## **ORIGINAL PAPER**

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# Adaptations to fasting in the American mink (*Mustela vison*): nitrogen metabolism

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Abstract The aim of this study was to investigate the adaptations of protein metabolism to seasonal fasting in an actively wintering boreal carnivore. Fifty farm-bred male American minks Mustela vison were divided into a fed control group and four experimental groups fasted for 2, 3, 5 or 7 days. The responses of nitrogen metabolism to wintertime food deprivation were determined by measuring the rate of weight loss, the tissue total protein concentrations and the plasma amino acid, urea, ammonia, uric acid and total protein levels. The mink has relatively poor adaptations to food deprivation, as it is not able to prolong phase II of fasting with fat as the major metabolic fuel. Instead, the species has to derive a part of its energy requirements from the breakdown of body proteins. The end product of protein catabolism-urea- accumulates in its circulation, and the mink may not be able to recycle urea-N. Although the mink can still have a high body fat percent at the end of the 7-day fast, it appears to enter phase III of fasting with stimulated proteolysis during this period.

**Keywords** American mink · Fasting · *Mustela vison* · Nitrogen metabolism · Starvation

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#### Introduction

The seasonal adaptations of nitrogen metabolism of mammals have not been targets of intensive research. Species inhabiting polar and boreal regions are attractive study subjects for such investigations, as they experience extreme seasonal fluctuations in temperature, photoperiod and food availability in their natural habitats. Until now, only the adaptations of over-wintering bears (Ursus spp.) have been focuses of several investigations (Nelson et al. 1973, 1975; Lundberg et al. 1976; Hissa et al. 1998). Ursids are able to recycle urea-N for the de novo synthesis of amino acids (AAs) and proteins and they have evolved the ability to bear, give birth and nurse cubs while fasting during the winter sleep (Nelson et al. 1983). In addition to bears, a boreal canid, the raccoon dog (Nyctereutes procyonoides), can maintain its protein catabolism constant for at least two months during wintertime fasting (Mustonen et al. 2004).

In addition to mammals spending the cold season in sleep or hibernation, northern carnivores that stay active throughout the year are interesting models to study the seasonal adaptations of protein metabolism. The adaptations of particular medium-sized (the blue fox Alopex lagopus, A-M Mustonen et al., unpublished data) and large canids (the gray wolf *Canis lupus*, DelGiudice et al. 1987) have been previously studied in this context. Fasting induces an increase in the plasma concentrations of particular essential AAs in the blue fox suggesting that its energy requirements derive partly from body proteins during fasting. In spite of this, blue foxes do not enter phase III of fasting characterized by stimulated protein catabolism during a repeated 22-day wintertime fast. The gray wolf experiences fasting-induced decreases in serum urea-N concentrations and in urinary urea:creatinine ratios, which suggests that this species also is able to preserve body proteins during food deprivation.

The American mink *Mustela vison* (Schreber, 1777) is a semi-aquatic mustelid predator originating from North America (Dunstone 1993). Due to human intervention and superior colonizing abilities, it presently thrives in most European countries, in vast areas of Russia and even in Japan and South America. The species is an interesting model to study the adaptations of nitrogen metabolism, as the northernmost mink populations inhabit the Palearctic and Nearctic tundra, where the animals may experience periods of involuntary food scarcity during the cold season. Feral minks have been noted to lose up to 39% of body mass (BM) during late winter and spring in association with mating season and food shortage (Smal 1991). In the absence of human interference, food availability is a major factor determining population densities of the species. Compared to the larger canids studied previously in this context, the mink is a smaller mustelid with rather poor adaptations to fasting (Mustonen et al. 2005). For this reason the species can offer novel information about the seasonal adaptations of energy metabolism in carnivores.

