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Continuous Melatonin Treatment and Fasting in the Raccoon Dog (*Nyctereutes procyonoides*) – Vernal Body Weight Regulation and Reproduction

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ABSTRACT—The raccoon dog (*Nyctereutes procyonoides*) is a canid omnivore with marked seasonal changes in its body adiposity. The aim of this study was to investigate the roles of melatonin, leptin, ghrelin and growth hormone (GH) in weight regulation and reproduction of the species. Sixteen raccoon dogs were treated with continuous-release melatonin implants in Aug 2000 and in Feb 2001 (the MEL group) and 16 animals were sham-operated (the SHAM group). Half of the raccoon dogs were fasted between Nov 27th 2000 and Jan 25th 2001. The autumnal results have been previously published and this paper reports the vernal data. The leptin concentrations of the SHAM females were high before the mating season, decreased before estrus, increased during gestation and reduced after parturition. The MEL females had higher leptin concentrations than the SHAM females in early March, whereas the MEL males had lower leptin concentrations than the SHAM males in late March. Also the ghrelin and GH concentrations of the SHAM females decreased before estrus. Continuous melatonin treatment advanced the vernal rise in the ghrelin concentrations and the vernal drop and the subsequent rise in the GH concentrations of the females. Melatonin also increased their body mass indices from July to Aug 2001, indicating that it triggers the autumnal accumulation of fat in the species.

Key words: ghrelin, growth hormone, leptin, melatonin, *Nyctereutes procyonoides,* raccoon dog

INTRODUCTION

Leptin is a peptide hormone secreted mainly by the white adipose tissue (Zhang *et al*., 1994). Leptin concentrations of humans and laboratory rodents correlate positively with body adiposity (Maffei *et al*., 1995) being rapidly suppressed by fasting and increased by re-feeding (Hardie *et al*., 1996; Kolaczynski *et al*., 1996). Exogenous leptin reduces food intake, body mass (BM) and adiposity in genetically obese *ob/ob* mice (Pelleymounter *et al*., 1995). In starvation leptin concentrations fall disinhibiting the production of hypothalamic neuropeptide Y (NPY) resulting in energy preservation. This neuroendocrine response to fasting can be blunted by exogenous leptin (Ahima *et al*., 1996). Leptin may play roles in the regulation of puberty, menstruation, pregnancy and lactation of mammals (for review see Moschos *et al*., 2002).

Ghrelin is a newly discovered signal peptide secreted in the gastrointestinal tract and hypothalamus (Kojima *et al*., 1999; Date *et al*., 2000a; Lu *et al*., 2002). Its secretion is regulated by cholinergic neurons (Sugino *et al*., 2003). Ghrelin stimulates growth hormone (GH) secretion (Kojima *et al*., 1999; Date *et al.,* 2000b), reduces fat utilization and increases food intake and BM gain (Tschöp *et al*., 2000). Its concentrations are increased by fasting and reduced by re-feeding and obesity (Tschöp *et al*., 2000, 2001). An abrupt ghrelin secretion peak can be observed shortly before feeding (Sugino *et al*., 2002a, b). Ghrelin antagonizes leptin action in the hypothalamus by activating the NPY pathway (Nakazato *et al*., 2001; Shintani *et al*., 2001). GH inhibits adipocyte differentiation, reduces triacylglycerol accumulation and increases lipolysis (Richelsen, 1997). Leptin administration can reverse the fasting-induced suppression in GH release by preventing the inhibitory action of NPY on GH secretion (Vuagnat *et al*., 1998).

The raccoon dog or *tanuki* (*Nyctereutes procyonoides*, Gray 1834) is a common omnivore in southern and central Finland originating from eastern Asia (Siivonen, 1972). The species gains BM as subcutaneous fat in the autumn (Kor-

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honen, 1987). Its BM is highest in Nov–Dec and decreases thereafter due to the utilization of fat stores during the winter. In the northernmost areas of their geographical distribution, wild raccoon dogs spend the coldest part of the winter in shallow winter sleep with occasional arousal and food intake during the warmer periods (Siivonen, 1972). In the early autumn, plasma leptin and GH concentrations of the raccoon dog are low but ghrelin concentrations relatively high (Nieminen *et al.*, 2002). Leptin and GH concentrations peak simultaneously in late Oct and decline rapidly thereafter. In the winter, leptin and GH concentrations are high but the ghrelin concentrations low. Leptin, ghrelin and GH may work in synergy to increase lipolysis during the coldest part of the winter. Fasting for two months in mid-winter does not affect the concentrations of these hormones.

In captivity, the raccoon dog mates in Feb–March (Valtonen *et al.*, 1977). Testicular recrudescence begins in early Nov and mature spermatozoa are produced from Dec to April (Xiao, 1996). Blood testosterone concentrations are highest in the early breeding season, and the testes remain quiescent between May and Aug. 17β-Estradiol concentrations are highest during proestrus and early estrus, decrease postcoitally and remain low during the early pregnancy (Valtonen *et al.*, 1978). Thereafter the concentrations rise slightly between days 13–26 of gestation and reduce towards term. Blood progesterone concentrations are low during proestrus and increase during estrus. Progesterone reaches its maximum values during the first half of pregnancy and decreases thereafter towards parturition. Gestation lasts 59–64 d and the average litter size is 5 cubs (Valtonen *et al.*, 1977).

Melatonin is an important factor controlling the seasonal rhythms of the raccoon dog (Xiao, 1996; Nieminen *et al.*, 2002). Subcutaneous melatonin implants raise their circulating melatonin concentrations and induce physiological changes like those that occur under short photoperiod. Autumnal melatonin treatment advances the autumn moult and testicular recrudescence (Xiao, 1996) as well as seasonal changes in appetite and in leptin and GH concentrations of the raccoon dogs (Nieminen *et al.,* 2002). Similar treatment in spring slows testicular regression, stimulates the growth of underfur and inhibits initiation of guard hairs (Xiao, 1996).

