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Short communication

Body-weight changes are clearly reflected in plasma concentrations of leptin in female mink (*Mustela vison*)

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The mink is a seasonal breeder with a propensity for seasonal fatness, and it is very responsive to changes in energy supply. The objectives of the present study were first, to validate a multi-species leptin assay for mink (*Mustela vison*) plasma, and second, to evaluate how plasma leptin and insulin concentrations responded to energy restriction and body-weight loss and refeeding with restoration of body reserves. The study was performed with six very fat yearling females (initial mean body weight 1451 (SD 119) g, i.e. approximately 300 g more than for a female in normal body condition). The animals were fed in restricted amounts (about 35 % metabolizable energy requirement for maintenance) in order to reach a very lean body condition. The target weight of 800 g was reached after about 1 month of restriction. The animals were then refed *ad libitum* until almost complete weight recovery. Blood samples were taken on days 1, 24, 34 (end of restriction), 44, 55 and 71 (end of experiment) and analysed for plasma concentrations of leptin and insulin. Three females were mated on day 44. Leptin and insulin concentrations mirrored each other and clearly reflected changes in body weight. Significant ($P<0{\cdot}001$) Pearson correlation coefficients of 0·75 (leptin–insulin), 0·72 (leptin–body weight) and 0·59 (insulin–body weight) were found. Two of the three females that were mated gave birth to normal litters. It was concluded that the leptin assay yielded acceptable results for animals with body weight:fat content within the range investigated here, and that plasma leptin reflected body fat mass.

Energy supply: Food restriction: Refeeding: Body fat mass

Since the discovery of leptin, the protein product of the obesity gene, knowledge of its diverse functions has evolved from the initial concept of being a satiety factor (Zhang *et al.* 1994) to, among others, being a chronic homeorhetic regulator of metabolism (Houseknecht & Portocarrero, 1998). The main site of leptin production is white adipose tissue, and in human subjects and rodents circulating leptin concentrations closely reflect body fat mass, but also recent energy intake: similarly to insulin, it decreases during fasting and increases during refeeding. Insulin may be the hormone that regulates leptin secretion in response to changes in energy intake, because alterations in insulin precede those of leptin (Havel, 2000). Moreover, the sympathetic nervous system plays a key role in regulation of the fasting-induced decrease in leptin production (Trayhurn *et al.* 1998). Leptin influences energy metabolism and leptin administration to *ob/ob* mice leads to increased body temperature, physical activity and heat production. Pair-feeding experiments have shown that chronic leptin treatment resulted in decreases in body weight that were larger than could be explained by only decreased food intake. It appears that leptin prevents the normal decrease in heat production resulting from restriction of energy supply (Baile *et al.* 2000; Havel, 2000).

Although the information on leptin's role in metabolism has increased during recent years, results from domestic

Abbreviations: BW, body weight; ME, metabolizable energy.

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animal species are still limited. There are results supporting the relation between leptin and body fat mass–plane of nutrition (contemporary and obese swine, Ramsay *et al.* 1998; lactating sows, Estienne *et al.* 2000; cattle and sheep, Blache *et al.* 2000; Delavaud *et al.* 2000; Erhardt *et al.* 2000; cats, Backus *et al.* 2000). However, in general, relations seem weaker than reported for laboratory rodents and human subjects. Repeated-measures data from domestic animals exposed to a period of food restriction and then refeeding are to our knowledge lacking. We decided to perform this study on a strict carnivore and seasonal breeder, the mink (*Mustela vison*). Minks accrete large fat depots during the autumn, depots which are mobilized during the winter and spring (Tauson, 2001). The objectives of the present study were to evaluate if a multi-species leptin assay could be validated for and used to monitor plasma leptin concentrations in mink, and if so, to evaluate how a period of food restriction followed by refeeding affected animal body weights and plasma concentrations of leptin and insulin.