This study has also a practical goal. The mink is reared for the fur industry and it has been observed that the high fat percent of farmed animals causes detrimental effects on their reproductive performance (Joergensen 1985). Fasting procedures of 1–8 weeks have been shown to be safe for fur-bearing canids and fasts of shorter duration could decrease obesity also in farm-bred mink if they can be applied safely to mink farming. The aim of this study was to investigate the characteristics of nitrogen metabolism of the mink in response to wintertime food deprivation. The specific aims were to find out: (1) how tissue protein concentrations of the mink change in response to fasting, (2) how these changes are reflected in plasma concentrations of total proteins and total AAs, (3) how individual essential and nonessential AAs respond to food withdrawal, (4) how fasting influences plasma levels of end products of protein catabolism as well as to demonstrate (5) if the species is able to prolong phase II of fasting or if it is forced to enter phase III of starvation after 2-7 days without food.

#### **Materials and methods**

Fifty farm-bred brown male minks born between 8–18 May 2003 were selected for the study. They were housed in standard wire cages ( $85\times31\times45$  cm) with wooden nest boxes ( $27\times31\times38$  cm). The cages were suspended above the ground in an unheated barn at the Juankoski Research Station fur farm, Juankoski, Finland ( $63^{\circ}N$ ;  $28^{\circ}E$ ). The animals were kept at natural temperature (-8.1 to  $+2.9^{\circ}C$ ) and photoperiod, but the effects of wind were absent. They were fed with commercial fur animal diets (12.3% proteins, 8.7% fat, 13.8% carbohydrates, 6,510 kJ kg fresh weight<sup>-1</sup>) according to common farming practices and water or ice was available ad lib.

The fasting experiments were conducted between Dec 10–16, 2003. The minks were randomly assigned into

five groups as follows; group 1: fed control animals (n=10), group 2: animals fasted for 2 days (n=10), group 3: animals fasted for 3 days (n=10), group 4: animals fasted for 5 days (n=10) and group 5: animals fasted for 7 days (n=10). None of the animals died during the food deprivation periods. Instead, they were alert and physically active and their locomotor ability remained normal throughout the fasts. Previously Bjornvad et al. (2004) have food-deprived minks up to 10 days. The fed control group was fasted overnight (16 h) before the sacrifice. The BMs of the control group were measured before the last meal and 16 h after the removal of food at sampling. The fasted animals were weighed 22 h after the removal of the remains of their last meal on the first day of fasting and at the end of the fasting period at sampling. Water was available ad lib. during food deprivation. The experiment was approved by the Animal Care and Use Committee of the University of Joensuu.

After the fasting trials the minks were sacrificed with an electric shock and blood samples were obtained with cardiac punctures. Electrocution leading to cardiac arrest is a recommended method for sacrificing fur animals (Council of the European Union 1993). Blood samples were taken with aseptic needles into test tubes containing EDTA and centrifuged at 1,000 g to obtain plasma. The livers were dissected and samples were taken from the quadriceps muscle of the left hind thigh. All samples were immediately frozen in liquid nitrogen and stored at  $-40^{\circ}$ C.

Plasma AA, urea and ammonia (NH<sub>3</sub>) concentrations were analyzed at the Laboratory of Oulu University Hospital (Oulu, Finland) with ion-exchange chromatography (Biochrom 20 Amino Acid Analyzer, Pharmacia Biotech Ltd., England). The plasma creatinine, total protein (TP) and uric acid (UA) concentrations were determined with the Creatinine Colorimetric, Total Protein Biuret and Uric Acid Enzymatic Colorimetric Methods purchased from the Randox Laboratories Ltd. (Crumlin, UK). Also the CK NAC-activated Creatine Kinase EC 2.7.3.2 reagents were purchased from the Randox Laboratories. For the actual measurements the Technicon RA-XT analyser (Swords, Ireland) was used. The liver and muscle total protein contents were measured spectrophotometrically (Lowry et al. 1951).

Multiple comparisons were performed with the SPSSprogram using the one-way analysis of variance (ANOVA) followed by the post hoc Duncan's test. Comparisons between two study groups were performed with the Student's t test for independent samples. The p value less than 0.05 was considered statistically significant. The results are presented as the mean  $\pm$  SE.

