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High leptin in pregnant mink (*Mustela vison*) may exert anorexigenic effects: a permissive factor for rapid increase in food intake during lactation

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The role for leptin in food intake regulation in the mink, a polytocous seasonal breeder with altricial young, was investigated in pregnant and lactating dams and data were related to quantitative energy metabolism measurements and plasma concentrations of other important metabolic hormones. A total of nine mink dams were measured in consecutive 1-week balance periods, each including a 22 h measurement of heat production by means of indirect calorimetry, and blood was sampled at weekly intervals throughout gestation and during lactation weeks 1–4. Intake of metabolisable energy (ME) was high and energy balance was positive until the first third of true gestation. During mid- and late gestation ME intake decreased ($P<0{\cdot}001$) while heat production remained almost constant, resulting in negative energy balance and the loss of body weight. From late gestation until lactation week 4, ME intake increased by 3·5 times, but weight loss continued. Plasma concentrations of leptin were approximately doubled during the last two-thirds of true gestation ($P<0{\cdot}01$), demonstrating a clear gestational hyperleptinaemia. Concentrations declined rapidly after parturition and then remained stable. Insulin was independent of leptin, with low concentrations coincident with hyperleptinaemia. Also, concentrations of thyroid hormones declined during gestation, probably reflecting the low food intake. Hyperleptinaemia concomitant with low ME intake, negative energy balance and mobilisation of body reserves suggested an anorexigenic effect of leptin in pregnant mink. This suppression of food intake in late gestation might be permissive for the rapid increase in food intake occurring after parturition.

Energy metabolism: Substrate oxidation: Thyroid hormones: Insulin: Insulin-like growth factor-1

Various strategies are applied among species for securing a maximum reproductive outcome. For seasonal breeders the situation is complicated by the necessity to survive a period with a scarcity of food and still maintain sufficient body reserves to support reproduction. The mink (*Mustela vison*) is a polytocous seasonal breeder with one annual breeding season in March in the Northern hemisphere. Similar to other seasonal breeders, it has annual cycles of changes in food intake and body weight (Hansen *et al.* 1991), body weights decreasing by 10–15 % during the winter months in *ad libitum*-fed dams (Korhonen & Niemälä, 1998). The breeding mink therefore faces the problem of conceiving after a period of declining body reserves. Furthermore, the reproductive performance of mink is highly responsive to energy supply, especially in yearling females (Tauson, 1993), so the pregnant dam may have to support growth and viability of a large number of fetuses. The dam gives birth to altricial young after a gestation comprising a short embryonic diapause and then a true gestation period of 31 d (Murphy & Douglas, 1992).

The role of leptin in the regulation of food intake, body weight and energy homeostasis has, since its discovery (Zhang *et al.* 1994), gained increasing recognition (for reviews, see Baile *et al.* 2000; Havel, 2000; Schwartz *et al.* 2000; Frübeck, 2001). A role for leptin in the regulation of reproduction (for a review, see Cunningham *et al.* 1999) has been proposed.

It has been suggested that the regulation of leptin in pregnancy probably is an outcome of cross-talk between the placenta, fetus and maternal adipose tissue (Henson & Castracane, 2000), and that it is involved in the control of maternal nutrient availability and fetal energy homeostasis. It may even cause the predetermination of a body

Abbreviations: BEFIMP, before implantation; CHO, carbohydrate; FT_4, free thyroxine; GEST1/3, first third of true gestation; GEST2/3, second third of true gestation; GEST3/3, last third of true gestation; HE, heat production; IGF-1, insulin-like growth factor-1; IMP, about the time of implantation; LACTW1, first week of lactation; LACTW2, second week of lactation; LACTW3, third week of lactation; LACTW4, fourth week of lactation; LW, live weight; ME, metabolisable energy; OXCHO, quantitative oxidation of CHO; OXF, quantitative oxidation of fat; OXP, quantitative oxidation of protein; RN, retained N; T_3, triiodothyronine; TT_3, total triiodothyronine; T_4, thyroxine; TT_4, total thyroxine; UN, urinary N.

