

#### Laboratory Methods

*Fiber-Type and Number Analysis.* Thin sections (5–6 μm) from the frozen muscle biopsies were serially cut using an American Optical Histostat freezing microtome knife at –20°C, and then mounted on glass slides. Cutting continued until five consecutive high-quality slides for each bear muscle were obtained. These sections were air-dried overnight and then stained for myofibrillar ATPase activity and NADH activity (see Tinker [1995] for complete protocol). The best slide, based on the quality and evenness of the staining, was selected from each group of five for analysis.

Using a compound microscope, a videoscope, a desktop computer, and morphometry software known as Flexible Image Processing System (FIPS; Wirsam Scientific and Precision Equipment, Ltd., Johannesburg, South Africa), each video image of the entire muscle cross section was visually divided into four subimages and enlarged at 6.3 magnification; these subimages were stored on a floppy disk for analysis. The diameter and cross-sectional area of approximately 300–400 fibers was measured. All fibers from each subimage were classified as either Type I (slow oxidative) or Type II (fast glycolytic or fast, oxidative, glycolytic) according to the classification method of Peter et al. (1972). (The staining protocol used in this study does not differentiate between the various Type II fibers, e.g., fast glycolytic or fast, oxidative, glycolytic.) Fiber-type ratios (percentages of each fiber type) were calculated for each muscle, and mean cross-sectional area and fiber diameter were calculated for each muscle using the FIPS morphometry software. Additionally, total number of fibers for each muscle sample was determined by adding the number of fibers analyzed from each of the four subimages. Each subimage consisted of approximately 975  $\mu\text{m}^2$ ; therefore, the total area of all four subimages was approximately 3,900  $\mu\text{m}^2$ , or 3.9  $\text{mm}^2$ .

**Protein Concentration and Water Content.** Protein concentration of each muscle was determined by a modified Bradford (Bradford 1976) colorimetric assay (see Tinker [1995] for complete protocol), and percent nitrogen of each muscle sample was measured by a carbon-hydrogen-nitrogen ignition method using a Carlo Erba model 4500 C/N analyzer. Total nitrogen (mg) was calculated based on the dry weight of the sample. Muscle water content was determined by weighing before and after drying in a Vertis freeze dryer for 24 h.

**CS Activity.** CS activity was determined using a colorimetric assay (Shepherd and Garland 1969) on wet-weight samples weighing approximately 20–40 mg (see Tinker [1995] for complete assay protocol).

**Statistical Analyses.** A paired *t*-test with repeated measures design was used to detect significant differences in mean values of the measured parameter. A standard *t*-test was used for nonrepeatable measurements, for example, muscle-fiber cross-sectional areas. One-tailed tests were performed when measured variables were expected to either increase or decrease, but not both; otherwise two-tailed tests were used. Percentage data were normalized before analysis using the arcsin-square root transformation. All alpha-levels are at 0.05 unless otherwise noted.

## Results

### Sampling Intervals

The mean sampling interval for all bears was 123 d ( $\pm 13.7$ ); the shortest interval between samples was 108 d and the longest

interval was 152 d. Bears were resampled in the spring in approximately the same order as they were handled in the fall in an effort to maintain fairly equal intervals between samples. Fall measurements were made very soon after the bears entered their dens; spring measurements were made shortly before the bears left their dens. Change refers to the difference between these two measurements during hibernation. Numbers in parentheses are standard errors of the mean.

### Body Mass Loss

The mean percent body mass loss for each bear during hibernation was 24.3% ( $\pm 6.1\%$ ) and ranged from 15% to 33% (Fig. 1). These values are consistent with expected body mass loss for bears during hibernation.

