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Dietary cholesterol enhances torpor in a rodent hibernator

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Abstract Dietary cholesterol can affect both body lipid composition and steroid hormone concentration. We investigated whether a diet rich in cholesterol influences torpor patterns of hibernating chipmunks (*Tamias amoenus*) and, if so, whether these changes are better explained by diet-induced changes in body lipid composition or the concentration of testosterone, which at high levels inhibits torpor. Two groups of chipmunks were maintained either on a cholesterol diet (rodent chow containing 10% cholesterol) or a control diet (rodent chow) during pre-hibernation fattening and throughout the hibernation season. Torpid chipmunks on the cholesterol diet had significantly lower minimum body temperatures (-0.2 ± 0.2 vs $+0.6 \pm 0.2$ °C), lower metabolic rates (0.029 ± 0.002 ml O_2 $g^{-1}h^{-1}$ vs 0.035 ± 0.001 ml O_2 $g^{-1}h^{-1}$), and longer torpor bouts at −1 °C (6.8 ± 0.5 vs 4.1 ± 1.0 days) than chipmunks on the control diet. Dietary cholesterol resulted in a significant increase in blood plasma cholesterol (sevenfold), liver cholesterol content (6.9-fold) and liver triglyceride content (3.5-fold) in comparison to controls. In contrast, dietary cholesterol had no detectable effect on the concentration of plasma testosterone, which was very low in both groups. Since torpor was deeper and longer in animals on the cholesterol diet our study suggests that torpor patterns of chipmunks were either directly affected by the dietary cholesterol or via changes in body lipid composition.

Key words Dietary cholesterol · Hibernation · Metabolic rate · Rodent · *Tamias amoenus* · Testosterone · Torpor bouts · Thermoregulation · Yellow-pine chipmunk

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Abbreviations *MR* metabolic rate · *PUFA* polyunsaturated fatty acids · *SFA* saturated fatty acids · T_a air temperature · T_b body temperature · $\dot{V}O_2$ rate of oxygen consumption

Introduction

Many hibernating mammals lower their body temperature (T_b) during periods of torpor close to or even below 0 °C (Barnes 1989; Geiser and Ruf 1995). This dramatic decline in T_b requires physiological and biochemical adjustments. Tissue function, cellular metabolism and homeostasis can only be maintained if cell membrane structure and function are not interrupted at low temperatures. Cellular membranes of hibernators appear to maintain permeability at lower temperatures than non-hibernators (Willis 1982), which appears to be partially due to differences in membrane lipid composition (Aloia 1988; Geiser 1990).

Cell membrane structure and function are affected by cholesterol content (Yeagle 1985). Cholesterol reduces the flexibility of acyl chains in membrane phospholipids above the temperature of the lipid phase transition, enhances the flexibility below their phase transition, and broadens the phase transition (Blume and Hillmann 1986). The effect of cholesterol on membrane fluidity of hibernating animals has not been determined (Aloia 1988). However, it is known that tissue cholesterol content (Esher et al. 1973), cholesterol/phospholipid ratios of body tissues and cellular membranes (Montaudon et al. 1983, 1986) and plasma cholesterol/high density lipoprotein cholesterol ratios (Russom et al. 1992) are higher in hibernating mammals than in non-hibernating individuals. Because cholesterol content of cell membranes is strongly affected by diet, the structure and function of membranes should in turn be affected by dietary cholesterol (McMurchie 1988). Since dietary fatty acids influence torpor patterns apparently via changes in body lipid composition (Geiser and Kenagy 1987; Geiser 1993; Frank 1992; Florant et al. 1993;

Frank and Storey 1996) it seems possible that dietary cholesterol may also show an effect. This is important to know because in the wild some hibernators are known to consume diet items that are rich in cholesterol and related compounds prior to the hibernation season (Tevis 1953; Cork and Kenagy 1989; Boonstra et al. 1990).