The aim of this study was to investigate the roles of leptin, ghrelin and GH in vernal weight regulation and reproduction of the raccoon dog. We also monitored the effects of exogenous melatonin and prolonged wintertime fasting on these hormonal parameters.

MATERIALS AND METHODS

Thirty-two farm-bred raccoon dogs (16 males, 16 females) born between May 23rd and 27th 2000 were randomly divided into two groups. On Feb 8th 2001 half of the animals received a continuous-release melatonin implant (12.0 mg PRIME-X® melatonin implant, Wildlife Pharmaceuticals, Fort Collins, CO, USA) inserted into the interscapular subcutaneous tissue under sterile conditions (the MEL group). The control group was sham-operated (the SHAM group). The MEL group had been treated with similar melatonin implants also on Aug 16th 2000 as a part of a previous experiment (Nieminen *et al.*, 2002). The difference between the plasma melatonin concentrations of the SHAM and the MEL groups was still significant on Oct 25th 2000 (25±4.3 vs. 73±8.6 pg ml^{-1}, t-test, $p<0.01$) but not on Jan 25th 2001 (59±34.4 vs. 70±10.5 pg ml^{-1}, t-test, $p>0.05$). In addition, half of the SHAM and half of the MEL animals had been fasted for two months from Nov 27th 2000 to Jan 25th 2001 (the fed and the fasted animals).

The animals were housed in male-female pairs (both sexes of the same treatment) in an enclosure under roof in cages (150×107×70 cm) with wooden nestboxes (70×45×40 cm with straw) in natural temperature and photoperiod. They were fed with commercial fur animal diets. The amount of feed offered varied seasonally, as the appetite and energy expenditure of raccoon dogs are age- and season-dependent (Feb 2nd–Feb 18th; 410 kcal $animal^{-1}$ d^{-1}, Feb 19th–April 1st; 320 kcal $animal^{-1}$ d^{-1}, April 2nd–April 30th; 350 kcal $animal^{-1}$ d^{-1} and May 1st–Aug 6th; individual feeding for the lactating females according to the number of cubs). Water or ice was available *ad lib.* All the procedures conformed to the Helsinki Convention.

Blood samples were drawn from a superficial vein of the left hind leg under sterile conditions every 2–3 weeks during the morning hours before the animals were fed. Females that gave birth were not sampled for three weeks after parturition to avoid disturbance. BMs were measured at each blood sampling. Body mass indices (BMIs) reflecting body adiposity were calculated by the formula: BM (kg)/[Body length (m)]^3 (Nieminen *et al.,* 2002). This formula strongly correlates ($r_s=1.000$, $p<0.01$) with the obesity index (BM (g)×100/[0.026×Body length (cm)^3]) empirically derived for the species by Korhonen *et al.* (1982). Body lengths from the tip of the nose to the anus were measured on Jan 10th 2001, which was after the animals had obtained their adult body lengths. Voluntary energy intake of the pairs was measured approximately every third week by providing them with *ad lib.* feeding followed by the weighing of the uneaten food 24 hr later. On March 27th 2001, the males were sacrificed with an electric shock after the cessation of their mating season, and their final 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). The females were sacrificed on Aug 7th 2001 with the same procedure. If not stated otherwise, the results discussed concern both sexes.

Leptin concentrations were measured with the Multi-Species Leptin RIA kit (Linco Research, St. Charles, MO, USA; intra- and interassay variations 2.8–3.6 and 6.5–8.7% CV) and plasma ghrelin concentrations with the Ghrelin (Human) RIA kit (Phoenix Pharmaceuticals, Belmont, CA, USA; <5 and <14% CV). GH concentrations were determined with the hGH Human Growth Hormone Double Antibody kit (DPC, Los Angeles, CA, USA; 1.5–5.9 and 1.8–8.3% CV). Diurnal melatonin concentrations were measured with the Melatonin-RIA kit (DLD Diagnostika GmbH, Hamburg, Germany; 4.3–7.4 and 11.7–12.1% CV) to verify the release of melatonin from the implants. Progesterone concentrations of the females were determined during their reproductive season between early March and late May with the Spectria Progesterone [^{125}I] Coated Tube Radioimmunoassay kit of Orion Diagnostica (Espoo, Finland; 2.9–5.8 and 4.7–5.1% CV). All the peptide hormone assays were validated such that serial dilutions of the raccoon plasma showed linear changes in BB_0^{-1} values that were parallel with the standard curves produced with human peptides (Fig. 1a–c).

Multiple comparisons were performed with the one-way analysis of variance (ANOVA) followed by the *post hoc* Duncan's test using the SPSS-program. Also the two-way ANOVA using melatonin treatment as one factor and fasting as another was performed.