### Protein Use and Muscle-Tissue Water Content

**Bradford Assay.** Results from the Bradford assay of peptide bonds indicated that there was a significant decrease in skeletal muscle protein concentration during hibernation in both the gastrocnemius muscle (mean change,  $-17.2 [\pm 6.83]$   $\text{mg g}^{-1}$  dry

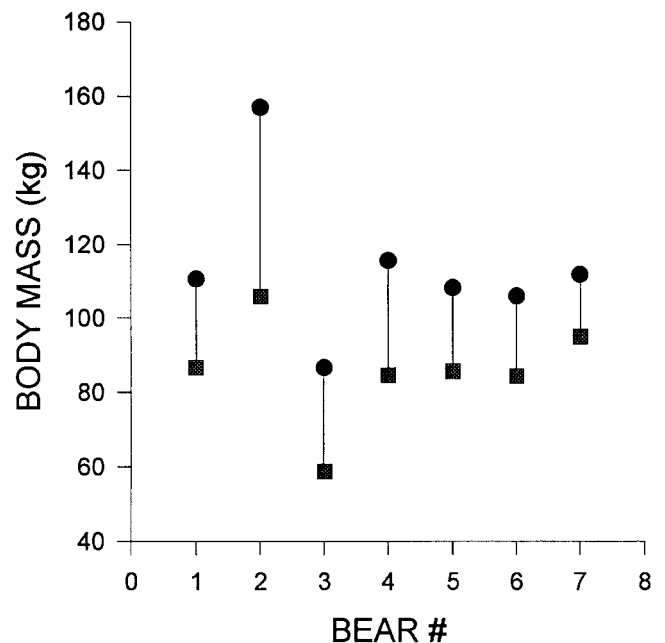


Figure 1. Body mass for each bear during hibernation. Fall weights were obtained during November and December (circles) and spring weights during March and April (squares), shortly before the bears emerged from their dens. Mean mass loss for all bears was 27.8 kg ( $\pm 10.4$  kg) and ranged from 16 kg (bear 9) to 51.2 kg (bear 2).

weight,  $P = 0.045$ ) and the biceps femoris muscle (mean change,  $-44.6 [\pm 13.59] \text{ mg g}^{-1}$  dry weight,  $P = 0.017$ ; Fig. 2).

**Carbon/Hydrogen/Nitrogen Assay.** Total nitrogen did not significantly change in either the gastrocnemius or the biceps femoris during hibernation ( $P = 0.28$  and  $P = 0.15$ , respectively; Fig. 3).

**Muscle-Tissue Water Content.** Percent water content of biceps femoris increased significantly during hibernation (mean change,  $2.68\% [\pm 0.87\%]$ ,  $P = 0.02$ ), while water content in the gastrocnemius did not differ significantly ( $P = 0.42$ ; Fig. 4).

#### *Muscle-Fiber-Type Composition, Number, and Cross-Sectional Area*

For muscle-fiber-type composition analysis, only six bears were sampled because one of the muscle biopsy samples was considered an outlier; all other fiber analyses were performed on samples from seven bears. There was a significant increase in the percentage of fast-twitch versus slow-twitch muscle fibers in the biceps femoris (mean change,  $9.9\% [\pm 4.5\%]$ ,  $P = 0.03$ )

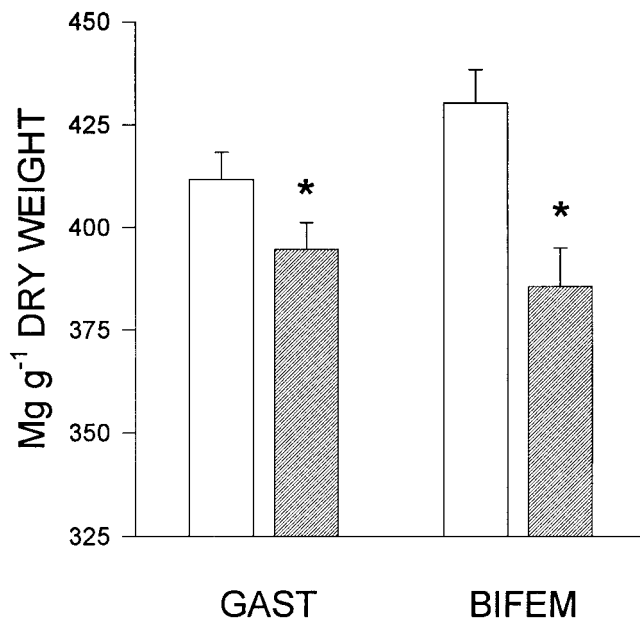


Figure 2. Changes in protein concentration ( $\text{mg g}^{-1}$  dry weight) during fall (open bars) and spring (hatched bars) in the gastrocnemius (GAST) and biceps femoris (BIFEM) muscles of seven bears using the Bradford protein assay. Asterisk depicts a significant reduction in protein content during hibernation. Gastrocnemius mean change in concentration:  $-17.2 \text{ mg g}^{-1}$  dry weight ( $\pm 6.83$ ),  $P = 0.045$ ; biceps femoris mean change in concentration:  $-44.6 \text{ mg g}^{-1}$  dry weight ( $\pm 13.59$ ),  $P = 0.017$ . Vertical lines represent one standard error.

