Exogenous Melatonin Elevates the Plasma Leptin and Thyroxine Concentrations of the Mink (*Mustela vison*)

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Eight male and eight female minks were given exogenous melatonin as subcutaneous implants. The plasma leptin and thyroxine concentrations were measured. The leptin concentrations showed clear seasonal variations and differences between the experimental groups. In September most of the control females had undetectable plasma leptin concentrations, but the melatonin-treated females had detectable concentrations significantly higher than the leptin levels of the controls. Most of the males had undetectable leptin concentrations, too. In October the plasma leptin levels had increased significantly in all the groups except the control males. The melatonin-treated minks had significantly higher leptin levels than the controls. There was a significant rise in the thyroxine levels from September to October and the melatonin-treated groups had significantly higher thyroxine levels than the controls. The effects of exogenous melatonin are very pronounced in the mink. Melatonin elevates the plasma leptin and thyroxine levels possibly by direct and indirect mechanisms.

Introduction

The mink (*Mustela vison,* Schreber, 1777) is a nearctic mammal introduced to Finland originally for fur industry. Escaped individuals have bred successfully in Finnish nature and the mink has become a common small carnivore in the Nordic countries. The mink adapts easily to different semiaquatic environments. It has an elongated body shape and a high basal metabolic rate (Iversen, 1972). The mink mates in March and gives birth in May.

Melatonin is a crucial factor controlling the seasonal cycles of body weight, moults and testicular activity of the mink. The increasing melatonin secretion after the summer solstice is the photoperiodic signal to the autumnal weight gain as well as to the autumn moult (Valtonen *et al.,* 1995). The melatonin secretion decreases in October before the testicular recrudescence in males and in November in females (Valtonen *et al.,* 1992). The high melatonin secretion of males in January coincides with the time of the maximum secretion of testosterone. Melatonin levels decrease in both sexes in March, the time of nidation in females, testicular involution in males and the initiation of the spring moult. Subcutaneous melatonin implants raise the circulating melatonin concentrations and stimulate the seasonal changes occurring under short day photoperiod including the autumn moult (Allain and Rougeot, 1980), increased appetite weight gain and deposition of fat (Valtonen, 1997) as well as increased testicular weight (DiGregorio and Murphy, 1987).

Leptin is a novel hormone discovered by the positional cloning of the murine *obese* (*ob*) gene (Zhang *et al.,* 1994). Leptin is secreted mainly by the white adipose tissue (WAT). Leptin secreted by the WAT provides a feedback signal informing the central nervous system about the energy reserves of the body stored as fat (Collins *et al.,* 1996). In the hypothalamus leptin inhibits the production of the neurotransmitter neuropeptide Y, which is responsible for the neuroendocrine response to fasting including a raised level of food consumption, reduction of thermogenesis and elevated levels of plasma corticosteroids (Stephens *et al.,* 1995). Leptin levels in the plasma of humans and rodents are positively correlated to the amount of fat present in the body measured as the body mass index (BMI) (Maffei *et al.,* 1995). In the garden dormouse (*Eliomys quercinus*) exogenous melatonin has been shown to augment the expression of the leptin producing *ob* gene (Ambid *et al.,*

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1998). Leptin also seems to be a trigger for the onset of puberty (Chehab *et al.*, 1997). In nature, when the availability of sufficient nutrition diminishes, e.g. in the winter, the falling leptin concentrations induce the behavioural and neuroendocrine response to fasting which is inhibited by exogenous leptin (Ahima *et al.*, 1996).

Thyroxine (T_4) is closely involved in the control of the basal metabolic rate, growth and the seasonal energy balance of boreal mammals (see Korhonen, 1987). The thyroid activity of mammals is controlled e.g. by the ambient temperature and photoperiodism (Dempsey and Searles, 1943) and possibly by thyroid-testis interactions (Boissin-Agasse *et al.*, 1981). Experiments with rodents suggest an inhibitory action of the pineal gland on the neuroendocrine-thyroid axis (Vriend, 1983).

The objective of this study is to investigate the roles of endogenous seasonal cycles and melatonin in the regulation of the plasma leptin and thyroxine levels of minks and to study the interactions between melatonin, leptin and thyroxine on the minks' seasonal preparation for wintering.