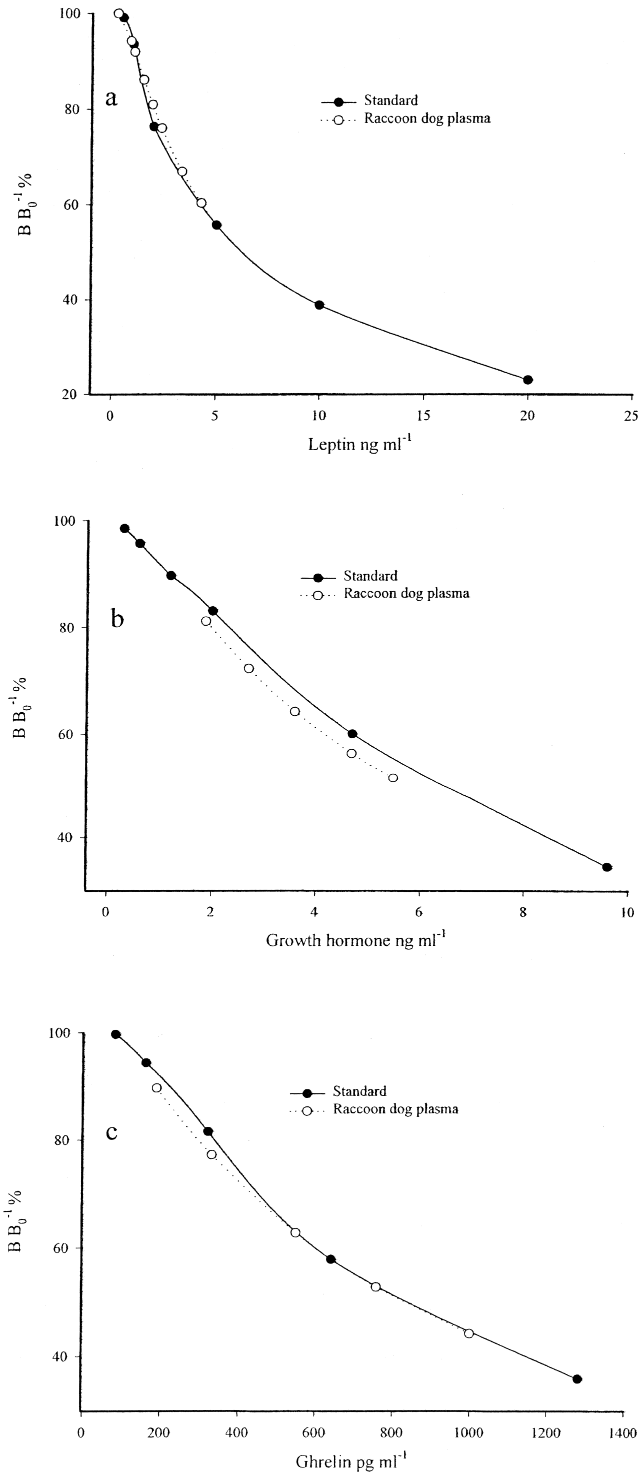

Fig. 1. Standard curves for leptin (a), growth hormone (b) and ghrelin (c) and the corresponding dose-response curves for serial dilutions of raccoon dog plasma (leptin: 25, 50, 75, 100, 150, 200, 250 and 300 μl; growth hormone: 200, 300, 400, 500 and 600 μl; ghrelin: 5, 10, 25, 50 and 75 μl), B=sample or standard binding, B_0=maximum binding.

Comparisons between two study groups were performed with the Student's t-test and the Mann-Whitney U test for parametric and nonparametric data. The normality of distribution and the homogeneity of variances were tested with the Kolmogorov-Smirnov test and the Levene test. Correlations were calculated with the Spearman Correlation Coefficient (r_S). P value less than 0.05 was considered to be statistically significant. The results are presented as the mean±SE. As the two-way ANOVA revealed no statistically significant interactions between the melatonin treatment and fasting, the data for the SHAM and the MEL groups have been pooled across the feeding regimes (the fed and the fasted animals), when reporting the melatonin-induced effects on the variables. In a similar way, when the fasting-induced changes are reported, the data for the fed and the fasted groups are composed of both the MEL and the SHAM animals.

RESULTS

The plasma melatonin concentrations

The diurnal plasma melatonin concentrations of the SHAM group were about 10 pg ml^{-1} throughout the study and there were no seasonal differences in the concentrations. The melatonin concentrations of the MEL group were higher than those of the SHAM group from Feb 20th (244±43.4 vs. 9±1.8 pg ml^{-1}, t-test, $p<0.01$) to the end of the experiment Aug 7th (86±19.3 vs. 10±1.7 pg ml^{-1}, t-test, $p<0.01$, the females only).

The changes in BMs, adiposity and food intake

Melatonin treatment did not affect the BMs of the raccoon dogs (see Fig. 2a for the females). The BMs of the fed raccoon dogs decreased gradually from Feb 8th to March 27th (t-test, $p<0.05$, Fig. 2b for the females). The BMs of the fasted raccoon dogs were stable and no vernal weight loss was observed. The fasted animals had lower BMs than the fed raccoon dogs from Feb 8th to March 7th (t-test, $p<0.01$). Sex had no influence on the BMs.

The BMIs of the female raccoon dogs reached their lowest mean value on April 11th, after which they gradually increased to the highest mean value on Aug 7th (Fig. 2c–d). The MEL females had higher BMIs than the SHAM females from July 24th (t-test, $p<0.05$) to Aug 7th (t-test, $p<0.05$, Fig. 2c). The fasted raccoon dogs had lower BMIs than the fed animals on Feb 8th (t-test, $p<0.01$, Fig. 2d for the females). The female raccoon dogs had higher BMIs than the males on March 27th (33.3±0.58 vs. 31.2±0.78, t-test, $p<0.05$).

The voluntary energy intake of the raccoon dogs increased from Feb 2nd (447±21 kcal d^{-1}) until the end of the experiment (1506±77 kcal d^{-1}, the females only, t-test, $p<0.01$). Melatonin had no effects on the vernal energy intake of the animals, but the fasted raccoon dogs consumed more energy than the fed animals from Feb 2nd (517±19 vs. 377±27 kcal d^{-1}) to March 15th (992±38 vs. 655±29 kcal d^{-1}, t-test, $p<0.01$), on April 25th (1060±31 vs. 770±64 kcal d^{-1}, t-test, $p<0.01$, the females only) and on June 26th (1704±53 vs. 1307±85 kcal d^{-1}, t-test, $p<0.01$, the females only).