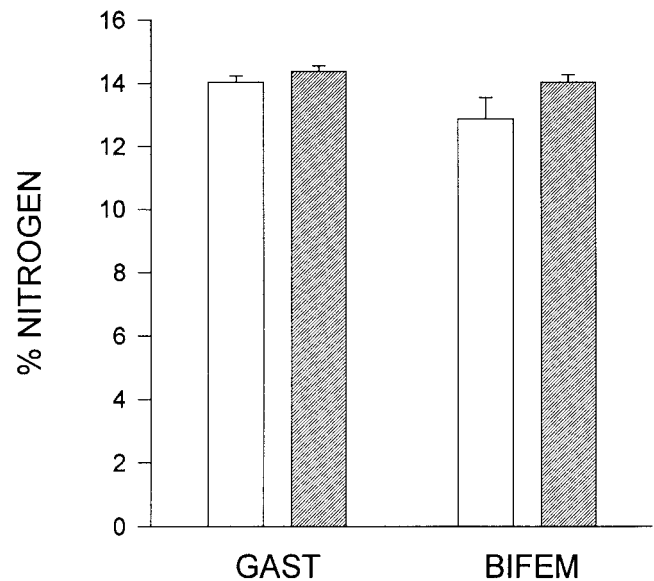


Figure 3. Fall (open bars) and spring (hatched bars) values for percent nitrogen in gastrocnemius (GAST) and biceps femoris (BIFEM) muscles of seven bears using the carbon/hydrogen/nitrogen tissue-ignition assay, which measures total percentage nitrogen in each sample. Neither the gastrocnemius nor the biceps femoris showed a significant change in percent nitrogen during hibernation using this assay;  $P = 0.28$  and  $P = 0.15$ , respectively. Vertical lines represent one standard error.

during hibernation. However, there was no significant increase in percentage of fast-twitch fibers in the gastrocnemius ( $P = 0.23$ ; Fig. 5).

Cross-sectional areas of fast-twitch muscle fibers in both the gastrocnemius and the biceps femoris were not statistically significantly altered during hibernation ( $P = 0.58$  and  $P = 0.48$ , respectively). Similarly, there was no significant change in cross-sectional area of slow-twitch muscle fibers in either the gastrocnemius ( $P = 0.18$ ) or the biceps femoris ( $P = 0.17$ ; Fig. 6). There was also no significant change in the mean number of total fibers counted (per  $3.9 \text{ mm}^2$ ) for either the gastrocnemius ( $P = 0.58$ ) or the biceps femoris ( $P = 0.63$ ; Fig. 7).

#### *CS Activity*

CS enzymatic activity decreased significantly in the biceps femoris muscles during hibernation (mean change,  $-8.41 \mu\text{mol g}^{-1} \text{ min}^{-1} [\pm 2.99]$ ,  $P = 0.015$ ). However, CS activity in the gastrocnemius muscle exhibited only a very slight and nonsignificant alteration during the hibernation period (mean change,  $-1.11 \mu\text{mol g}^{-1} \text{ min}^{-1} [\pm 2.95]$ ,  $P = 0.72$ ; Fig. 8).

## Discussion

### *Body Mass Measurements*

Bears lost an average of 24.3% of their total body mass during hibernation. Notably, bear 7, the only bear sampled that was