Materials and Methods

For this study 16 male and 16 female sapphire minks of the Siikasalmi Research Station of the University of Joensuu were used. The animals were born in early May 1999. The follow-up period was from September 15th to November 16th 1999. The animals were housed under roof under normal fur farm conditions exposed to natural temperature (Fig. 1) and photoperiod. All the minks were farmborn and caged singly in standard farm cages (30 cm*72 cm*42 cm) with wooden nestboxes. The shadowhouses were aligned in the north-south direction and all the animals were housed at the eastern side of the building. The animals were randomly assigned to four groups, each consisting of 8 individuals. One of the male and the female groups was treated with exogenous melatonin. The other two groups were the control groups. The melatonin implants were introduced in late July.

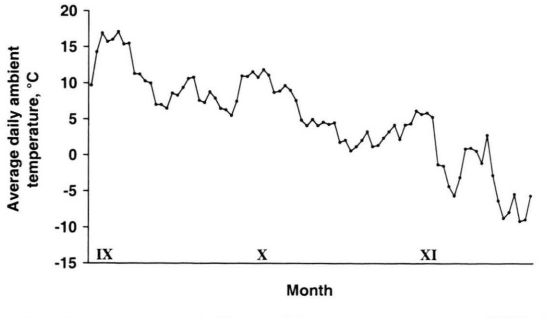

Fig. 1. The average daily ambient temperature (°C) in the study area from Sep 1st to Nov 21st.

For this study we used the PRIME-X® implants manufactured by Wildlife Pharmaceuticals, Fort Collins, Colorado, USA. They are used in the fur industry to accelerate the growth and priming of the winter fur by 4–6 weeks (Valtonen, 1997). Melatonin is one of the least toxic substances known and no harmful effects on the health of the animals have been demonstrated. The implant contains 2.7 mg of melatonin in a silastic matrix. The capsules were administered with a sterile syringe into the interscapular subcutaneous tissue. The amount of melatonin liberated from the 2.7 mg capsules used on the mink is about 10 μg/day during a 90-day experiment (Terry Cairns, Wildlife Pharmaceuticals, Fort Collins, Colorado, USA, personal communication 2000).

All the animals were fed *ad libitum* with fresh mink feed, consisting mainly of slaughter offal, fish and cereals slightly less than the standards of the Finnish Fur Breeders' Association. The feed was manufactured by the local feed kitchen containing about 16 000 kJ/kg dry matter metabolizable energy, of which 46% was from protein, 33% from fat and 21% from carbohydrates. The feed was supplemented with vitamins and minerals. Water or snow was available *ad libitum*. During the follow-up period the amount of feed eaten was measured for a period of three days in a ten-day interval and the energy consumption kJ/animal/day was calculated. The animals were weighed once a week and the length of the animals was measured at the end of the follow-up period.

All the animals used in this experiment were normal fur producing animals, and as melatonin implants are used to prime the winter fur the melatonin-treated animals were sacrificed five weeks earlier than the controls because their winter fur was already adequate to be used. The blood samples were taken three times from the control groups at three- and five-week-intervals and twice from the melatonin groups. The sampling period began in September as this study concentrated on

the physiological changes associated with the onset of wintering. The samples were taken in the morning hours before the animals were fed. The first two samples of the control groups and the first samples of the melatonin group were drawn from the nail of the hind leg cut by aseptic scissors. For the last samples the animals were killed by electric shock and the blood samples were taken by a cardiac puncture with aseptic needles. The blood samples were centrifuged immediately at 1000 g to obtain plasma, which was frozen in liquid nitrogen and subsequently stored at −20 °C.

The plasma leptin concentrations were measured using the radioimmunoassay (RIA) method (Ma *et al.*, 1996). We used the *Multi-species leptin radioimmunoassay (RIA) kit*® developed by Linco Research, St. Charles, Missouri, USA (Anon, 1999a). An anti-human-leptin antibody was used, which has cross-reactivity to the leptin of many mammals. The leptin had been iodinated with ^{125}I. The plasma thyroxine concentrations were also measured using the RIA method. We used the *Spectria* T4 (^{125}I) coated tube radioimmunoassay kit* of Orion Diagnostica, Espoo, Finland (Anon, 1999b).