Dietary cholesterol is not only incorporated into tissues and cellular membranes, it is also a precursor of steroid hormones (Tepperman and Tepperman 1987). Steroid hormones, particularly testosterone, have been shown to inhibit hibernation in rodents (Goldman et al. 1986). Moreover, it has been suggested that the increase of testosterone before rodents become reproductive in spring may result in termination of the hibernation season (Darrow et al. 1988).

Because cholesterol in cell membranes may be important for deep hibernation, but on the other hand may be used for testosterone production, which is known to prevent animals from entering torpor, we were interested in whether dietary cholesterol enhances or inhibits hibernation. Since dietary cholesterol could be used either as membrane molecules or as a testosterone precursor, we also measured the content of cholesterol and triglycerides in the blood and liver and the concentration of plasma testosterone to obtain an indication of their enrichment in the body and to provide a possible explanation for our physiological findings. The yellow-pine chipmunk, *Tamias amoenus* (50 g) was used as experimental animal because they are reliable hibernators and are known to ingest diet items that contain cholesterol in the wild (Tevis 1953).

Materials and methods

Twelve chipmunks, *T. amoenus*, were caught on 6–8 September in the Cascade Mountains near Fish Lake, Chelan County, Washington. They were transported to the University of Washington, divided into two groups ($n = 6$ each) of matched body mass and sex and kept individually in cages at an air temperature (T_a) of 23 ± 1 °C (SD) with light from 0600–1800 hours. Animals were fed ad libitum with Purina rodent laboratory chow 5001 until 18 September. From 18 September until the end of the experiment the control group was maintained on the rodent chow (one of these individuals died on 28 December), whereas the experimental group was fed rodent chow containing 10% cholesterol powder (added by weight; grade 95%; Sigma, St. Louis). The small dilution of other nutrients in the cholesterol diet can be easily compensated by a small increase in food intake and should have little effect on the nutritional state of the animals. Moreover, the physiological variables were measured during hibernation when animals consumed almost no food and thus should not be adversely affected. On 27 October, after the animals had been on their respective diets for 40 days, T_a was reduced from 23 to 4 ± 1 °C. The animals were exposed to a range of T_a since torpor bout length is generally strongly temperature dependent and hibernaculum temperatures change with season (Wang 1978; Young 1990). Torpor bouts are known to increase with a fall of T_a above the species-specific set point for T_b that is metabolically defended during torpor, but decrease at T_as below the set point (Geiser and Kenagy 1987, 1988). The thermal regime the animals were exposed to during the hibernation season was: 27 October to 10 December, T_a 4 ± 1 °C; 11 December to 10 January, T_a 2 ± 1 °C; 11 January to 7 February, T_a 0.5 ± 1 °C; 8 February to 22 February, T_a 6 ± 1 °C; 23 February to 8 March, T_a −1 ± 1 °C. After 8 March T_a was raised again to 2 °C.

From 11 December to 8 March, when torpor bouts of *T. amoenus* do not change with the season when T_a is constant (Geiser et al. 1990), the effect of exposure to T_as both above and below the set point for T_b on torpor bout length was determined. To achieve this, animals were exposed to T_as of 2–0.5 and finally to −1 °C after the set point for T_b had been determined in the two experimental groups. This low T_a was selected because it is within the range experienced by hibernators in the wild and would elicit metabolic defence in one experimental group, but not the other. The duration of torpor bouts was measured by observing daily between 0900 and 1000 hours the displacement of sawdust which occurs during arousal. Sawdust was placed on the back of torpid individuals on the day they were first observed in torpor and replaced after each arousal. Since arousals are not randomly distributed during a day and usually occur in the afternoon (Körtner and Geiser 1996; Pohl 1996), our measurements are a reliable indicator of how many days animals were torpid. The mean duration of all undisturbed torpor bouts of all hibernating individuals at each T_a was used to determine the mean torpor bout duration for the two diet groups.