#### Results

The decrease in the BMs of the minks was significant already after 2 days of fasting and the minks fasted for

Table 1 Effects of fasting on the body mass (BM), the absolute and relative liver, kidney and fat pad weights and on the plasma urea: creatinine ratios of the minks (mean  $\pm$  SE)

	16 h	2 days	3 days	5 days	7 days
InitialBM (g)	$2719\pm99$	$2636\pm89$	$2623\pm95$	$2608\pm99$	$2795\pm90$
FinalBM (g)	$2702\pm98$	$2559\pm92$	$2375 \pm 100*$	$2338 \pm 97*$	$2448\pm92$
BM change (g)	$-17 \pm 4$	$-77 \pm 9*$	$-248 \pm 17*$	$-270 \pm 9*$	$-347 \pm 13*$
BM change (%)	$-0.6 \pm 0.15$	$-3.0 \pm 0.37 *$	$-9.7 \pm 0.84*$	$-10.5 \pm 0.49 *$	$-12.5 \pm 0.59 *$
Liver mass (g)	$59 \pm 2$	$61 \pm 2$	$59 \pm 2$	$63 \pm 3$	$72 \pm 5*$
Liver mass (% BM)	$2.2 \pm 0.04$	$2.4 \pm 0.06*$	$2.5 \pm 0.12*$	$2.7 \pm 0.09*$	$3.0 \pm 0.17 *$
Kidneys mass (g)	$10.5 \pm 0.31$	$11.5 \pm 0.39$	$10.8\pm0.39$	$10.0 \pm 0.41$	$11.3 \pm 0.33$
Kidneys mass (% BM)	$3.9\pm0.09$	$4.5 \pm 0.19*$	$4.7 \pm 0.35*$	$4.3 \pm 0.16*$	$4.6 \pm 0.17*$
Subcutaneous fat (g)	$860 \pm 58$	$788\pm60$	$720\pm56$	$706 \pm 57$	$763\pm48$
Subcutaneous fat (% BM)	$31.5 \pm 1.0$	$30.4 \pm 1.4$	$29.8 \pm 1.4$	$30.1 \pm 1.4$	$31.0 \pm 1.0$
Omental fat (g)	$47 \pm 7$	$37 \pm 5$	$35\pm 6$	$32\pm4$	$34 \pm 4$
Omental fat (% BM)	$1.7 \pm 0.20$	$1.4 \pm 0.16$	$1.4 \pm 0.22$	$1.4 \pm 0.16$	$1.4 \pm 0.16$
Retroperitoneal fat (g)	$40\pm 6$	$29 \pm 3$	$22 \pm 3*$	$24 \pm 3*$	$23 \pm 3*$
Retroperitoneal fat (% BM)	$1.4 \pm 0.19$	$1.1 \pm 0.10$	$0.9 \pm 0.12*$	$1.0 \pm 0.12^*$	$0.9 \pm 0.11*$
Mesenteric fat (g)	$86 \pm 10$	$70 \pm 11$	$55 \pm 11*$	$56 \pm 7*$	$64 \pm 9$
Mesenteric fat (% BM)	$3.1\pm0.30$	$2.7\pm0.37$	$2.2\pm0.40$	$2.3 \pm 0.28$	$2.5\pm0.33$
Intra-abdominal fat (g)	$180 \pm 21$	$143\pm19$	$118 \pm 20*$	$118 \pm 14*$	$128 \pm 17$
Intra-abdominal fat (% BM)	$6.6 \pm 0.64$	$5.5 \pm 0.62$	$4.7 \pm 0.75$	$5.0 \pm 0.55$	$5.1 \pm 0.60$
Total fat (g)	$1040\pm75$	$931\pm76$	$837\pm73$	$829\pm70$	$891\pm60$
Fat (% BM)	$38.1 \pm 1.52$	$35.9 \pm 1.82$	$34.6 \pm 2.00$	$35.3 \pm 1.84$	$36.1 \pm 1.20$
Urea-creatinine ratio	$19.0 \pm 1.81$	$25.9\pm4.00$	$38.5 \pm 6.20*$	$26.7 \pm 2.60$	$27.9 \pm 1.86$