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weight set point imprint to be carried forward postnatally (Frübeck, 2001). Leptin may be an important growth factor for the fetus, or it may signal energy status between mother and fetus (Caprio *et al.* 2001; Mostyn *et al.* 2001).

Lactation is usually more energy demanding than gestation, especially in polytocous species. In cases when food supply alone cannot meet energy requirements, body energy reserves will be mobilised for milk production. The balance between food intake and body condition during gestation and food intake and performance during lactation is therefore of paramount importance for both the pregnant or lactating dam and her offspring. After parturition plasma concentrations of leptin decline, and it has been suggested that low leptin concentrations contribute to stimulate food intake and milk production (Brogan *et al.* 1999; Woodside *et al.* 2000) and possibly also cause lactational anoestrous (Woodside *et al.* 2000; Caprio *et al.* 2001).

The pregnant mink usually exhibits a low food intake during the part of gestation when fetal growth is rapid. This results in negative energy balance in late gestation (Tauson *et al.* 1994), and after parturition the body must adapt to producing a high milk output (Fink *et al.* 2001) in order to support the rapid weight gain of which mink kits are capable (Tauson, 1994).

The mink dam selected for high litter size and with a high milk yield could therefore serve as a suitable model for studies of the energetic demands on high-producing polytocous species and how they are able to sustain production performance by balancing intake of food with mobilisation of body reserves. The objective of the present study was therefore to investigate the quantitative energy and protein metabolism of pregnant and lactating mink dams and the hormonal regulation of these processes, with special emphasis on the role of leptin in the regulation of food intake and energy balance.

Materials and methods

Animals, housing and management

A total of nine 2-year-old mink dams of the standard brown genotype (Nes *et al.* 1987) were used. The animals were raised and bred at the experimental farm of the Royal Veterinary and Agricultural University. The dams were mated to non-experimental males on two consecutive days, starting on 17 March. The dams were then transferred to the laboratory, where they were kept in metabolism cages (for a description, see Jørgensen & Glem-Hansen, 1973) equipped with devices for quantitative collection of feed residues and excreta and for measuring water intake. Before expected parturition nest-boxes were put into the cages, and a bottom screen with finer meshes (6·4 × 25·4 mm) was used. When the animals gave birth, the date and number of live and stillborn kits were recorded and the kits were weighed. Kits born during the daytime were weighed as soon as parturition was considered to be completed, and kits born during the night were weighed the following morning. Some cross-fostering took place; two dams giving birth to two and three kits were given kits so that they suckled litters of five, and from one dam giving birth to ten kits three were removed. The animals were kept under natural daylight conditions (end of March until end of May; 55°N, 16°E). After a 1-week adaptation period, the experiment was carried out in consecutive 1-week balance periods, each including a 22 h respiration experiment by means of indirect calorimetry. The experiment lasted from shortly after breeding until approximately 4 weeks after parturition. The experiment ended before the kits started to consume significant amounts of other food than their mothers' milk.

Ethical approval

The experimental procedures followed Danish National Legislation and the guidelines approved by the member States of the Council of Europe for the protection of vertebrate animals (Anonymous, 1986).

Diets and feeding

Throughout the experiment the animals were given free access to food, i.e. the daily feed allowances were adjusted so that there were some food residues. Conventional wet mink diets purchased from a commercial mink food producer (Stårup Fodercentral, Højby, Sjælland, Denmark) were used. From the start of the experiment (end of March) until parturitions started (late April) a gestation-period diet was used, and from then on until the end of the experiment a lactation-period diet was fed. The diets were purchased on single occasions, weighed into plastic bags containing daily portions, and then immediately frozen. The food was taken out of the freezer the day before use and was thawed overnight. The dietary composition, the analysed chemical composition of the diets and the calculated percentages of metabolisable energy (ME) derived from protein, fat and carbohydrates (CHO) are reported in Table 1.