The metabolic rate (MR, measured as rate of oxygen consumption, $\dot{V}O_2$) was determined in all torpid individuals between 11 and 20 December. Two individuals were measured on a particular day. For these experiments each torpid animal on day 2–4 of a torpor bout at T_a 2 °C was transferred from its cage to a 2-l respiratory vessel at the same temperature. Animals were suspended in a plastic mesh hammock to maximise heat exchange with the surrounding air. After MR had stabilised at T_a 2.0 ± 0.5 °C, T_a was slowly decreased (< 0.02 °C min^{-1}) until each animal increased MR to prevent a further drop in T_b. The T_b at this point was immediately measured (within 10 s) by 3 cm rectal insertion of a 36-gauge thermocouple (calibrated to the nearest 0.1 °C) and designated as minimum T_b. Although the minimum T_b was determined rectally it should provide an accurate estimate of the hypothalamic set point for T_b since rectal and hypothalamic temperatures in small hibernators during steady-state torpor are known to be virtually identical (Heller and Colliver 1974). The T_a at which MR began to increase during cooling to prevent a further fall of T_b was called minimum T_a (Geiser and Kenagy 1987).

$\dot{V}O_2$ (STPD) was monitored continuously after removal of H_2O and CO_2 from the air stream with an Applied Electrochemistry S-3A O_2 analyzer using the set-up described in Hill (1972). Flow rates of dried inlet air were controlled and measured with a Brooks thermal mass flow controller. The T_a in the respirometer was measured continuously by thermocouple and recorded along with $\dot{V}O_2$ on a Leeds and Northrup Speedomax 250 recorder. To check the calibration of the thermocouples, readings of T_a were also taken to the nearest 0.1 °C with a digital thermometer.

Hibernating animals were decapitated on 20–24 March, when they were on day 2 or 3 of a torpor bout at T_a 2 °C. Blood was collected in heparinised centrifuge tubes, centrifuged for 5 min at 1000 *g* and the plasma was removed and stored at −30 °C. A section of the liver was removed, chopped into fine pieces with scissors and rinsed repeatedly to remove blood. Livers were then homogenised and frozen at −30 °C.

Total cholesterol and triglycerides were quantified in isopropyl alcohol extracts using a Technicon AutoAnalyser II system (Northwest Lipid Research Center, Seattle, Lipoprotein Laboratory). Samples were diluted with isopropyl alcohol in the presence of zeolite mixture which extracts the lipid from the protein and removes interfering substances. Total cholesterol in the extract was quantified by a Liebermann-Burchard reagent assay; triglycerides were hydrolised with potassium hydroxide coupled with 2,4 pentanedione and were quantified fluorometrically (Kessler and Leder 1965; Warnick et al. 1982). The coefficient of variation was $< 4.2\%$ for triglycerides and $< 1.2\%$ for cholesterol assays.

Plasma levels of testosterone were measured by radioimmunoassay after partial purification on diatomaceous earth/glycol microcolumns. Plasma samples were equilibrated with approximately 2000 cpm of tritiated testosterone (for subsequent determination of

losses owing to the extraction and chromatography procedure) and then extracted in 5 ml of redistilled dichloromethane. Organic extracts were then dried under a stream of nitrogen, reconstituted in 0.5 ml of 10% ethyl acetate in iso-octane and transferred to the microcolumns. Steroid extracts were eluted from the columns in increasing concentrations of ethyl acetate in iso-octane as described by Wingfield and Farner (1975). Extracts were dried under N_2, taken up in phosphate buffered saline and assayed as described previously (Ball and Wingfield 1987). Recoveries, and intra-assay variation were within limits described by Wingfield and Farner (1975). All lipid and hormone assays were conducted within 2 months of tissue preparation.

Statistical comparisons between means were made using a Student's *t*-test for equal or unequal variances as appropriate. Numerical values are expressed as mean ± 1 standard error (SE) if not stated otherwise.