\*Significant difference compared to the control group fasted for 16 h (P < 0.05)

7 days lost about 350 g during the fasting period (ANO-VA, F = 145.571, df = 4, P < 0.0004; Table 1). The relative liver and kidney masses increased during fasting for 2– 7 days (t test, F = 1.288-5.965, df = 9.758-18, P < 0.0004-0.049). The muscle and liver protein concentrations decreased due to fasting for 5–7 days (t test, F = 1.742-8.289, df = 10.493-18, P < 0.0004-0.013; Fig. 1). The minks fasted for 5 days had higher plasma TP (t test, F = 30.633, df = 11.055, P < 0.016) and UA concentrations (t test, F = 77.807, df = 9.550, P < 0.009) than the controls (Fig. 1–2). Creatine kinase or creatinine did not respond clearly to food deprivation, but the plasma urea: creatinine ratio was highest in the minks fasted for 3 days (ANOVA, F = 3.613, df = 4, P < 0.019; Table 1).

The minks fasted for 2 days had lower plasma concentrations of arginine (Arg), asparagine (Asn), carnosine, isoleucine (Ile), glycine (Gly), leucine (Leu), phenylalanine (Phe), proline (Pro), serine (Ser), taurine (Tau), valine (Val), total AAs and nonessential AAs than the fed animals (*t* test, F=0.009-4.418, df=8.632-10, P < 0.004-0.047; Fig. 3a). Three days of fasting decreased the plasma levels alanine (Ala), Arg, citrulline (Cit), Gly, Pro, Ser, total AAs and nonessential AAs, but increased the concentrations of 3-methylhistidine (3-MH) and urea (*t* test, F=0.310-6.301, df=6.286-10, P < 0.002-0.043; Figs. 2, 3b). The minks fasted for 5 days had lower plasma concentrations of Ala, Arg, Asn, Cit, glutamine (Gln), Gly, Pro, Ser, total AAs and

**Fig. 1** The liver, muscle (mg  $g^{-1}$ ) and plasma (mg ml<sup>-1</sup>) total protein concentrations of the American mink during 2–7 days of fasting (mean + SE). \* *Asterisk* denotes significant difference compared to the fed control group (*t* test, P < 0.05)

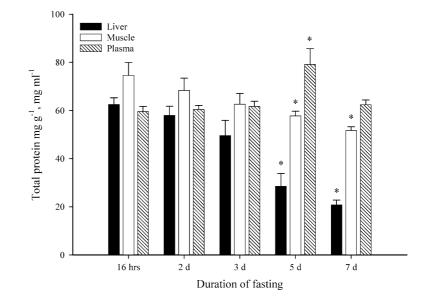
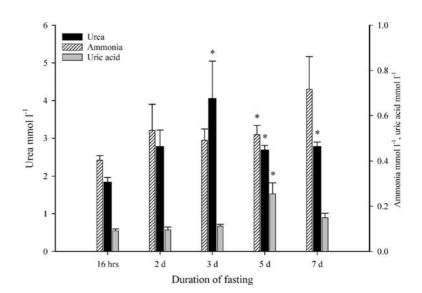


Fig. 2 The plasma urea, ammonia and uric acid (mmol  $l^{-1}$ ) concentrations of the American mink during 2–7 days of fasting (mean + SE). \* *Asterisk* denotes significant difference compared to the fed control group (*t* test, P < 0.05)



nonessential AAs, whereas the levels of  $\alpha$ -aminobutyrate, 3-MH, NH<sub>3</sub> and urea were higher than in the fed group (*t* test, F = 0.035 - 5.689, df = 6.003-10, P < 0.0004 - 0.042; Figs. 2, 3c). Seven days of fasting led to lowered concentrations of Ala, Asn, Cit, Gln, Pro, Ser and nonessential AAs but elevated levels of urea (*t* test, F = 0.015 - 3.208, df = 8-10, P < 0.0004 - 0.049; Figs. 2, 3d).