The plasma leptin concentrations

The plasma leptin levels of the males did not fluctuate during their study period Feb 8th–March 27th 2001 (Table 1).

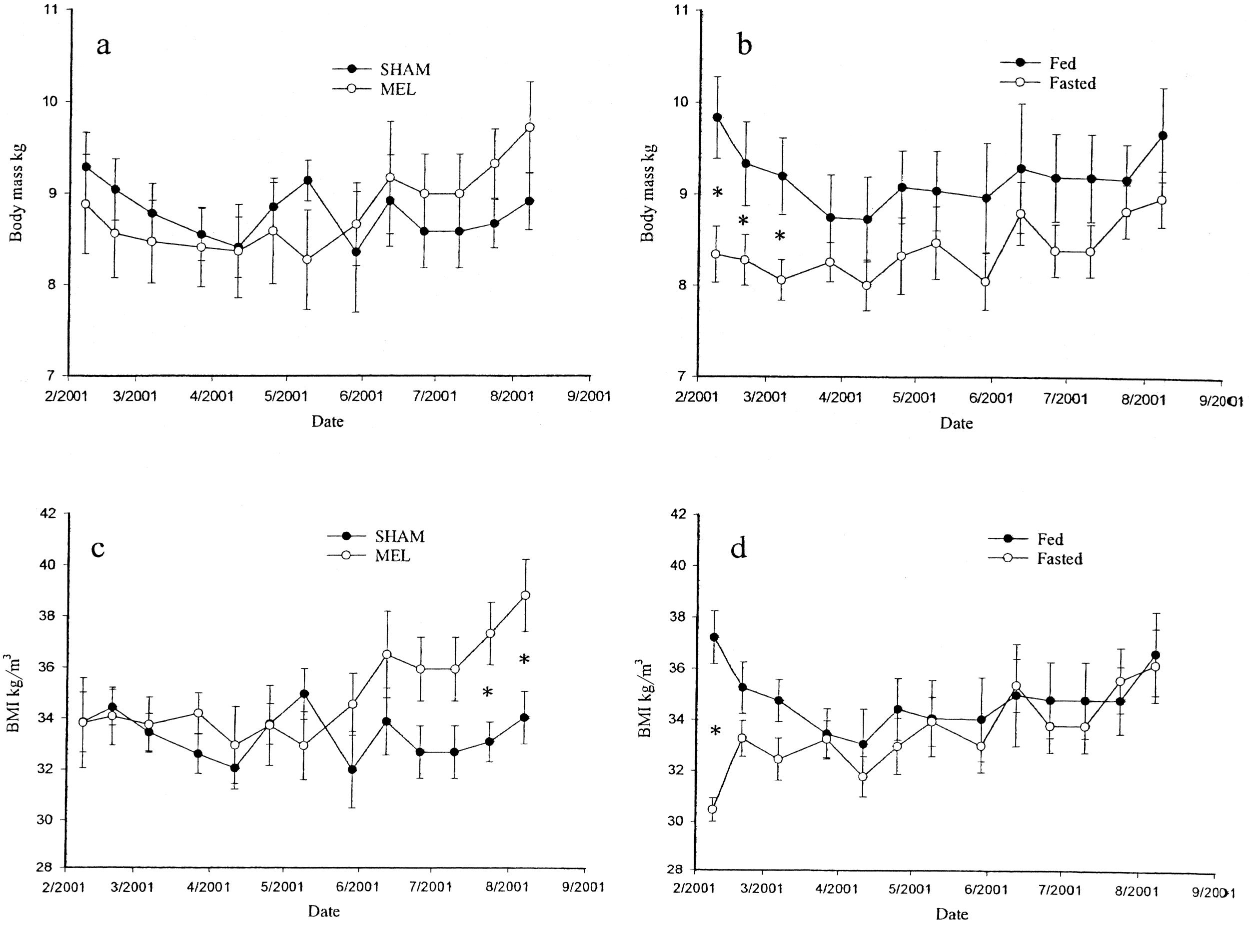

Fig. 2. Effects of melatonin and fasting on the body mass (kg, a–b) and body mass index (BMI; kg/m^3, c–d) of the female raccoon dogs (mean±SE). The MEL group was treated with melatonin implants on Aug 16th 2000 and on Feb 8th 2001. The fasted animals were not fed from Nov 27th 2000 to Jan 25th 2001. * difference between the MEL and the SHAM females (c) or between the fed and the fasted females (b, d).

Table 1. Leptin, growth hormone and ghrelin concentrations (ng ml^{-1}) of the sham-operated (SHAM), melatonin-treated (MEL), fed and fasted male raccoon dogs during their vernal study period Feb 8th–March 27th 2001 (mean±SE).