Results

The seasonal change of body mass was similar between *T. amoenus* on the control and animals on the cholesterol diet (Fig. 1). Body mass at capture (8 September) was 49.5 ± 1.0 g in control animals and 49.3 ± 0.9 g in cholesterol-fed animals. Body mass rose by about 25–30% during pre-hibernation fattening, and fell below values at capture at the end of the hibernation season (Fig. 1). The decrease in body mass of both diet groups was more pronounced in the early part of the hibernation season than later in the hibernation season when the cholesterol-fed group lost less mass than the controls.

Physiological variables in hibernating *T. amoenus* were affected by the cholesterol diet. Animals on the cholesterol diet had a significantly lower mean minimum T_b, which was metabolically defended during torpor, than animals on the control diet (Fig. 2; $P < 0.01$), demonstrating that the set point for T_b was lower in the cholesterol-fed group. The mean minimum T_b of animals on cholesterol was −0.2 ± 0.2 °C ($n = 6$) and the lowest individual minimum T_b was as low as −0.8 °C. The mean minimum T_b in animals on the control diet was +0.6 ± 0.2 °C ($n = 6$) and none of these individuals lowered its T_b below 0 °C. The minimum T_a, at which the increase in metabolic rate was first observed during the cooling procedure, also differed between the diet groups (Fig. 2; $P < 0.01$). Thus, animals on the cholesterol diet showed a better cold tolerance than those on the control diet. Moreover, the MR measured at $T_a = 2$ °C (i.e. at a T_a at which torpid individuals of both diet groups did not defend their T_b) in animals on the cholesterol diet (0.029 ± 0.002 ml O_2 $g^{-1}h^{-1}$, $n = 6$) was significantly lower than that of animals on the control diet (0.035 ± 0.001 ml O_2 $g^{-1}h^{-1}$, $n = 6$, $P < 0.05$, not shown). Body mass during these measurements was 50.1 ± 2.1 g ($n = 6$) in animals on cholesterol diet and 53.4 ± 2.2 g ($n = 6$) in animals on control diet and these means were statistically indistinguishable ($P > 0.1$).

The response of torpor bout duration to lowering of T_a also differed between diet groups (Fig. 3). Animals on the control diet had lengthened torpor bouts when T_a was lowered from 2 to 0.5 °C but shortened torpor bouts when T_a was lowered from 0.5 to −1 °C. In contrast, animals on the cholesterol diet already showed maximum torpor bouts at T_a 2 °C, and lowering of T_a from 2 to 0.5 and to −1 °C had little or no effect on the duration of torpor bouts (Fig. 3). At T_a −1.0 °C, which was slightly below the minimum T_a of animals on control diet (i.e. they were metabolically defending their T_b) but well above the minimum T_a of animals on cholesterol diet (i.e. they were not defending their T_b), torpor bouts lasted 4.1 ± 1.0 ($n = 4$) and 6.8 ± 0.5 days

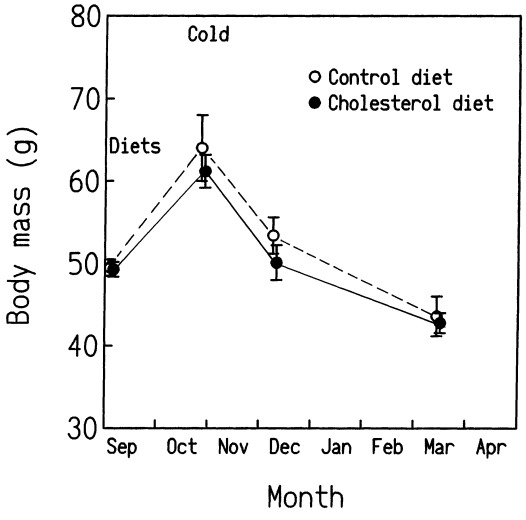

Fig. 1 Seasonal change of body mass in *Tamias amoenus* maintained on the two different diets. Different diets were fed from 18 September; animals were exposed to cold (4 °C) on 27 October. Values are means ± SE for $n = 6$ each with the exception of controls ($n = 5$) in March