### Discussion

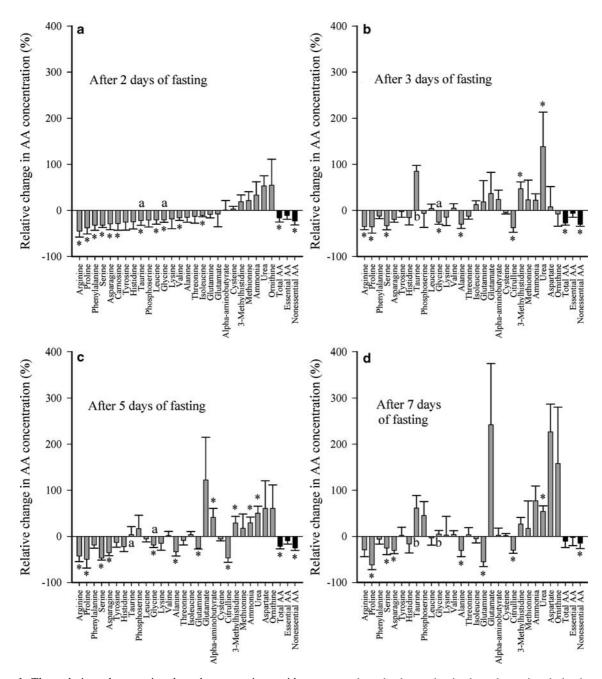
Mammals adapted to long-term seasonal fasts are able to maintain critical organ function, reduce metabolic rate and locomotor activity, stimulate mobilization of stored lipids and promote gluconeogenesis and ketogenesis, which provide fuel for glucose-dependent tissues during food deprivation. A substantial difference between successful fasting and starving is the ability to conserve body protein, the fuel whose depletion limits survival during fasting (Castellini and Rea 1992). During the first few days of fasting (phase I), there is a limited rate of BM loss and nitrogen excretion, whereas hepatic glycogen stores are depleted and fat utilization increases. Phase II involves stimulation of lipid mobilization and preservation of proteins, whereas proteolysis and rate of weight loss increase during phase III. Mammals such as bears, seals and hibernating rodents are able to prolong phase II of fasting with stimulated fat oxidation for several months. In contrast, the non-fasting adapted species are rapidly forced to enter phase III of fasting, which occurs when 30-50% of body protein has been wasted. Long-term fasting is not a routine part of the life history of the mink, and the results of the present study demonstrate that its responses to withstand long-term wintertime food deprivation are very different from the adaptations shared by naturally fasting mammals.

Significant decreases were observed in the liver (54–67%) and muscle protein concentrations (23–31%) of the minks after 5–7 days of fasting. These results indicate a net loss of tissue proteins during total food deprivation.

The mink presumably utilizes its body proteins as sources of metabolic energy, Krebs cycle intermediates and nitrogen during a negative energy balance. Previous studies on other carnivores have reported stable (American black bear U. americanus: Koebel et al. 1991; the raccoon dog: A-M Mustonen et al., unpublished data) or decreased muscle protein concentrations during seasonal fasting (Am. black bear: Tinker et al. 1998). In contrast to the mink, these species utilize passive wintering strategies, and despite the inactivity and the possible loss of muscle proteins during fasting, their locomotor ability seems to be normal at arousal. As the muscle samples of the present study were dissected from the quadriceps muscle of the hind thigh, the mink appears to utilize muscle proteins critical for producing body locomotion during a negative energy balance.