Balance and respiration experiments

The quantitative collection of food residues and excreta was carried out once daily between 08.30 and 12.00 hours, and the total amount from each period was stored at −18°C until analysis. Urine was collected in bottles containing 10 % (w/v) H_2SO_4 (5 ml). The screen for faeces collection and the urine collection funnel were rinsed with anhydrous citric acid (10 g/l) at the end of the daily collection procedure.

Each balance period included a 22 h respiration experiment by means of indirect calorimetry in an open-air circulation system. The respiration chamber (760 litres) was designed so as to permit the direct insertion of the metabolism cage into the chamber. The animals were moved to the respiration chamber at least 1 h before the start of the experiment to adapt to the surroundings. The temperature and relative humidity were kept at 15 to 18°C and 0·65 to 0·75, respectively. Air from the chambers was analysed every third min for concentration of O_2 and CO_2. O_2 was analysed by a paramagnetic analyser (Magnos 4G; Hartmann & Braun AG, Frankfurt, Germany), and CO_2 was analysed with the IR principle (Uras 3; Hartmann & Braun AG).

Table 1. Dietary composition and analysed chemical composition (g/kg) of the gestation- and lactation-period diets

	Gestation period	Lactation period
Fish offal	450	390
Industrial fish	250	300
Poultry wastes	110	75
Hb	50	60
Barley	30	60
Wheatbran	30	20
Fish-meal + maize gluten meal	15	15
Wheatgerm	7·5	–
Soya fibre	7·5	15
Soya oil	5·0	20
Vitamin and mineral mixture*	2·5	2·5
Water	to make 1000	to make 1000
Analysed chemical composition		
DM	326·4	319·3
Ash	31·2	35·9
Crude protein	170·0	158·8
Fat	47·5	48·6
Carbohydrate	77·7	76·1
Gross energy (MJ/kg DM)	22·8	22·1
Calculated percentage contribution to ME†		
Protein	54	53
Fat	38	35
Carbohydrate	9	12
ME (MJ/kg DM)	15·07	15·53

ME, metabolisable energy.
* Containing (per kg): vitamin A, 840 mg; vitamin D_3, 7 mg; α-tocopherol, 24 000 mg; vitamin B_1, 10 000 mg; vitamin B_2, 4800 mg; vitamin B_6, 3200 mg; D-pantothenic acid, 3200 mg; niacin, 8000 mg; choline chloride, 40 000 mg; folic acid, 120 mg; biotin, 80 mg; vitamin B_{12}, 8 mg; para-amino-benzoic acid, 800 mg; zinc bacitracin, 8000 mg; Fe, 20 g; Zn, 12·5 g; Mn, 6·2 g; Cu, 1·0 g.
† Calculated from analysed chemical composition and ME factors of 18·42 kJ/g digestible protein, 39·76 kJ/g digestible fat and 17·58 kJ/g digestible carbohydrate (Hansen *et al.* 1991).

Weighing and blood sampling of animals

The dams were weighed when the animals entered the laboratory, at the start of each balance period and at the end of the experiment. Mink kits were weighed at birth and then weekly. Blood samples were taken mainly at the same time points as described earlier, the exception being the period about the time of expected implantation, when blood samplings were omitted in order not to disturb the implantation process (Tauson, 1991). During blood sampling the animals were held in a conventional mink trap and blood was withdrawn by puncture of Vena cephalica antebrachii (Blixenkrone-Møller *et al.* 1987). Anaesthesia was not given in order to avoid interference with hormone release. To circumvent diurnal variation in hormone concentrations, blood was sampled from 09.00 to 11.30 hours. Blood was collected in heparinised tubes, and the separated plasma was stored in plastic tubes at −18°C until assay.

Analytical procedures, food and excreta

The diets and faeces were analysed for DM by evaporation at 100°C to constant weight. Ash was determined by combustion at 525°C for 6 h, N was determined by the micro-Kjeldahl technique with the Tecator-Kjeltec system 1030 (Tecator AB, Höganäs, Sweden), crude protein was calculated as N × 6·25, fat was determined by petroleum–diethyl ether extraction after HCl hydrolysis and gross energy was determined by the use of an adiabatic bomb calorimeter. CHO were calculated by the difference. Urine was analysed for gross energy and N.