After sacrification all the animals were weighed and their length was measured. The BMIs reflecting the amount of WAT in the body were calculated from these data by the formula: weight (kg)/ length (m^3). The same formula is used for human infants and toddlers as their body surface in relation to body weight is greater than in adult humans (weight (kg)/ length (m^2)). For the statistical analysis a one-way analysis of variance (ANOVA) was performed followed by a *post hoc* Duncan's test ($p<0.05$). The ANOVA was carried out using the SPSS-program. The paired comparisons were done using a Student's *t*-test for unpaired data ($p<0.05$). The results are expressed as the mean ± SE.

Results

The weight gain and energy consumption of the animals

There were significant differences in the weight gain between the study groups. At the beginning of the experiment the weights of the female minks treated with melatonin were significantly higher than the weights of the controls, but the controls soon reached the same mean weight. No significant differences were found in the lengths or BMIs of the female minks at the time when the last samples were taken (Table I). The melatonin and control groups of the male minks showed significant differences in their body weights for the whole of the follow-up period, the melatonin-treated males being significantly heavier. Also the mean BMI of the males treated with melatonin was significantly higher than the mean BMI of the male controls, but this may be due to the fact that the control males were sacrificed 5 weeks later than the males treated with melatonin (Table I). The melatonin-treated males were also significantly shorter that the controls. This difference was evidently caused by the later sacrification time of the controls. As

Table I. Mean plasma leptin (ng/ml) and plasma thyroxine concentration (nmol/ml), body mass index (BMI, kg/m^3) and body length (cm) of minks (mean ± SE).

		Control females	Melatonin females	Control males	Melatonin males
Leptin, ng/ml	**Sep 22nd**	0.00±0.00	0.95±0.22 †	6.79±3.81	0.47±0.23
	Oct 12th	1.46±0.37 **a** *	7.82±0.77 **b** *	2.50±1.23 **a**	7.80±1.10 **b** *
	Nov 16th	2.82±0.74		2.39±0.56	
Thyroxine, nmol/ml	**Sep 22nd**	23.60±1.53 **b**	26.86±0.95 **b**	16.72±2.12 **a**	22.58±1.49 **b**
	Oct 12th	28.88±2.02 **a, b**	36.65±1.99 **c** *	25.52±1.15 **a** *	34.91±3.11 **b, c** *
	Nov 16th	27.77±3.15		25.87±2.67	
BMI, kg/m^3		21.72±0.43 **a**	22.08±0.53 **a**	21.76±0.52 **a**	27.54±0.85 **b**
		Nov 16th	**Oct 12th**	**Nov 16th**	**Oct 12th**
Body length, cm		42.13±0.30 **a**	41.88±0.67 **a**	50.38±0.38 **c**	47.50±0.50 **b**
		Nov 16th	**Oct 12th**	**Nov 16th**	**Oct 12th**

Values with dissimilar superscripts are significantly different from each other (one-way ANOVA, $p < 0.05$).
* values significantly different from the previous values of the same group (unpaired Student's *t*-test, $p < 0.05$)
† values significantly different from the controls (unpaired Student's *t*-test, $p < 0.003$)

the mink is a dimorphic species, the differences between the male and female groups were quite large.

All the experimental groups showed a decrease in energy consumption as the autumn progressed (Fig. 2). The female groups showed significant differences at all the three periods of measurement ($p<0.05$). The control females consumed feed and energy at a higher rate than the melatonin-treated females. The control males, however, consumed feed and energy at a lower rate than the males treated with melatonin. The difference was statistically significant at the first period of measurement ($p<0.05$). The energy consumption of the control males was fairly stable until the beginning of October, after which their energy consumption reduced steeply.

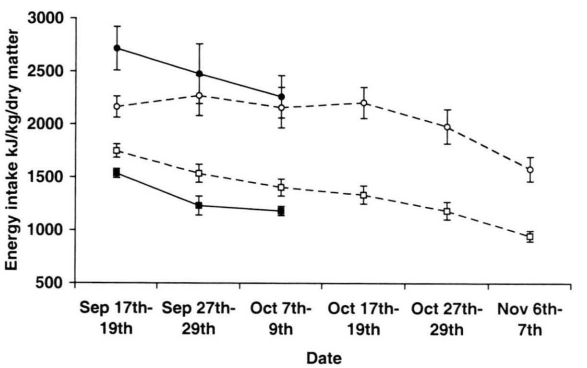

Fig. 2. Variations in energy intake (kJ/animal/day) of minks (mean ± SE) ○ = control males, n=8, ● = melatonin males, n = 8, □ = control females, n = 8, ■ = melatonin females, n = 8.