		SHAM	MEL	Fed	Fasted
Leptin	Feb 8th	2.2±0.13	2.1±0.09	2.3±0.12	2.0±0.07
	Feb 20th	2.1±0.19	2.1±0.19	2.0±0.26	2.2±0.06
	Mar 7th	2.2±0.12	2.0±0.19	2.4±0.17	1.9±0.08†
	Mar 27th	2.3±0.13	1.9±0.09*	2.2±0.15	2.0±0.09
Growth hormone	Feb 8th‡	1.0±0.14	1.2±0.05	1.2±0.07	1.0±0.13
	Feb 20th	1.7±0.21	1.5±0.06	1.9±0.18	1.4±0.07
	Mar 7th	1.6±0.12	1.1±0.26	1.7±0.09	1.0±0.24†
	Mar 27th‡	0.7±0.15	0.9±0.20	0.9±0.16	0.6±0.17
Ghrelin	Feb 8th	1.5±0.31	1.5±0.16	1.7±0.24	1.4±0.21
	Feb 20th	1.4±0.33	2.0±0.10	2.0±0.05	1.6±0.23
	Mar 7th	1.4±0.32	1.6±0.08	1.5±0.07	1.5±0.20
	Mar 27th	1.3±0.28	1.5±0.16	1.7±0.04	1.3±0.18

* differs from the values of the SHAM males
† differs from the values of the fed males
‡ differs from the values of Feb 20th when all the male data are pooled together
(t-test, $p<0.05$)

The leptin concentrations of the MEL males were lower than those of the SHAM males on March 27th (t-test, $p<0.05$). The fasted males had lower leptin concentrations than the fed males on March 7th (t-test, $p<0.05$). The male raccoon dogs had higher leptin concentrations than the females on Feb 20th (2.1 ± 0.13 vs. 1.6 ± 0.10 ng ml^{-1}, t-test, $p<0.01$).

The leptin concentrations of the females decreased from Feb 8th to Feb 20th (t-test, $p<0.01$) and increased thereafter to the peak values on April 11th (the MEL females, t-test, $p<0.05$) or on April 25th (the SHAM females, t-test, $p<0.01$, Fig. 3a). Thereafter the leptin concentrations of the females decreased until May 29th and subsequently

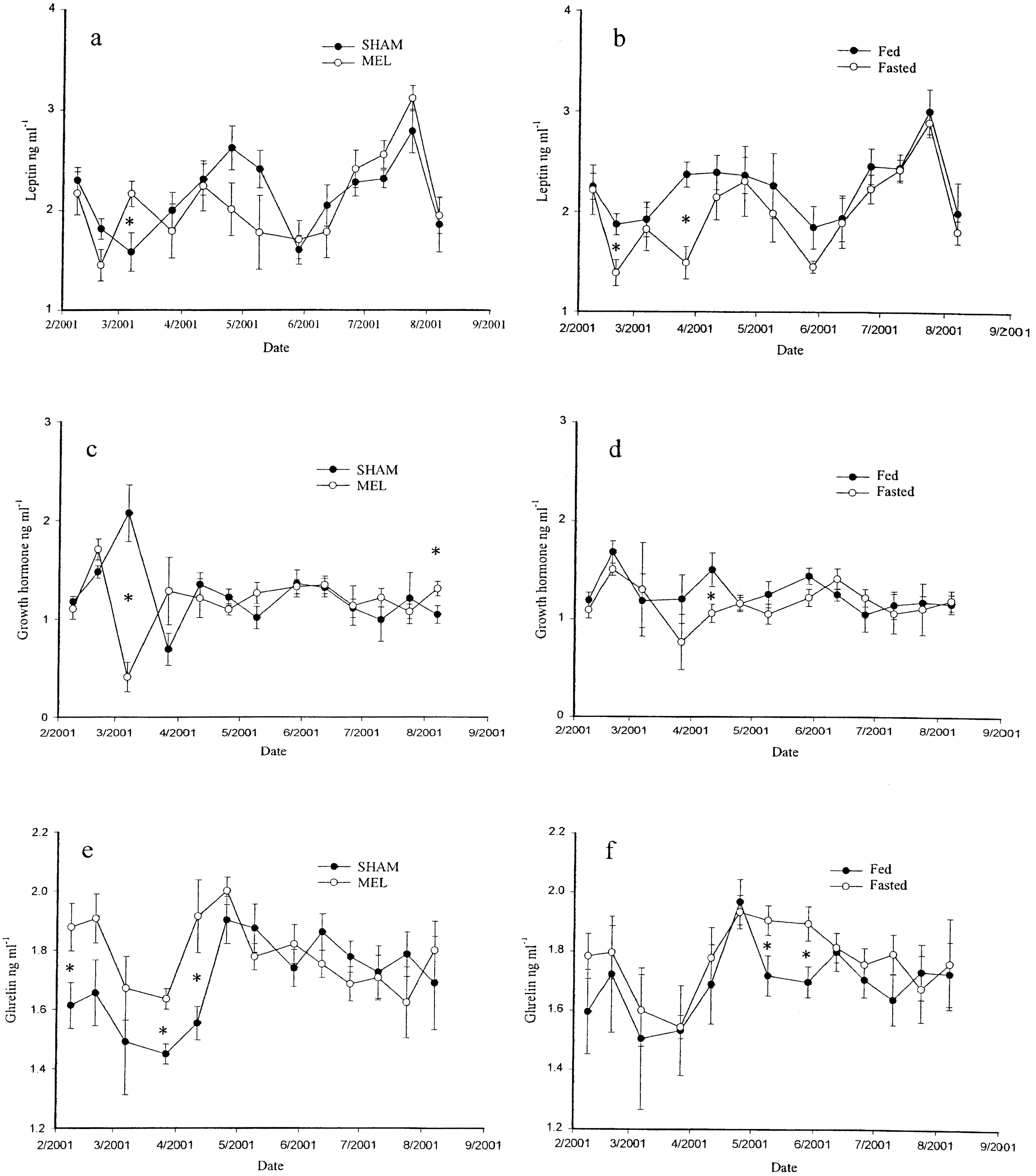

Fig. 3. Effects of melatonin and fasting on the plasma leptin (ng ml^{-1}, a–b), growth hormone (ng ml^{-1}, c–d) and ghrelin concentrations (ng ml^{-1}, e–f) of the female raccoon dogs (mean±SE). The MEL group was treated with melatonin implants on Aug 16th 2000 and on Feb 8th 2001. The fasted animals were not fed from Nov 27th 2000 to Jan 25th 2001. * difference between the MEL and the SHAM females (a, c, e) or between the fed and the fasted females (b, d, f).

increased until July (t-test, $p<0.01$). Another clear decrease in the leptin concentrations of the females was observed from July 24th to Aug 7th (t-test, $p<0.01$). The MEL females had higher leptin concentrations than the SHAM females on March 7th (t-test, $p<0.05$, Fig. 3a), and the fasted females had lower leptin concentrations than the fed females on Feb 20th (t-test, $p<0.05$) and on March 27th (t-test, $p<0.01$, Fig. 3b).