Previous studies on carnivores have reported stable or decreased TP and total AA concentrations during fasting (black bear: Nelson et al. 1973; raccoon dog: Mustonen et al. 2004; blue fox: A-M Mustonen et al., unpublished data). The plasma TP concentrations of the minks remained stable during fasting with a transient increase after 5 days without food. The total AA concentrations, on the other hand, decreased between days 2-5 of fasting but increased to the levels of the fed animals after 7 days. The ability of the mink to maintain its plasma TP concentrations and to increase the total AA levels to the control level at the end of the fast suggests tissue proteolysis. The mink conforms to the previous data on the actively wintering blue fox with relatively stable plasma concentrations of essential AAs during a wintertime fast (A-M Mustonen et al., unpublished data). On the contrary, some carnivore species have shown stable plasma concentrations of nonessential AAs (A-M Mustonen et al. 2004, unpublished data), the levels of which clearly decreased in the fasting mink.

The minks of the present study showed permanent decreases in the plasma concentrations of Pro, Ser (days 2–7), Arg, Gly (2–5), Ala and Cit (3–7) during fasting. Similar fasting-induced changes have been previously



**Fig. 3 a–d** The relative changes in the plasma amino acid concentrations (%) of the American mink during 2–7 days of fasting compared to the control group (mean + SE). *Asterisk* denotes significant difference compared to the fed control group, dissimilar letters indicate significant differences between the fasted groups (*t* test, P < 0.05)

observed in raccoon dogs and blue foxes (A-M Mustonen et al. 2004, unpublished data). Moreover, several decreases (Asn, Ile, Leu, Phe, Tau, Val) documented in the mink plasma after 2 days of fasting have been observed also in the fasting raccoon dog (Mustonen et al. 2004). In the mink, however, these changes were transitory and no longer present after 3 days without food. The plasma concentrations of 3-MH increased in the fasted minks during days 3–5 of food deprivation further supporting the hypothesis that the animals had to utilize their body protein pool. 3-MH is a constituent of actin and myosin in skeletal muscle and it is not reutilized after muscle protein breakdown but excreted in the urine (Young and Munro 1978). Thus, an increased plasma 3-MH level has been used as an index of myofibrillar protein breakdown during fasting (Hissa et al. 1998; Mustonen et al. 2004) and, for instance, the raccoon dog and the blue fox (A-M Mustonen et al. 2004, unpublished data) have decreased or stable plasma 3-MH levels during phase II of fasting.

A fasting-induced increase in circulating Gln concentrations has been observed in several canids such as the domestic dog *Canis familiaris* (Miller et al. 1983), the raccoon dog (Mustonen et al. 2004) and the blue fox (A- M Mustonen et al., unpublished data). In the dog, the liver becomes a net producer of Gln during fasting (Miller et al. 1983). Gln could be also synthesized in muscles from other AAs after protein breakdown and subsequently released into the bloodstream (Goldberg and Chang 1978). This AA is an important carrier of carbon, nitrogen and energy, plays roles in hepatic urea synthesis, renal ammoniagenesis and liver and kidney gluconeogenesis, serves as respiratory fuel for many cells, and its amide-N is used in the biosynthesis of several AAs, purine and pyrimidine nucleotides and amino sugars (Curthoys and Watford 1995; Mathews and van Holde 1996). For this reason, elevated blood Gln concentrations may be required for successful prolonged fasting. However, the mink does not experience a fasting-induced increase in the blood Gln levels. In fact, its Gln concentrations decreased from the control levels during days 5–7 without food. It is possible that fastinginduced hyperglutaminemia is a response shared by different carnivore families and the inability of the liver of the mink to release sufficient amounts of Gln into circulation may increase its demand for muscle proteolysis during fasting.