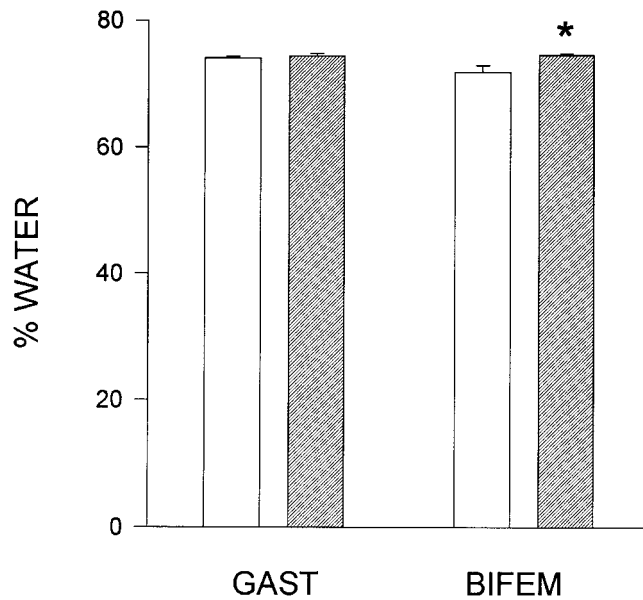


Figure 4. Mean percentage water of gastrocnemius (*GAST*) and biceps femoris (*BIFEM*) during fall (*open bars*) and spring (*hatched bars*) for muscle samples from seven bears following freeze-drying for protein concentration analysis. Weights were obtained before drying and again after 24 h in the freeze-dryer for both fall and spring samples. Asterisk depicts a significant increase in water content in biceps femoris during hibernation. Vertical lines represent one standard error.

without cubs and therefore not lactating during the study period, lost a smaller percentage of body mass (15%) than any of the bears with cubs and considerably less than the average of the seven bears.

#### Protein Use during Hibernation

There are conflicting reports of protein use during periods of inactivity by both hibernators and nonhibernators. Some studies (Musacchia et al. 1989; Steffen et al. 1991) suggest that no change in protein concentration occurs in hibernating ground squirrels (*Spermophilus lateralis*). Notably, these animals arouse and feed at regular intervals during hibernation, which may replenish much of the protein catabolized during torpor. However, other investigators have reported that protein concentration decreases during hibernation, as well as in specific muscles during hind limb suspension (Steffen and Musacchia 1984; Wickler and Hoyt 1990). In our study, 13 of 14 muscle biopsies taken during the spring from the gastrocnemius and biceps femoris indicated a net loss of protein concentration when compared with corresponding biopsies taken during fall sampling. While these results support our hypothesis that lactating female black bears use a significant amount of protein during hibernation, they are contradictory to previous studies that did

not identify any net loss in bear skeletal muscles during early and late hibernation (Nelson 1973, 1980; Lundberg et al. 1976; Koebel et al. 1991).

For example, while Koebel et al. (1991) and Lundberg et al. (1976) did not observe a loss in muscle protein, Lundberg et al. (1976) found that protein turnover, a measure of both protein catabolism and protein synthesis, increases three- to fivefold in captive bears during hibernation. This statement seems to imply that net protein loss does not occur during hibernation in bears. Since it has been shown that bears do not eat or drink during hibernation (Nelson 1973), the only source of free amino acids that could be used for protein synthesis would have to be from protein breakdown. Therefore, if protein degradation increases during hibernation, it follows that protein synthesis would also have to increase in order to provide an exact protein replacement. Urea recycling could potentially assist in achieving this nitrogen balance. The recycling of nitrogen (from urea hydrolysis and hepatic amino acid synthesis) back into structural proteins has been offered as an explanation for the apparent lack of protein loss during hibernation (Lundberg et al. 1976). This scenario suggests,