Materials and methods

Six yearling mink dams of the standard brown genotype (Nes *et al.* 1987) were used. The animals originated from our experimental herd and were housed under conventional conditions, i.e. in non-insulated sheds under natural daylight conditions (55°N, 12°E, mid February–late April). The animals were fed on a wet feed mixture based on fish, fish and animal by-products, heat-treated barley, animal fat and vegetable oil. Water was available *ad libitum* at all times. The diet contained 318 g DM and 168 g crude protein/kg. The metabolizable energy (ME) content was 5020 kJ/kg (15·8 MJ/kg DM). Before the experiment the animals had been fed close to *ad libitum*. Animals were weighed in mid February and six animals considered as very fat were assigned to the experiment. Their initial mean body weight (BW) was 1451 (SD 119) (range 1289–1646) g. From then on the animals were weighed once per week until the end of the experiment in late April. For a period of 34 d the animals were fed in restricted amounts in order to reach a target weight of 800 g, corresponding to a lean body condition. The daily food supply was 50 g providing 250 kJ ME, and assuming a ME requirement for maintenance of 527 kJ/kg$^{0·75}$ (Chwalibog *et al.* 1980); this corresponded to approximately 35 % ME requirement for maintenance at the start of the experiment, increasing to about 55 % ME requirement for maintenance at the target weight of 800 g. This level of restriction was justified by the fact that carnivores with seasonal cycles of fat accretion and mobilization may experience prolonged periods of low food intake or even fasting when living in the wild, and these animals had substantial body reserves available for mobilization. After the period of restricted feeding the animals were refed *ad libitum* for a period of approximately the same length as the restriction period. Blood samples were collected by venepuncture into heparinized tubes on days 1, 24, 34, 44, 55 (only three animals; see later) and 71 of the experiment. After separation the plasma was stored at −40°C.

Leptin was determined by radioimmunoassay (Multi-species leptin radioimmunoassay kit; Linco Research, Inc., St Charles, MO, USA). Serial dilutions of a mink plasma sample containing 5·5 ng leptin/ml produced a displacement curve parallel to the human leptin standard curve. All samples were run in one assay and the within-assay CV estimated from the precision profile was <10 % for concentrations between 1 and 10 ng/ml. The CV of a human leptin quality control sample provided with the kit was 6 % (mean value 4·2 ng/ml).

Plasma concentrations of insulin were determined by radioimmunoassay (Pharmacia insulin radioimmunoassay; Kabi-Pharmacia, Uppsala, Sweden) previously validated for mink plasma (Fink *et al.* 1998). The within-assay CV estimated from the precision profile was <15 % for concentrations between 3 and 240 μU/ml. The between-assay CV for three control samples were 2 % (mean value 13 μU/ml), 5 % (mean value 42 μU/ml) and 3 % (mean value 109 μU/ml).

The last 2 weeks of the restriction period coincided with the most intensive part of the breeding season, whereas after 10 d of refeeding the breeding season was almost at an end. Since mink females not tried for mating early in the season may be willing to mate very late in the season (Elofson *et al.* 1989) the dams were exposed to males on day 44 (30 March) and three of them mated.

Statistical analyses of BW, leptin and insulin data were performed by means of Statistical Analysis System (SAS®; SAS Institute Inc., Cary, NC, USA) procedure MIXED for repeated-measures data (Littell *et al.* 1996), using a model with day of experiment as fixed effect and autoregressive order 1 as covariance structure. Furthermore, SAS® linear regression procedure REG was used to calculate a regression equation for leptin and BW, and Pearson's correlation coefficients between BW, leptin and insulin were calculated by means of procedure CORR (Version 6, 1985; SAS Institute Inc.).

Experimental procedures conformed to national Danish legislation.

Results

After only 2 weeks of restriction, the mean BW value was 226 g below the initial 1451 g ($P=0·003$). On day 24, the mean BW value was 432 g below the initial value ($P<0·001$), and BW reached its nadir of 813 (SD 123) (range 620–950) g approximately 1 month after restriction had started. Body weights had then decreased by on average 638 g ($P<0·001$). In response to refeeding, the animals regained BW rapidly, the increase during the first 10 d being 333 g ($P<0·001$), and after 3 weeks the animals had regained 595 g and reached BW not significantly different from the initial values ($P>0·5$). During the last 2-week period the BW remained stable (Fig. 1(a)).

Plasma leptin concentrations averaged 3·7 ng/ml, ranging from 3·0 to 5·5 ng/ml, at the start of the experiment. The highest value was recorded for the heaviest female. Plasma leptin concentrations had reached their nadir of 1·4 (SD 0·22) ng/ml, significantly below the initial concentrations ($P<0·001$), by only day 24 and remained on this level until the end of the restriction period, although BW continued to decrease. During the first 10 d of refeeding plasma concentrations of leptin increased, but not