The plasma leptin concentrations

There were clear seasonal fluctuations in the leptin concentrations of the minks. At the time of the first measurement, in the middle of September, most of the values were below detection level with great intergroup variance. The results did not follow the normal distribution, and the variances between the experimental groups were uneven. Consequently, the medians instead of averages were used for the interpretation of the results in some cases. All the control females had undetectable values of plasma leptin (median 0.00 ng/ml) (Table I). Most of the melatonin-treated females, however, had detectable values of plasma leptin, the median being about 1.12 ng/ml. The difference between the study groups was statistically significant ($p<0.003$). Most of the control males had undetectable levels of plasma leptin (median 0.00 ng/ml), but there were three individuals with exceptionally high values. Also the values of the melatonin-treated males were mostly undetectable. The difference between the male groups was not statistically significant.

At the second time of leptin measurements (October 12th) the mean plasma leptin levels of the animals had increased significantly. The control females now had leptin concentrations significantly higher than at the previous measurement ($p<0.005$). The melatonin-treated females also had significantly higher leptin levels than at the previous measurement ($p<0.0004$), and the leptin levels were significantly higher than the levels of the control females, too ($p<0.05$ Table I). The control males had higher leptin concentrations than at the time of the previous measurement, but the difference was not statistically significant. The males treated with melatonin showed a 17-fold rise in their leptin levels, and the difference between the control and the melatonin groups was statistically significant ($p<0.05$) as was the difference between the consecutive measurements of the melatonin group ($p<0.0004$). The melatonin groups were sacrificed in October, and only the control groups were sampled in November. The leptin concentrations of the control groups did not change significantly from October to November.

The differences in the leptin concentrations between the males and the females were not significant at any of the measurements. The correlation between the plasma leptin levels and the BMIs of the control animals was significant ($r_S=0.572$, $p<0.05$) in November but not at the other measurements (Fig. 3). The correlation between the plasma leptin and thyroxine levels was significant in the melatonin-treated animals in October ($r_S=0.650$, $p<0.01$) but not at other times (Fig. 4).

The plasma thyroxine concentrations

In September the thyroxine levels of the melatonin-treated females were higher than those of the control females, but the difference was not statistically significant (Fig. 5, Table I). The thyroxine levels of the melatonin-treated males, however, were significantly higher than the thyroxine con-

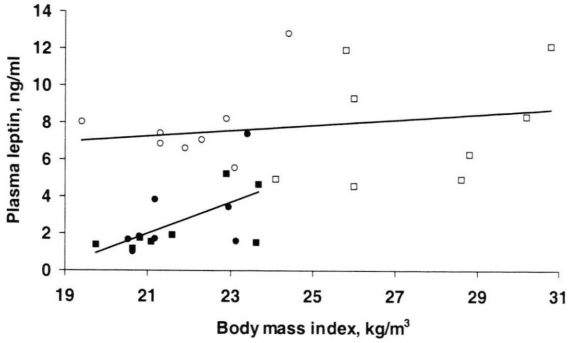

Fig. 3. The relation between plasma leptin concentration (ng/ml) and body mass index (kg/m^3), ○ = melatonin females, n = 8, □ = melatonin males, n = 8, Oct 12th, r_s = 0.109, ● = control females, n = 8, ■ = control males, n = 8, Nov 16th, r_s = 0.572, statistically significant correlation $p < 0.05$.

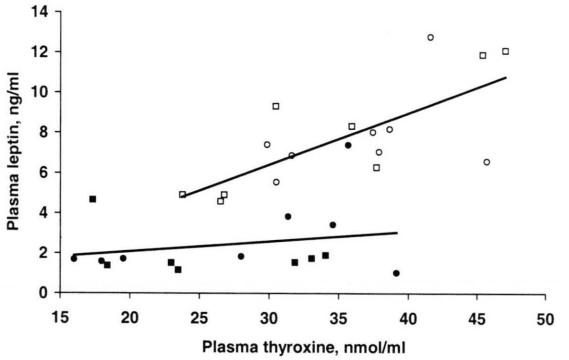

Fig. 4. The relation between leptin (ng/ml) and thyroxine (nmol/ml) concentrations Oct 12th ● = control females, n = 8, ■ = control males, n=7, r_s = 0.150, ○ = melatonin females, n = 8, □ = melatonin males, n = 8, r_s = 0.650, statistically significant correlation $p < 0.01$.