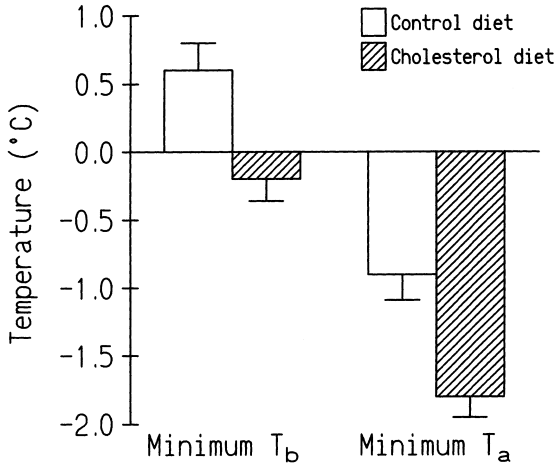

Fig. 2 The minimum body temperature (T_b), which was metabolically defended by torpid *Tamias amoenus* on the two diets and the corresponding minimum air temperature (T_a) at which the increase of the metabolic rate was observed. Both the minimum T_b and T_a differed significantly between the two diets ($P < 0.01$, *t*-test). Values are means with SE for $n = 6$ each

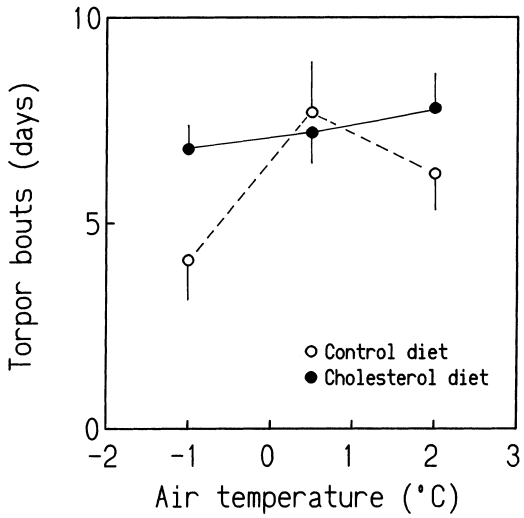

Fig. 3 Duration of torpor bouts at different air temperatures in *Tamias amoenus* on the two different diets. Torpor bouts of individuals on the control diet were strongly affected by the change in temperature, whereas individuals on the cholesterol diet were only slightly affected. Significant differences between the two diet groups were observed at T_a −1 °C ($P < 0.025$, *t*-test); at this T_a animals on control diet were thermoregulating, while those on the cholesterol diet because of their lower minimum T_a were not. Values are means with SE for $n = 6$ with the exception of controls ($n = 4$) at T_a −1 °C

($n = 6$), respectively, and these means differed significantly ($P < 0.025$). Moreover, one animal on the control diet refused to hibernate at the low T_a of −1 °C although it hibernated at higher T_as, whereas all individuals on the cholesterol diet hibernated at this low T_a. At T_as of 0.5 and 2.0 °C, the duration of torpor bouts did not differ between the two diet groups, regardless of whether all the controls or only the four that displayed torpor at T_a −1.0 °C were considered.

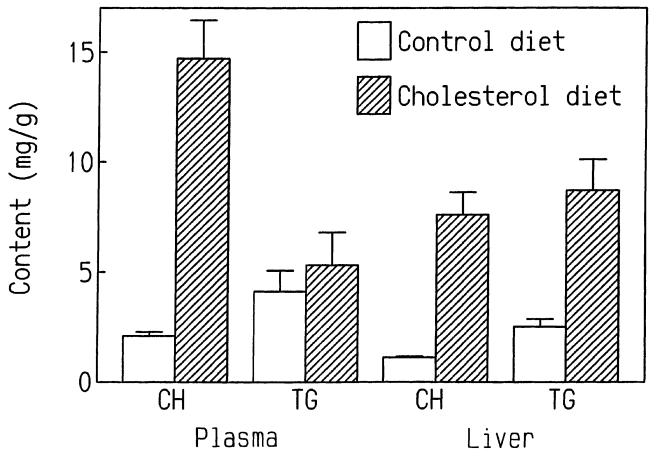

Fig. 4 Content of cholesterol (*CH*) and triglycerides (*TG*) in blood plasma and liver (calculated from wet weight) of *Tamias amoenus* on cholesterol ($n = 6$) and control ($n = 4$) diets. Significant differences were observed between the two diet groups ($P < 0.01$, *t*-test) except for blood triglycerides. Values are means with SE