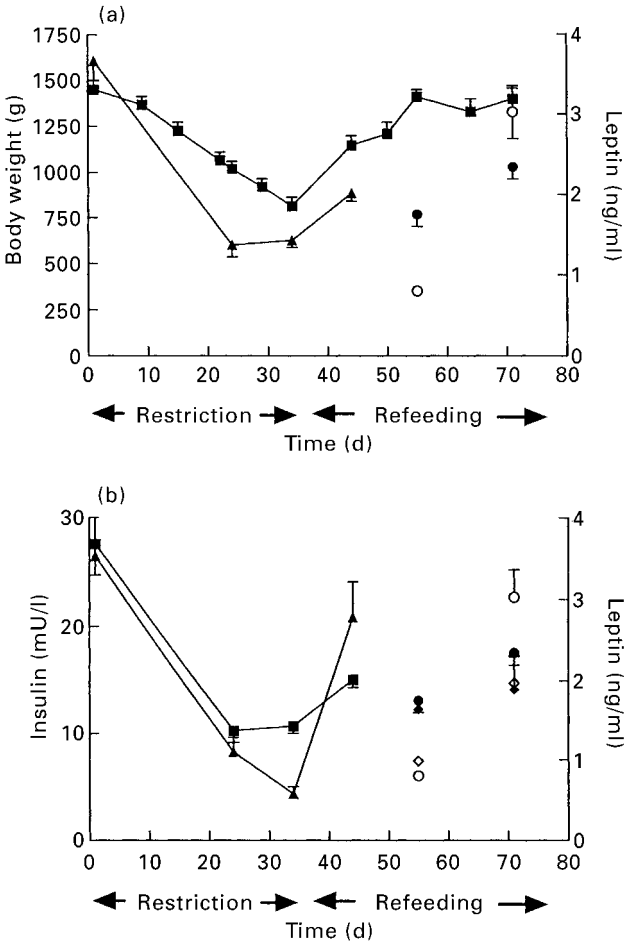

Fig. 1. (a) Body weights and plasma concentrations of leptin (■, body weight; ▲, leptin, all; ●, leptin, pregnant; ○, leptin, barren) and (b) plasma concentrations of leptin and insulin (■, leptin, all; ●, leptin, pregnant; ○, leptin, barren; ▲, insulin, all; ◆, insulin, pregnant; ◇, insulin, barren) in six female mink during a period of restricted feeding (days 1–34) and during refeeding (days 35–71). On day 55 only three mated females were sampled, one turned out to be barren and two were pregnant. Days 55 and 71 results are presented separately for females that were pregnant and for those that were not mated (*n* 3) or barren (*n* 1). Values are means with standard errors represented by vertical bars.

significantly ($P=0{\cdot}08$). On day 55 of the experiment, blood samples were only taken from the three mated females, and leptin concentrations of only 1·4 ng/ml were recorded. At the end of the experiment, leptin concentrations were significantly increased in response to refeeding ($P<0{\cdot}001$), but still significantly below the initial values ($P=0{\cdot}01$) (Fig. 1). A second order regression equation for the relation between plasma leptin and BW was found:

$$\text{Leptin (ng/ml)} = 5{\cdot}25\ (\text{SEM}\ 2{\cdot}039) - 0{\cdot}009\ (\text{SEM}\ 0{\cdot}0036) \times \text{BW (g)} + 5 \times 10^{-6}\ (\text{SEM}\ 1{\cdot}6 \times 10^{-6}) \times \text{BW}^2\ \text{(g)},$$

where *n* 33, RSD 0·625, R^2 0·64, CV 28·7.

Plasma insulin concentrations mirrored those of leptin (Fig. 1(b)) although the magnitude of the response was greater: insulin decreased by more than 20 μU/ml during restriction ($P<0{\cdot}001$), and then during the first 10 d refeeding it increased by more than 15 μU/ml to an average value not significantly different from the initial concentration ($P=0{\cdot}15$). The samples taken from the three mated females on day 55 showed low plasma insulin concentrations, not significantly different from those found during restriction ($P>0{\cdot}15$). Similar to leptin, insulin concentrations recorded by the end of the experiment were significantly below the initial values ($P=0{\cdot}004$).

Pearson correlation coefficients between leptin and insulin (0·75), leptin and BW (0·72) and insulin and BW (0·59) were all statistically significant ($P<0{\cdot}001$).