centrations of the control males ($p<0.05$). The mean thyroxine concentrations of all the experimental groups rose in October and the rise was statistically significant ($p<0.05$) except in the control females. In both the females and the males the melatonin-treated groups had thyroxine levels that were significantly higher than those of the control groups ($p<0.05$). The thyroxine levels of the controls did not change significantly from October to November.

There was a significant difference between the thyroxine concentrations of the females and the males at the time of the first measurement ($p<0.004$). As the thyroxine levels of the male groups raised more sharply than the levels of the

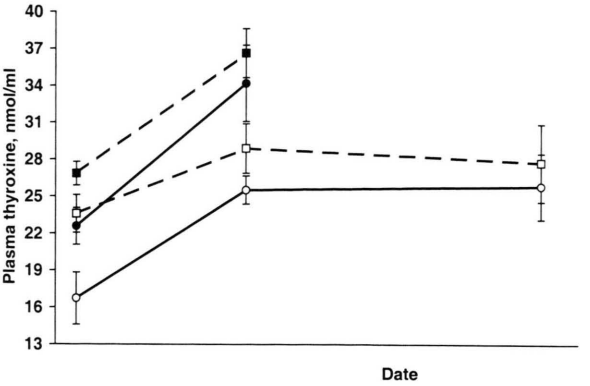

Fig. 5. Plasma thyroxine concentration of minks (mean ± SE), □ = control females, ■ = melatonin females, ○ = control males, ● = melatonin males.

females this difference levelled off and there were no significant differences between the sexes at later measurements.

Discussion

Exogenous melatonin has previously been shown to elevate the weight of minks in the autumn (DiGregorio and Murphy, 1987; Valtonen *et al.*, 1995). In the females the differences in the weight between the study groups were significant only at the time of the first measurement. This probably represents the remains of a difference that could have been detected at an earlier point of time as the minks generally gain weight in the late summer and the early autumn. The mean weight of the melatonin-treated males was, however, significantly higher than the weight of the control males at all the three measurements. The control males continued to gain weight longer than the females until they entered the phase of weight loss at the same time as the control females. Thus the effects of exogenous melatonin on the body weight are very pronounced in both the male and the female minks but the sexes react differently. It has been reported also in the Syrian hamster (*Mesocricetus auratus*) that the effects of exogenous melatonin on body weight and energy metabolism are even more pronounced in females than in males (Bartness and Wade, 1984). Our results may also be associated with higher dose of melatonin administration to females due to their lower body weights.

In female minks the uptake of energy is the lowest in December (Korhonen, 1990) and in males in February (Korhonen *et al.,* 1989). The melatonin-treated females may have consumed more energy than the controls just after the implantation of the melatonin capsules, because animals treated with melatonin were heavier at the beginning of the follow-up period. The implantation of melatonin capsules leads to an increased appetite during the following eight weeks (Valtonen, 1997). As early as in the middle of September, however, the melatonin females consumed significantly less energy than the control females, which reached the lower level of energy intake a few weeks later. This suggests that melatonin indeed accelerated the seasonal fall in energy consumption. The melatonin-treated males, however, used food and consumed energy at a higher level than the controls. The energy consumption of the melatonin-treated males was very high in September, about 8 weeks after the implantation of the melatonin capsules. Generally, our findings confirm the previous results of the seasonal changes in the weight of minks as well as the general effects of exogenous melatonin treatment.

The most important determinant of the plasma leptin concentrations of humans and rodents is considered to be the amount of fat present in the body and there is a clear positive correlation between the plasma leptin level and the BMI (Maffei *et al.,* 1995). The average plasma leptin concentrations are about 7.5 ng/ml in lean humans, 31.3 ng/ml in obese individuals and 12.5 ng/ml in mice. The leptin levels of the control minks were very low compared to these values. The multi-species leptin radioimmunoassay kit that we used was not, however, specific to the leptin of the minks, and our values might in fact be lower than the absolute values.