Changes in thermal physiology of hibernating *T. amoenus* were accompanied by changes in body lipid content (Fig. 4). The cholesterol content of blood plasma was sevenfold greater in animals on cholesterol diet than in animals on the control diet (Fig. 4; $P < 0.01$). Plasma triglyceride content was similar in the two diet groups. In liver tissue, both cholesterol content and triglyceride content was much greater in animals on cholesterol diet than in controls (Fig. 4; $P < 0.01$).

The concentration of testosterone in the blood plasma of hibernating *T. amoenus* was very low in both dietary groups. Mean plasma testosterone concentration was 0.29 ± 0.17 ng·ml^{-1} ($n = 6$) in animals on the cholesterol diet and 0.10 ± 0.01 ng·ml^{-1} ($n = 4$) in controls and these means did not differ significantly.

Discussion

Our results provide the first evidence that dietary cholesterol can affect some physiological variables of hibernating mammals. These diet-induced physiological differences were accompanied by pronounced differences in tissue cholesterol and triglyceride content. It is therefore possible that they were caused by the diet-induced differences in the composition of body lipids. Because both diet groups contained similar levels of plasma testosterone and these were very low in comparison to reproductive sciurids, it is unlikely that the observed dietary effect on torpor patterns was caused by testosterone.

Changes in torpor patterns of hibernating chipmunks on the cholesterol diet were similar to changes in rodent hibernators maintained on diets rich in essential polyunsaturated fatty acids (PUFA) (Geiser and Kenagy 1987; Frank 1992; Florant et al. 1993; Geiser 1993). Body temperatures in hibernators on diets rich in PUFA showed about 2–3 °C lower minimum T_bs, longer torpor bouts and lower metabolic rates than animals on diets low in PUFA or rich in saturated fatty acids (SFA) and a reduction of about 1 °C in comparison to individuals maintained on rodent chow (Geiser and Kenagy 1987; Frank 1992; Florant et al. 1993; Geiser 1993). Since physiological changes observed here were similar to those in the fatty acid diet studies, it is possible that similar mechanisms were involved. As for dietary PUFA, dietary cholesterol may have affected physical properties of tissues and cellular membranes. Since an increase in cholesterol decreases or even abolishes phase transitions of membranes (Blume and Hillmann 1986) it is possible that the most likely increased cholesterol content of cell membranes in animals on cholesterol diet resulted in a less temperature-sensitive structure and allowed the normal physiological function of cells and tissues at lower temperatures than in the control animals. An alternative explanation is that the activity of

membrane-associated proteins, rather than or in addition to the physical membrane lipid properties, was modulated by membrane cholesterol content (Yeagle 1985).

But how can blood and liver cholesterol and triglyceride content influence thermoregulation and torpor patterns? It is likely that the pronounced diet-induced changes in composition of tissue lipid composition observed here were not directly or only partially responsible for the diet-induced physiological changes, but only reflect compositional changes in other tissues, organs and in particular cellular membranes, which show a much bigger diet-induced compositional change in hibernators than in non-hibernators (Geiser 1990). The most likely candidate for causing changes in thermoregulation is the nervous system. It is possible that alterations of neural tissue composition, as observed in chipmunks after manipulation by dietary fatty acids (Geiser 1990), may explain the shift in set point for T_b. Such modulations may occur via changes in membrane receptor activity, which is known to be affected by their lipid environment (Loh and Law 1980). It has been demonstrated that cholesterol content of brain myelin increases in winter (Robert et al. 1982) when the set point for T_b is low, which lends some support to our interpretation. Moreover, cholesterol content increases in lung surfactants during daily torpor (Orgeig et al. 1996) suggesting that high amounts of cholesterol in body lipids may be important for function at low T_b in endotherms.