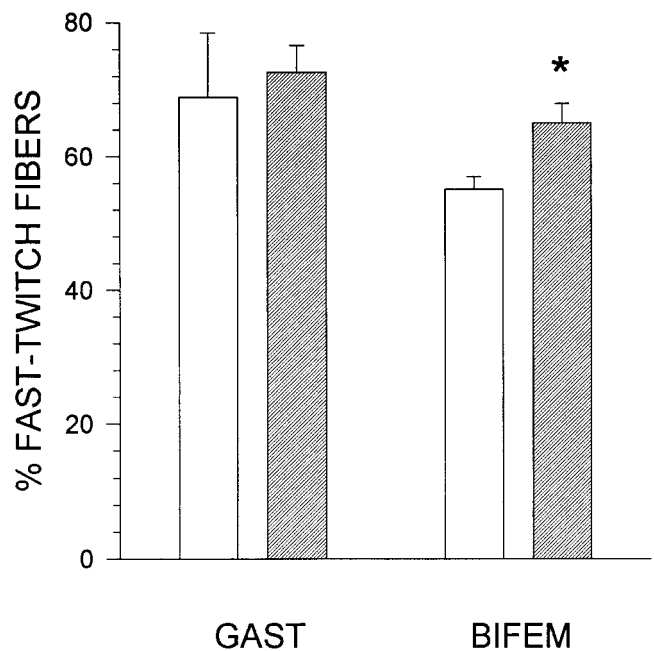


Figure 5. Percentages of fast-twitch fibers for fall (*open bars*) and spring (*hatched bars*) for gastrocnemius (*GAST*) and biceps femoris (*BIFEM*) muscles of six bears. Asterisk depicts a significant increase in the percentage of fast-twitch fibers during hibernation and the reciprocal decrease in percentages of slow-twitch fibers for the same period (only fast-twitch fibers are represented in the figure). Mean increase in percentage of fast-twitch fibers in the gastrocnemius was  $3.56 (\pm 5.0)$  ( $P = 0.23$ ); in biceps femoris mean increase was  $9.9 (\pm 4.5)$  ( $P = 0.03$ ). Vertical lines represent one standard error.

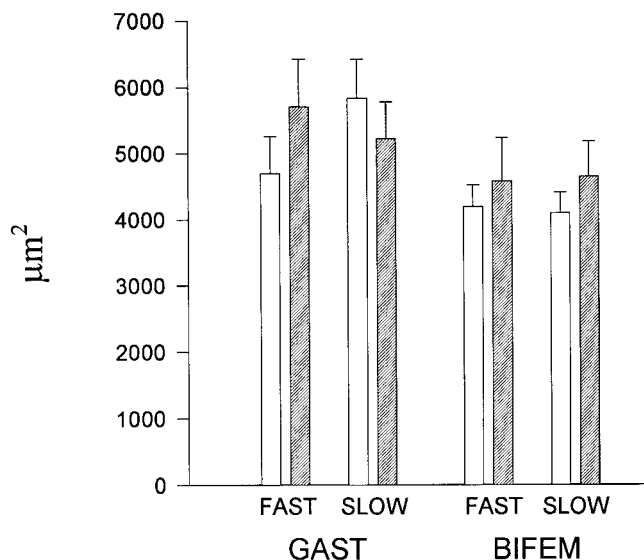


Figure 6. Mean cross-sectional areas ( $\mu\text{m}^2$ ) of both fast- and slow-twitch fibers for fall (open bars) and spring (hatched bars) in gastrocnemius (GAST) and biceps femoris (BIFEM) muscles for seven bears. Neither fast- nor slow-twitch muscle exhibited a significant change in fiber area during hibernation ( $P = 0.18$  and  $P = 0.17$  for gastrocnemius and biceps femoris, respectively). Vertical lines represent one standard error.

however, that an extremely efficient (almost 100%) process of nitrogen recycling is needed to achieve high protein turnover with no protein loss, a highly unlikely condition.

Our results from the carbon/hydrogen/nitrogen assay suggest that the mean percent nitrogen from both the gastrocnemius and biceps femoris was not significantly reduced during hibernation. Protein catabolism is characterized not just by a reduction in muscle protein but also by an increase in free amino acids, creatinine, ammonia, and urea as metabolic end products. A reduction of peptide bonds (the results of Bradford assay) without a concomitant reduction in total nitrogen (the results of carbon/hydrogen/nitrogen assay) may represent a high protein turnover. We identified these end products by carbon/hydrogen/nitrogen analysis in our biopsy of muscle and associated vasculature, but not the liver, where intermediates may accumulate. Our data suggest that some of the nitrogen from catabolized protein was being identified as other nitrogenous end products or intermediates because of a flux of protein and nitrogen at any point in time with a very rapid turnover in the liver. This is in partial agreement with Lundberg et al. (1976) in that it suggests an increase in muscle protein catabolism and a high mobilization of nitrogen in overwintering bears, but unlike Lundberg et al. (1976) and Koebel et al. (1991), we identified a significant loss of muscle protein over this period, similar to protein losses during hibernation by arctic ground squirrels (*Citellus undulatus*; Galster and Morrison 1976), which, like bears, typically do not feed during the

hibernation period. However, the magnitude of this loss was lower than that reported for other animals exposed to protracted immobilization (Desplanches et al. 1987; Thomason and Booth 1990).