The correlation between the BMIs and the leptin levels of the control groups was significant in November, but not at the other sampling times (Fig. 3). The leptin levels of the controls were also much lower than the leptin levels of the melatonin-treated females with the same BMI values. Recent studies on the Syrian hamster also reveal that the leptin levels of female hamsters are not simply determined by the amount of WAT (Schneider *et al.,* 2000). The same is true in the common shrew (*Sorex araneus*), which has its highest leptin levels in the mid-winter, when its body weight is the lowest (Nieminen and Hyvärinen, 2000). The metabolism of the mink is strongly affected by endogenous circannual cycles and by changes in the photoperiod. These effects seem to overshadow the function of the WAT mass as the regulator of leptin secretion at some points of the minks' seasonal physiological changes.

There was a significant rise in the plasma leptin levels of the minks between the first two times the blood samples were taken. The rapid rise of plasma leptin levels in early October could be seen in all the experimental groups, but the rise was much steeper in the melatonin-treated animals than in the controls indicating that exogenous melatonin had elevated the leptin levels.

The thyroxine levels of minks rise during the autumn and spring moults and fall during the late autumn after the moult (Boissin-Agasse *et al.,* 1981). We observed these changes, too. In the females the thyroxine levels rose earlier than in the males, but the differences were levelled out by the time of the second measurement. In rodents the effects of melatonin or of a short photoperiod on the thyroid function are mostly inhibitory (Vriend, 1983) but some data exist suggesting that the thyroid activity of rats is reduced during a long photoperiod (Dempsey and Searles, 1943). In the silver fox (*Vulpes vulpes*) exogenous melatonin loweres the plasma thyroxine concentrations (Forsberg and Madej, 1990). In the minks, however, we observed a significant rise of the plasma thyroxine concentrations in the autumn due to exogenous melatonin treatment (Fig. 5, Table I).

There was a significant correlation between the plasma leptin and thyroxine levels of the melatonin-treated animals in October. This may be due to the fact that exogenous melatonin elevated both the leptin and the thyroxine concentrations. Yet there might be some interplay of these two hormones, as it has been demonstrated that the leptin secretion of murine adipocytes is also elevated *in vitro* by triiodothyronine (T_3) (Yoshida *et al.,* 1997). It has also been reported *in vivo* that leptin raises the levels of serum thyroxine (T_4) and prothyrotropine releasing hormone mRNA in the paraventricular nucleus lowered by fast (Ahima *et al.,* 1996, Légrádi *et al.,* 1997). The interactions between melatonin and the thyroid axis are probably species-specific and closely associated with the seasonal cycles of body weight, moult and reproduction.

The mechanism of the rise of plasma leptin concentrations by exogenous melatonin remains unclear. There might be a direct effect of melatonin on the expression of the *ob* mRNA supported by the finding that the leptin levels but not the BMIs of the female groups differed from each other. The mechanism could also partly be the indirect stimulation of feeding efficiency and the weight gain due to the shortened photoperiod experienced by the minks. This leads to an increase in the amount of fat present in the body to secrete more leptin and thus could elevate the plasma leptin levels, too. The effects on the plasma thyroxine levels could in the same way be both direct and indirect, mediated by the acceleration of the autumn moult-associated rise in the thyroxine levels.

Our results show marked seasonal changes in the plasma leptin and thyroxine levels of the minks as well as a stimulatory effect of melatonin on the plasma leptin and thyroxine concentrations. The mink is an animal with strong seasonality and photoperiodism unlike laboratory rodents or humans. The induction of leptin and thyroxine production by melatonin should be regarded in this context. The neuroendocrine response to fasting triggered by the fall of leptin levels in the winter is a part of this web of survival mechanisms (see Ahima *et al.*, 1996). The shortening photoperiod increases the endogenous melatonin secretion, which is a signal to the autumnal weight gain and the storage of fat. The stored fat in the white adipose tissue informs the central nervous system via leptin about the amount of energy available for different purposes, such as wintering or reproduction. After the onset of wintering the food is scarce and the animals are forced to enter a phase of energy preservation. Then it is reasonable to allow the neuroendocrine response to fasting to take place triggered by a fall of the plasma leptin levels. Instead of WAT mass the circannual endogenous rhythms of the mink may be the main regulators of leptin secretion in the autumn.

Acknowledgements

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