When the data from the specific phases of individual reproductive cycles of the females were pooled together, their leptin concentrations were higher before the mating season than during the proestrus period (t-test, $p<0.05$). The leptin concentrations measured before the mating season (t-test, $p<0.01$) and during gestation (t-test, $p<0.05$) were higher than the leptin concentrations measured from the females that had given birth but lost their litters. The females with lost litters also had lower leptin concentrations than the barren females (t-test, $p<0.05$). The leptin and progesterone concentrations of the SHAM females correlated positively with each other during their reproductive season March 7th–May 29th ($r_S=0.448$, $p<0.05$, Fig. 4), although there was significant variation for this data. When the whole data for the male and the female raccoon dogs throughout the study were analyzed together, their leptin concentrations correlated positively with the BMs ($r_S=0.281$, $p<0.01$) and the BMIs ($r_S=0.163$, $p<0.01$). They did not correlate with the ghrelin ($r_S=-0.063$, $p>0.05$) or GH concentrations ($r_S=-0.061$, $p>0.05$) or with the food intake ($r_S=-0.037$, $p>0.05$).

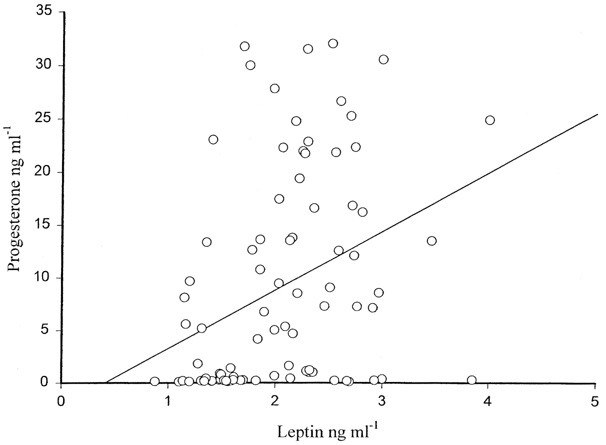

Fig. 4. Relation between the plasma progesterone (ng ml^{-1}) and leptin concentrations (ng ml^{-1}) of the SHAM female raccoon dogs between March 7th and May 29th 2001 ($r_S=0.448$, $p<0.05$).

The plasma GH concentrations

The plasma GH concentrations of the male raccoon dogs increased from Feb 8th to Feb 20th (t-test, $p<0.01$, Table 1). From Feb 20th to March 27th the concentrations decreased (t-test, $p<0.01$). Melatonin did not affect the GH concentrations of the males, but the fasted males had lower GH concentrations than the fed males on March 7th (t-test, $p<0.05$).

The GH levels of the female raccoon dogs increased between Feb 8th and 20th (t-test, $p<0.01$, Fig. 3c–d). The GH concentrations of the MEL females declined on March 7th (t-test, $p<0.01$), whereas a similar decrease was observed in the SHAM females three weeks later on March 27th (t-test, $p<0.01$, Fig. 3c). Thereafter the concentrations increased (t-test, $p<0.01$) and remained relatively stable until the end of the experiment. The rise occurred two weeks earlier in the MEL females, and they had higher GH concentrations than the SHAM females on Aug 7th (t-test, $p<0.05$). The fasted females had lower GH concentrations than the fed females on April 11th (t-test, $p<0.05$, Fig. 3d). When the whole data for the male and the female raccoon dogs throughout the study were analyzed together, their GH concentrations correlated positively with the BMs ($r_S=0.186$, $p<0.01$), but there was no correlation between the GH and ghrelin concentrations ($r_S=0.116$, $p>0.05$), the BMIs ($r_S=0.067$, $p>0.05$) or the food intake ($r_S=-0.134$, $p>0.05$).

The plasma ghrelin concentrations

There were no seasonal changes in the plasma ghrelin concentrations of the male raccoon dogs (Table 1). Melatonin or fasting did not affect their ghrelin concentrations, either. The ghrelin concentrations of the female raccoon dogs decreased from Feb 8th to March 27th (t-test, $p<0.05$, Fig. 3e). From March 27th the ghrelin concentrations of the SHAM females increased to peak values on April 25th (t-test, $p<0.01$). The concentrations remained relatively stable until the end of the experiment. The MEL females had higher ghrelin concentrations than the SHAM females on Feb 8th (t-test, $p<0.05$), on March 27th (t-test, $p<0.01$) and on April 11th (t-test, $p<0.05$). The fasted females had higher ghrelin concentrations from May 9th (t-test, $p<0.05$) to May 29th (t-test, $p<0.05$, Fig. 3f). When the data for the male and the female raccoon dogs throughout the study were analyzed together, their ghrelin concentrations did not correlate with the BMs ($r_S=-0.032$, $p>0.05$), the BMIs ($r_S=0.126$, $p>0.05$) or the food intake ($r_S=0.054$, $p>0.05$).