Of the three mated females, two gave birth after 48 and 49 d gestation to litters of four and eight kits respectively. The female that remained barren was the one that had reached the lowest BW (620 g) during restriction, and on day 55 the lowest concentrations of leptin (0·8 ng/ml) and insulin (7·4 mU/l) were recorded for this female. By the end of the experiment the two pregnant females had plasma leptin concentrations below those of the females that were not mated (*n* 3) or barren (*n* 1), but plasma insulin concentrations were similar for females that were pregnant, not mated or barren (Fig. 1(b)).

Discussion

Studies in sheep and cattle have shown that the multi-species leptin assay used in the present study underestimates plasma leptin concentrations when compared with a species-specific assay, and moreover, that results were unresponsive to changes in nutrition or adiposity (Blache *et al.* 2000; Delavaud *et al.* 2000; Ehrhardt *et al.* 2000). However, with the use of this multi-species assay significant feeding-level effects have been reported in sheep (Bocquier *et al.* 1998), in pigs (Barb, 1999) and in young mares (McManus & Fitzgerald, 2000). Similarly, age, and hence BW, affected pig serum leptin concentrations in one experiment but not in another (Qian *et al.* 1999), and in sows serum leptin was positively correlated with backfat thickness at farrowing (Estienne *et al.* 2000). Results in the literature on mink seem unreliable: Mustonen *et al.* (2000) and Nieminen *et al.* (2000) reported very low or even non-detectable plasma leptin concentrations without clear-cut relation to body condition. The animals used in the present study were exposed to a substantial variation in food supply, resulting in BW decreasing to about 55 % of initial values during restriction, and then almost completely recovering during refeeding. Plasma leptin concentrations reflected BW clearly, indicating that the assay produced acceptable results within the range of body fat content represented here, although they probably under-estimated true values.

Although we did not estimate body fat content, the fluctuations in BW in response to food restriction and refeeding must to a great extent have reflected changes in body fat stores, and thereby confirmed that circulating leptin monitors body fat mass in the mink as well as in rodents and human subjects (Havel, 2000) and cats (Backus *et al.* 2000). Moreover, plasma leptin concentrations followed the same pattern as plasma insulin, and there was a positive

correlation between plasma leptin and insulin. We have previously shown that insulin changes rapidly in mink in response to changes in energy supply (Fink *et al.* 1998; Tauson *et al.* 2000), which was confirmed here and fits the concept of insulin as regulator of energy-supply-induced changes in leptin secretion (Havel, 2000). However, food restriction may cause an increased sympathetic activity to the white adipose tissue (Migliorini *et al.* 1997), and this regulation mediated via catecholamines and β-agonists seems to be more pronounced than other regulatory factors (Trayhurn *et al.* 1998), so it cannot be excluded that this mechanism was the main cause of the decrease in plasma leptin during food restriction.

Metabolic status strongly influences reproductive performance, but still the question of exactly how nutrition regulates reproduction remains unanswered. Leptin is suggested to be a signal to the neuroendocrine reproductive system, and may serve as a metabolic gateway. It seems to be involved in the timing of puberty: exogenous-leptin-inhibited food restriction induced reproductive quiescence by restoring gonadotropin secretion (Cunningham *et al.* 1999; Foster & Nagatani, 1999). Plasma leptin concentrations and leptin gene expression seem to decrease when seasonal animals are exposed to short days (Djungarian hamster, Klingenspor *et al.* 1996; sheep, Bocquier *et al.* 1998), suggesting that effects of photoperiod may over-ride those of nutritional status–body fat content. Reproduction in mink is strictly regulated by photoperiod and reproductive outcome is very responsive to nutritional manipulation (Tauson, 2001). In extremely lean animals the pre-ovulatory luteinizing hormone surge and ovulation can be abolished (Tauson *et al.* 2000). None of the mated females was in such a lean condition at mating, but the female remaining barren had been in such condition by the end of the restriction period. Plasma leptin and insulin concentrations had increased since the end of the restriction period in all mated animals, but less so in the barren female. The gestational hyperleptinaemia commonly recorded during the last two-thirds of gestation in human subjects and rodents (Hoggard *et al.* 1998) could not be documented here, but the last blood sampling took place more than 20 d before delivery, i.e. during the first third of the true gestation, and therefore the role of the placenta for leptin production probably was limited. The results of this investigation are too limited to provide clear evidence of leptin's role in reproduction in the mink, but indicate that future research in this area might be rewarding.

In conclusion, our results suggest that plasma leptin concentrations in mink are very responsive to a varied energy supply and body condition of the animals. Therefore, the mink may serve as an excellent animal model for studies on leptin's role in nutrition–reproduction interactions in seasonally breeding animals.

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