It is interesting to estimate the amount of protein lost during the winter based on the range of protein reduction observed during this study in the gastrocnemius and biceps femoris ( $-65 \text{ mg g}^{-1}$  to  $-157 \text{ mg g}^{-1}$  wet weight, respectively). On the basis of preliminary measurements (H. H. Harlow and D. B. Tinker, unpublished data) obtained by bioelectric impedance analysis (Farley and Robbins 1993), bears in this study (average weight = 104 kg) had an average body fat content of approximately 39.2%. Assuming that their fat-free carcass contained approximately 53% skeletal muscle (Behnke et al. 1942), the losses in the gastrocnemius and biceps femoris would represent a whole-body seasonal loss of approximately 2.2 kg and 5.2 kg of protein, respectively. For sake of argument, if mixed skeletal muscle throughout the bear is used at a value intermediate to that of the gastrocnemius and biceps femoris (i.e., 3.7 kg), this use would account for about 13.3% of the total overwinter weight loss ( $100 \times 3.7 \text{ kg protein} / 27.8 \text{ kg total weight loss}$ ), assuming only minimal water loss from dehydration, as suggested in this study (Fig. 4). However, skeletal muscle may not be the only source of protein used during hibernation. For example, many vertebrates are believed to have labile protein reserves (Le Maho et al. 1981) and use specific com-

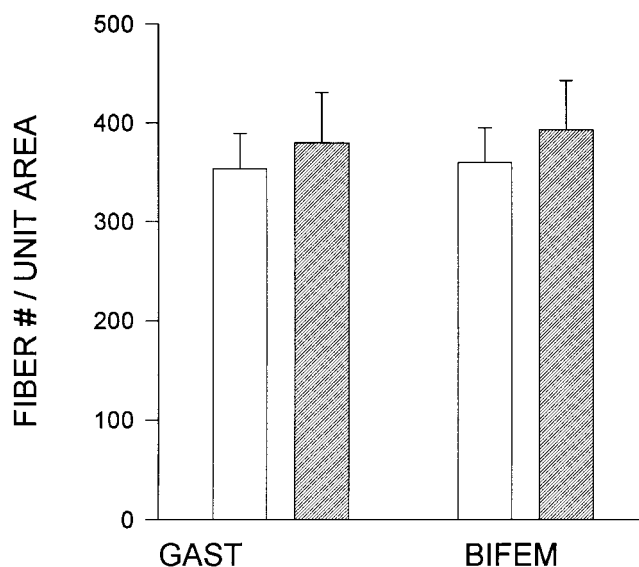


Figure 7. Number of muscle fibers per  $3,900 \mu\text{m}^2$  (in four video subimages) of combined fast- and slow-twitch muscle fibers during fall (open bars) and spring (hatched bars) in gastrocnemius (GAST) and biceps femoris (BIFEM) muscles for seven bears. There was no significant change in number of fibers per  $3,900 \mu\text{m}^2$  in either muscle during hibernation. Vertical lines represent one standard error.

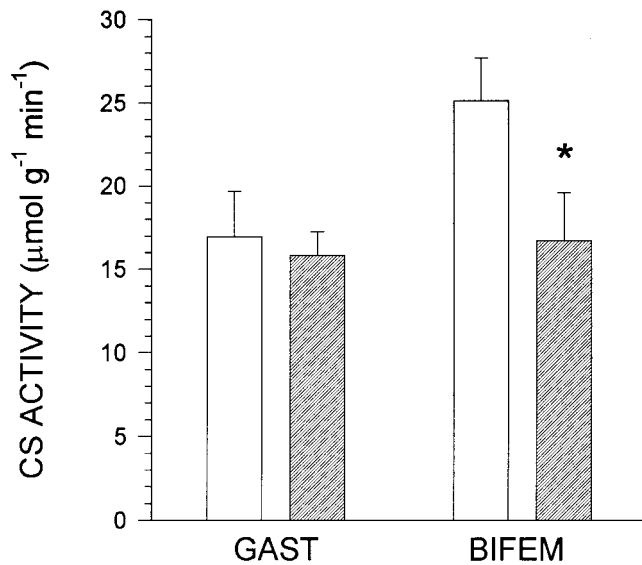


Figure 8. Mean CS activity for fall (open bars) and spring (hatched bars) in gastrocnemius (GAST) and biceps femoris (BIFEM) muscles of seven bears sampled. Asterisk depicts a significant decrease in CS activity during hibernation; mean decrease,  $-8.41 \mu\text{mol g}^{-1} \text{min}^{-1}$  ( $\pm 2.99$ ) ( $P = 0.015$ ). Vertical lines represent one standard error.