The diet-induced changes of the response of torpor bouts to a lowering of T_a are most likely a consequence of the shift in set point for T_b. The lowest T_a that animals were exposed to in this study was T_a −1 °C, which was selected because T_as below 0 °C are experienced in burrows of many hibernators in the second part of winter (Wang 1978; Barnes 1989; Young 1990). The T_a −1 °C was below the minimum T_a of animals on the control diet and elicited metabolic defence of T_b during torpor since the set point for T_b had been reached. Apparently as a consequence of the rise of metabolic rate in turn the duration of torpor bouts was reduced (Geiser and Kenagy 1988). However, this T_a of −1 °C was well above the temperature where regulation of T_b was induced in animals on the cholesterol diet. Thus, the relatively small changes in the diet-induced shift in the set point for T_b in the cholesterol group resulted in torpor bouts that were 66% longer than in the controls. The resulting reduced number of arousals in the cholesterol-fed animals together with the lower metabolic rates and the wider T_a range in which no energy is wasted for thermoregulation should result in substantial energy savings during hibernation. Since winter mortality in many small hibernators is caused by depleted fat stores, a reduction in energy expenditure should increase the chance of winter survival.

In contrast to body lipids, plasma testosterone concentration was not significantly affected by dietary cholesterol. Since concentration of both control and cholesterol groups were less than 10% of that in actively breeding animals (Barnes 1996) it is unlikely that they had a physiological effect. Testosterone inhibits torpor in hamsters, *Phodopus sungorus*, at a serum concentration of about 1.5 $ng \cdot ml^{-1}$ (Goldman et al. 1986), which is three- to tenfold of the values observed here. If the level of testosterone in the cholesterol group had been effective, an inhibition rather than enhancement of torpor would have occurred.

While our findings may be interesting from a physiological point of view, questions as to their ecological significance may be raised. Since an increased intake of diet items rich in essential PUFA occurs in heterothermic rodents during autumn (Tevis 1953; Wood 1993; Frank 1994), it can be argued that selection of food items rich in PUFA forms part of the preparation for torpor (Geiser and Kenagy 1987; Frank 1994). In contrast, it may appear unlikely that largely herbivorous rodents have access to cholesterol. However, there is some evidence that indicates that cholesterol is available to free-living rodents and perhaps may be sought. Ground squirrels, *Spermophilus lateralis*, and chipmunks, *Tamias* spp., do not exclusively consume plant material and not infrequently supplement their diet with animal flesh (Tevis 1953). Moreover, a major diet item of *Tamias* spp. and other sciurid rodents are fungi (Tevis 1953; Cork and Kenagy 1989) that contain mycosterol, which is structurally very similar to cholesterol (Gurr and James 1980) and also other plant sterols. While these examples may reflect opportunistic diet uptake, observations on Arctic ground squirrels, *Spermophilus parrvii*, strongly suggest diet selection. The species has been observed to prey actively on collared lemmings in late June/July and to consume the brain of their prey preferentially (Boonstra et al. 1990; Boonstra R, personal communication), which is rich in cholesterol. Arctic ground squirrels commence hibernation in early September and pre-hibernation fattening occurs during June/July when predation of lemmings has been observed. Arctic ground squirrels show extremely low T_bs during hibernation of about −3 °C and are able to prevent freezing of body fluids by supercooling (Barnes 1989). However, normal physiological function during hibernation at these extremely low temperatures may require a high content of cholesterol in tissues and cellular membranes.

Our study shows that the pattern of hibernation in chipmunks can be affected by dietary cholesterol. It suggests that in some species uptake of cholesterol may form part of the preparation for hibernation. Thus, the possible importance of dietary cholesterol should be considered in future studies on the nutritional ecology of hibernators.

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