The ghrelin-leptin ratios of the male and the female raccoon dogs increased from Feb 8th (0.7±0.06) to Feb 20th (1.1±0.10, t-test, $p<0.05$). Thereafter the ratios of the female raccoon dogs decreased until April 11th (0.8±0.08, t-test, $p<0.05$). Another peak was obtained in the females on May 29th (1.1±0.08, t-test, $p<0.01$), and thereafter their ratios reduced on July 24th (0.6±0.03, t-test, $p<0.01$) and increased on Aug 7th (1.0±0.09, t-test, $p<0.01$). Melatonin treatment increased the ghrelin-leptin ratios on Feb 20th (1.3±0.14 vs. 0.9±0.11, Mann-Whitney U test, $p<0.05$) and on May 9th (1.2±0.16 vs. 0.8±0.07, Mann-Whitney U test, $p<0.05$, the females only). Fasting did not affect the ghrelin-leptin ratios of the raccoon dogs. The female raccoon dogs had higher ghrelin-leptin ratios than the males on Feb 20th (1.3±0.14 vs. 0.8±0.10, t-test, $p<0.05$) and on March 27th (1.0±0.11 vs. 0.7±0.08, t-test, $p<0.05$).

DISCUSSION

Effects of exogenous melatonin

Half of the raccoon dogs were treated with continuous-release melatonin implants in Aug 2000 and in Feb 2001 to obtain elevated melatonin concentrations throughout the study. The release of melatonin from the implants did not follow any diurnal rhythm making the interpretation of the data more difficult. Continuous melatonin treatment affected the BMIs and the concentrations of weight regulatory hormones of the raccoon dogs, but had no effects on their BMs or food intake (see also Xiao, 1996; Nieminen *et al*., 2002). The higher BMIs of the MEL females in July–Aug indicate that continuous melatonin treatment advances the accumulation of fat in late summer. Increasing endogenous melatonin secretion after the summer solstice (Xiao, 1996) probably triggers the autumnal fat storage of the species.

Autumnal melatonin treatment advances the testicular recrudescence of male raccoon dogs and estrus of females (Xiao, 1996; Asikainen *et al*., 2003). In the same way, the autumn moult, the autumnal increase in the appetite and the changes in the concentrations of weight regulatory hormones are also advanced due to melatonin (Xiao, 1996; Nieminen *et al*., 2002; Asikainen *et al*., 2003). Vernal melatonin treatment, on the other hand, slows testicular regression, stimulates the growth of underfur and inhibits the growth of guard hairs (Xiao, 1996). Continuous melatonin treatment also induced changes to the vernal concentrations of the weight regulatory hormones. As the responses were sexually dimorphic, they may be associated with reproduction and with the advancement of the mating season induced by the autumnal implantation.

The leptin concentrations of the SHAM female raccoon dogs reflected their reproductive cycles. Their leptin concentrations were high before the mating season and decreased before estrus, which occurred during the later half of March. In female rodents, leptin accelerates the onset of puberty (Ahima *et al*., 1997; Chehab *et al*., 1997) but inhibits early follicular development (Kikuchi *et al*., 2001) and ovulation (Duggal *et al*., 2000). The male and female raccoon dogs experienced a rise in their leptin levels during the winter before the vernal part of the experiment started (Nieminen *et al*., 2002) simultaneously with the increasing plasma testosterone and luteinizing hormone levels connected to the approaching mating season (Asikainen *et al*., 2003). These findings conform to the hypothesis of high leptin concentrations functioning as a permissive metabolic gate for mammalian reproduction.

The leptin concentrations of the SHAM females increased during gestation from the end of March to the end of April. During the later half of pregnancy in May, the concentrations declined simultaneously with the progesterone levels (Asikainen *et al*., 2003). There was a positive correlation between the leptin and progesterone concentrations of the SHAM females during their mating season and pregnancy, as observed previously during the human menstrual cycle (Hardie *et al*., 1997). After giving birth (later half of May), the leptin concentrations of the SHAM females decreased. Also baboon (*Papio* sp.; Henson *et al*., 1999) and human pregnancies (Hardie *et al*., 1997) are associated with increased leptin concentrations, which decline after parturition.

There was no clear seasonality in the vernal leptin concentrations of the MEL females. This may be due to the great interindividual variation in the timing of estrus in this study group, as the MEL females gave birth between March 17th and May 2nd and the SHAM females delivered between May 15th and May 24th. The MEL females had, however, higher leptin concentrations than the SHAM females on March 7th, when the MEL females were pregnant. Thus their higher leptin concentrations may be considered as an indicator of the advancement of their mating season by the autumnal melatonin treatment (Asikainen *et al*., 2003). Exogenous melatonin has previously increased leptin concentrations in plasma of the mink (*Mustela vison*; Mustonen *et al*., 2000) and leptin gene expression in white and brown adipose tissues of the garden dormouse (*Eliomys quercinus*; Ambid *et al*., 1998).

The GH levels of the SHAM females were high before the mating season and declined before estrus. A similar decline was also observed in the MEL females three weeks earlier, but it could not be connected to their reproductive cycles. After the decline in the GH concentrations, the levels rose again, which occurred two weeks earlier in the MEL females. Melatonin has previously decreased GH secretion of the rat *in vitro* (Griffiths *et al*., 1987). It did not affect the GH levels of the male raccoon dogs, but there were seasonal changes in their GH levels, which decreased before the heat as observed also in the females. For this reason, the possible involvement of GH in raccoon dog reproduction cannot be excluded (see also Scanes and Harvey, 1995).