Fasting-induced proteolysis was also evidenced by the increased plasma concentrations of urea, NH<sub>3</sub> and UA after 3-7 days of fasting. The levels of urea, the primary end product of protein catabolism in mammals (Mathews and van Holde 1996), increased over two fold after 3 days of fasting and remained 46-51% higher than in the fed minks during the rest of the fasting period. The highest UA concentrations were documented after 5 days of fasting with a 1.8-fold increase and the highest NH<sub>3</sub> values after 7 days with a 78% increase compared to the control group. Generally, the circulating urea levels decrease during phase II of fasting in mammals with good adaptations to food deprivation (Nelson et al. 1973; Mustonen et al. 2004), and the  $NH_3$  and UA levels remain stable (Nelson et al. 1984; Mustonen et al. 2004). It is, however, known that bears and other mammals with prolonged natural fasts utilize proteins while fasting (grey seal Halichoerus grypus: Nordøy et al. 1990; European hedgehog *Erinaceus europaeus*: Cherel et al. 1995; polar bear U. maritimus: Atkinson et al. 1996), but their importance as an energy source could be of a higher magnitude in the fasting mink.

In addition to the increase in the plasma levels of urea, NH<sub>3</sub> and UA, the stable or increased plasma urea:creatinine ratios and the elevated plasma alanine and aspartate aminotransferase activities (Mustonen et al. 2005) support the hypothesis of the fasting-induced stimulation of proteolysis in the mink. As expected, the mink may not be able to recycle urea-N during fasting unlike suggested previously for e.g. ursids (Nelson et al. 1975) and pinnipeds (Pernia et al. 1980). In general, the rate of weight loss is stimulated during phase III of starvation, and also the weight loss of the minks appeared to increase between days 5–7 of fasting. Bjornvad et al. (2004) have presented similar data on BM changes of fasting minks as observed in this study. The data of

the present study suggest that the minks were in phase III of fasting after 7 days without food, although their body fat percent was as high as 36% at the end of the fast (Mustonen et al. 2005). It has been observed also in other species such as in fasting Microtinae voles (Mosin 1984) that animals can starve to death when still having considerable amounts of fat in their bodies.

Proteins are known to be the relatively most important energetic fuel for the mink, their oxidation being 39% of the total heat production when animals are close to energy balance (Tauson et al. 1997). This percentage is distinctively higher than e.g. for the laboratory rat *Rattus norvegicus* (10–15%) fed near maintenance level (Chwalibog et al. 1998). During restricted feeding, the oxidation of proteins decreases to 35% in the mink. As an obligate carnivore with high protein:carbohydrate ratio of the diet, the mink requires a high capacity for gluconeogenesis (Sørensen et al. 1995). Efficient use of AAs for the de novo synthesis of glucose could have enabled normoglycaemia during the 7-day fast in spite of the decreased liver glycogen concentrations (Mustonen et al. 2005).

The high demand for proteolysis may also derive from an inability to mobilize body lipid stores effectively. Lipolytic activity of the mink liver and adipose tissue decreases during fasting and accumulation of triacylglycerols results in fatty livers (Mustonen et al. 2005). It has been hypothesized that the species could have a limited capacity for ketogenesis due to insufficient  $\beta$ oxidation, which would lead to the accumulation of fat to the livers and impair the use of body lipids as metabolic fuel during a negative energy balance. It is also possible that ecophysiological reasons, e.g. a requirement of a thick subcutaneous fat layer for the semiaquatic life-style under boreal conditions, could lead to preservation of adipose tissue. It must be recalled that the animals of the present study were farm-bred minks, the body size and fat content of which can be higher than in wild animals (Schoenemann 2004). This could have affected the results e.g. by prolonging phase II of fasting.

In conclusion, the American mink has relatively poor adaptations to food deprivation, as it is not able to prolong phase II of fasting with lipids as the major metabolic fuel. Instead, the species derives a part of its energy requirements from body proteins during fasting and the end products of protein catabolism accumulate in its circulation. Although the mink can have a very high body fat percent at the end of the 7-day fast, it appears to enter phase III of fasting with stimulated proteolysis during this period. For this reason fasting procedures aiming to reduce the body fat content of the mink cannot be recommended for mink farmers.

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