partments, including the skin and viscera, as well as blood albumins (Garcia-Rodriguez et al. 1987). Bintz et al. (1979) determined that muscle (carcass) accounts for only about 52% of the protein catabolized by starving Richardson's ground squirrels (*Spermophilus richardsonii*), with the remainder provided from viscera, liver, skin, and fat. If these additional sources of protein are available to bears in the same proportions reported for squirrels, the amount of protein, as catabolized calculated from skeletal muscle, would approximately double (i.e., about another 3.7 kg), resulting in a total of about 7.4 kg of catabolized protein, or a ratio of about 3 : 1 of fat to protein catabolism. This ratio is higher than the 2 : 1 ratio proposed by Bintz and Mackin (1980) necessary to retain water balance by hibernating Richardson's ground squirrels, but lower than the 5 : 1 ratio proposed by Cherel et al. (1995) on nonlactating hibernating hedgehogs (*Erinaceus europaeus*). Therefore, we believe that while overwintering black bears do indeed use skeletal muscle protein, they may have more robust nonskeletal muscle protein reserves than those identified in other animals. These labile protein stores, together with skeletal muscle protein, would act as a source of water and nitrogen, especially during pregnancy and lactation. Alternately, the impact of skeletal muscle catabolism on locomotor capacity may be minimized by using specific muscle groups (Le Maho et al. 1981) that are not as critical for producing body movement and were not among the ones tested in this study. It is possible that if protein is being continually degraded and synthesized,

some amount of repartitioning of the proteins may be occurring; that is, proteins from skeletal muscles other than the ones we biopsied may be catabolized and some of the free amino acids synthesized into protein in the gastrocnemius and biceps femoris. If this in fact occurs, our sampling could underestimate actual muscle loss.

#### *Muscle-Fiber Transformation, Cross-Sectional Area, and CS Activity*

There was a significant decrease in percentage of Type I, slow-twitch, aerobic muscle fibers during hibernation in the biceps femoris but not the gastrocnemius, suggesting that some transformation from slow- to fast-twitch fibers in bears had occurred. However, the extent of this conversion was small compared with nonhibernator muscle disuse rodent models (Templeton et al. 1984; Thomason and Booth 1990) and certainly small compared with what would be expected of humans if confined for a comparable length of time (Appell 1990). Interestingly, the profile of this transformation was somewhat in between that of rodent hibernators and nonhibernator disuse models. When viewed collectively, there is a striking difference in CS activity in hibernating small mammals and in animals subjected to some form of suspension or immobilization. Most studies using rodent hibernators have reported a significant increase in CS activity during hibernation (Wickler et al. 1987; Thomason and Booth 1990; Wickler and Hoyt 1990; Steffen et al. 1991). However, hind limb suspension or immobilization models largely depict a decrease in CS activity in concert with a loss of slow oxidative muscle fibers during periods of muscle disuse (Booth 1977; Fell et al. 1985; Desplanches et al. 1987). Unlike other hibernator models, CS activity actually decreased in the biceps femoris from fall measurements in bears, which seems logical for the following reasons. First, as mentioned earlier, bears do not lower their body temperature to the same extent as more classic hibernators and do not undergo the violent periods of shivering thermogenesis during periodic arousals. Shivering is achieved primarily by Type I fibers and is thought to be the main process that results in the increase in CS activity in other rodent and insectivore hibernators (Wickler and Hoyt 1990; Wickler et al. 1991). Second, there is no supportive evidence that denning bears demonstrate periodic muscular activity such as violent shivering bouts or isometric contractions (Barnes et al. 1994). Third, a decrease in overall CS activity would be anticipated on the basis of the observed decrease in slow, oxidative muscle fibers, which use CS in aerobic metabolic processes.