The ghrelin concentrations of the raccoon dogs were low and relatively stable during the whole vernal study period compared to the more variable autumnal values (Nieminen *et al*., 2002). The ghrelin concentrations of the SHAM females decreased before estrus, but no such relationship could be observed in the MEL females. However, the ghrelin concentrations of the MEL females decreased simultaneously with the ghrelin levels of the SHAM females. Thereafter the ghrelin concentrations of the female groups increased in April, but the rise occurred two weeks earlier in the MEL females. Ghrelin may have a possible role in raccoon dog reproduction, as it is known to regulate luteinizing hormone and testosterone secretion of rodents (Furuta *et al*., 2001; Tena-Sempere *et al*., 2002). One of the main functions of ghrelin is considered to be stimulation of GH secretion (Kojima *et al*., 1999; Date *et al*., 2000b), but there was no positive correlation between the circulating GH and ghrelin concentrations of the raccoon dogs confirming our autumnal results (Nieminen *et al*., 2002).

Exogenous ghrelin is able to counteract the leptin-mediated inhibition of NPY production in the hypothalamus

(Nakazato *et al.*, 2001; Shintani *et al.*, 2001). Due to the antagonistic interactions of these peptides, we have previously used the ratio of circulating ghrelin and leptin as an approximation of the satiety state of the raccoon dog (Nieminen *et al.*, 2002). Their ghrelin-leptin ratios rose at the end of Feb as well as from April to June. In the autumnal part of the study, the ghrelin-leptin ratios correlated positively with the energy intake of the animals (Nieminen *et al.*, 2002). The vernal changes in the ratios cannot be explained by variations in the food intake of the animals, as the ratios fluctuated but the food intake increased systematically towards the autumn. The ghrelin-leptin ratios increased again at the end of the study. The high autumnal ghrelin-leptin ratios, possibly inducing seasonal hyperphagia, were also observed in juvenile raccoon dogs in Aug 2000 (Nieminen *et al.*, 2002).

It can be assumed that the autumnal melatonin implantation advanced the seasonal rhythms of reproduction and weight regulatory hormones of the raccoon dogs, but the vernal melatonin treatment advanced the accumulation of fat in the next autumn. Melatonin-induced differences in the vernal concentrations of weight regulatory hormones could be observed only during particular samplings, but in the case of GH and ghrelin melatonin seemingly advanced the vernal changes in their concentrations. This is supported by previous reports (Xiao, 1996; Nieminen *et al.*, 2002; Asikainen *et al.*, 2003) demonstrating clear melatonin-induced advancements in the seasonal rhythms of furring, reproduction and energy metabolism of the species. It is possible that the raccoon dog needs a long day signal before exogenous melatonin can induce physiological changes connected to wintering. Also in sheep (*Ovis aries*) and goats (*Capra hircus*), a period of long days is required before a short day melatonin signal will advance their reproductive cycles (Arendt, 1995).

Effects of wintertime fast

Half of the raccoon dogs of the MEL and the SHAM groups had been fasted for two months in Nov 2000–Jan 2001 to induce a winter sleep-like state to them. The locomotor activity of the fasted animals decreased by 30–50% (Nieminen *et al.*, 2002). It is also known from previous studies that a total fast induces a slight but significant suppression (0.5–1.5°C) to the rectal body temperature of raccoon dogs (Asikainen *et al.*, 2002). The fasted animals lost about 28% (3.1 kg) of their BM (*i.e.* fat) during the fast. After the fasting period, the BMs of the fed animals decreased in Feb-March and thereafter their weight loss leveled off. In contrast, the BMs of the fasted raccoon dogs remained stable during the vernal part of the experiment, and their appetite was higher than in the fed animals during the most food intake measurements.

Two months of fasting during the seasonal rest did not affect the leptin, GH or ghrelin concentrations of the raccoon dogs (Nieminen *et al.*, 2002). However, during the vernal study period, the fasted raccoon dogs had occasionally lower leptin and GH concentrations and higher ghrelin concentrations than the fed animals. As high leptin (Pelleymounter *et al.*, 1995) and GH concentrations (Andres *et al.*, 1991) can reduce food intake of mammals and high ghrelin levels increase the appetite (Tschöp *et al.*, 2000), the lower vernal leptin and GH concentrations and the higher ghrelin levels of the fasted raccoon dogs may be associated with their higher appetite compared to the fed animals. The lower leptin and GH concentrations together with the higher ghrelin levels could also be connected to lower rates of vernal fat mobilization, as leptin (Reidy and Weber, 2000) and GH (Richelsen, 1997) have lipolytic actions, but high ghrelin concentrations suppress the mobilization of fat (Tschöp *et al.*, 2000). It is difficult to determine the significance of ghrelin in the control of appetite of the raccoon dogs, as mammals experience a short and transient increase in plasma ghrelin concentrations before meals (Sugino *et al.*, 2002a, b). However, the blood samples of the raccoon dogs were taken several hours before their feeding and thus the increased basal ghrelin concentrations of the fasted animals could be connected to the replenishment of their fat stores. Also the fed raccoon dogs experienced nearly 20% wintertime and vernal weight losses but during a longer time span (Dec–March). The vernal mobilization of fat stores was presumably higher in the fed animals with higher concentrations of leptin and GH and lower levels of ghrelin in circulation.

In conclusion, continuous melatonin treatment advanced the vernal rise in the leptin and ghrelin concentrations and the vernal drop and the subsequent rise in the GH concentrations of the female raccoon dogs. It also increased their BMIs at the end of summer. As the melatonin-induced changes in the concentrations of weight regulatory hormones were sexually dimorphic, they may be associated with reproduction and with the advancement of the mating season by the autumnal implantation.

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