In the gastrocnemius, slow-/fast-fiber-type ratios and CS activity were essentially unchanged. These results agree with Koebel et al. (1991), who also found no significant alteration in CS activity in postdenning biopsies from gastrocnemius of captive black bears. The alteration of CS activity by overwinter-



ing black bears was, therefore, more like that of animals artificially immobilized than that of rodent hibernators. A study by Booth (1977) made an interesting observation regarding muscle position and CS activity that may help explain this observed difference between large and small mammal hibernators. Booth (1977) found that rat gastrocnemius (as well as the soleus muscles) exhibits an even greater decrease in CS activity when immobilized in a position that was flexed. It may be that the position assumed by rodent hibernators, and to a lesser extent bears during hibernation, helps retard muscle-fiber conversion and either stabilizes or, as in rodent hibernators, enhances CS activity.

Steffen and Musacchia (1984) have suggested that muscle atrophy results from a decrease in muscle cell size, rather than a reduction in the number of fibers. This idea is supported by a number of studies on both hibernating small mammals and animals subjected to various types of immobilization or suspension. For example, hibernating ground squirrels (*Spermophilus lateralis*; Musacchia et al. 1989) and immobilized laboratory rats (Nicks et al. 1989) experience up to a 42% decrease in cross-sectional area of muscle fibers, but no change in fiber number. Similarly, the mean number of fibers per unit area for black bear gastrocnemius and biceps femoris muscle in this study did not change during hibernation. However, unlike results from other disuse models, the mean cross-sectional area of the two muscles sampled from bears did not change over the 4-mo period, indicating no measurable atrophy of these skeletal muscles when muscle-fiber size is used as an index. It is important to point out that muscle-tissue weights taken before and after freeze-drying for protein concentration analysis showed that there was no change in water content of the gastrocnemius and only a small but significant increase in water content of the biceps femoris sampled during hibernation. These data suggest that dehydration was not a factor affecting muscle cell shape or size during winter.

It appears that bears in this study did not experience any significant muscle atrophy in terms of loss in muscle-fiber size or number during hibernation, and only a conservative loss in protein and temperate alteration of fiber-type ratios. Perhaps this moderate alteration of protein content and fiber transition and lack of reduction in cell size or number allowed bears to maintain muscle function for emergence in the spring. Possible explanations for this unique condition could involve biochemical processes of nitrogen recycling to retain skeletal muscle protein and periods of mild shivering or some type of isometric activity during denning not previously identified in captive bears to maintain muscle strength. These explanations seem logical since, with the exception of a moderate decrease in skeletal muscle protein concentration and moderately altered fiber type, bears apparently did not undergo the physiological changes common to other muscle disuse atrophy models. Hibernating bears must therefore be doing something different

in order to exhibit relatively normal muscle function following several months of inactivity. Monitoring of muscle activity of naturally hibernating bears using electromyogram telemetry and data recorders could be used to clarify this unique state.

### Conclusions

On the basis of the muscle biopsies obtained during early and late stages of denning and hibernation, black bears, and particularly lactating female bears, experienced some amount of protein loss during hibernation. This catabolized protein was presumably used as a source of metabolic energy, Krebs cycle intermediates, and replacement water for insensible water loss, as well as a source of nitrogen and water for cub production and growth. However, the magnitude of protein loss by bears (a parameter of muscle atrophy) was not as great as predicted from small mammal hibernator and immobilization disuse models.

Hibernating bears do not seem to completely fit the characterizations of any single muscle disuse model but apparently share some similarities of each of these models because of their unique, mild but continuous torpor period. In addition, when compared with human disuse models and rodent hind limb suspension models that exhibit profound atrophy in terms of muscle-fiber number, size, and reduction of Type I fibers, bears showed only limited muscle atrophy. There was no reduction in cross-sectional area or number of muscle fibers (indicators of muscle atrophy). The transformation of slow-twitch aerobic fibers to fast-twitch anaerobic fibers exhibited by these bears, similar to that in rodent hibernators, was lower than that observed for the extended periods of inactivity reported for other disuse atrophy models, but the concomitant decrease in CS activity was more characteristic of nonhibernating immobile mammals. Clearly, bears employ a unique physiological strategy in order to maintain muscle tone during extended periods of inactivity while in hibernation by demonstrating only moderate skeletal muscle protein loss and slow oxidative muscle-fiber transformation.

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