Analytical procedures, hormones and metabolites

Leptin. Plasma concentrations of leptin were determined by radioimmunoassay (Multispecies leptin radioimmunoassay kit; Linco Research Inc., St Charles, MO, USA) previously validated for mink plasma (Tauson & Forsberg, 2002). 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).

Thyroid hormones. Plasma concentrations of total triiodothyronine (TT_3) and total and free thyroxine (TT_4 and FT_4) were analysed by the use of a commercial chemiluminescence immunoassay (Amerlite; Johnson and Johnson, Amersham, UK). Serial dilutions of mink plasma with high concentrations of TT_3, TT_4 and FT_4 produced displacement curves parallel to the standard curves of the respective assays. The intra-assay CV for TT_3 were below 10 % for all concentrations, and the corresponding inter-assay CV were 21·8 % for samples with low concentrations (mean 1·1 nmol/l) and 10·2 % (mean 3·0 nmol/l) and 8·0 % (mean 6·1 nmol/l) for samples with medium and high concentrations, respectively. For TT_4 the intra-assay CV were 14·3 % for samples with low concentrations (mean 6·0 nmol/l), 6·0 % (mean 20·6 nmol/l) for medium concentrations and 4·1 % (mean 73·4 nmol/l) for samples with high concentrations, and the inter-assay CV were 26·1 % for low, 3·6 % for medium and 5·0 % for high concentrations. For FT_4 the intra-assay CV were below 10 % for all concentrations, and the corresponding

inter-assay CV were 18·8 % for samples with low concentration (mean 12·2 pmol/l) and below 10 % for samples with medium (mean 21·9 pmol/l) and high (mean 41·9 pmol/l) concentrations.

Insulin. Plasma concentrations of insulin were determined by radioimmunoassay (Pharmacia insulin RIA; Kabi-Pharmacia, Uppsala, Sweden). Serial dilutions of mink plasma containing high concentrations of insulin produced a dose–response curve parallel to the standard curve. The intra-assay CV for quality control samples were 5·4 % (mean 12 μU/l), 5·3 % (mean 42 μU/l) and 5·3 % (mean 117 μU/l). The corresponding inter-assay CV were 7·8, 2·2 and 6·7 %.

Insulin-like growth factor-1. The plasma concentration of insulin-like growth factor-1 (IGF-1) was determined by radioimmunoassay according to the manufacturer's recommendations (IGF-1, catalogue no. 53065; Incstar Corporation, Stillwater, MI, USA). Plasma was extracted with ODC-silica columns before assay. Serial dilutions of mink plasma with high concentrations of IGF-1 produced displacement curves parallel to the human standard curve. The intra-assay CV, calculated from the precision profiles of five assays, was below 12 % for IGF-1 concentrations between 2·9 and 82·5 nmol/l. The inter-assay CV for two control samples were 19 % (mean 10 nmol/l) and 21 % (mean 28 nmol/l). The minimum detectable level of IGF-1 was set to 2 nmol/l (average 10 % fall from '0'-binding of five assays).

Prolactin. Plasma was analysed for prolactin with an enzyme immunometric assay designed for canine prolactin (Milenia canine prolactin; Diagnostic Products Corporation, Los Angeles, CA, USA) as described by Xiao *et al.* (1995). The intra-assay CV calculated from the precision profiles was below 10 % for concentrations between 2 and 40 ng/ml. The inter-assay CV for a control sample with 4·7 ng/ml was 8·9 %. The detection limit according to the manufacturer's information was 0·5 ng/ml.

Glucose and fructosamine. Plasma concentrations of glucose and fructosamine were analysed in a computerised multichannel spectrophotometer (Cobas Mira; Hoffmann-La Roche & Co., Basel, Switzerland). The analysis of glucose was performed according to Bondar & Mead (1974) and fructosamine was analysed by measuring formazane at 550 nm (Baker *et al.* 1985). The inter-assay CV were 1·6 and 3 %, respectively.

Calculations

ME was calculated as ME = gross energy – energy in faeces – energy in urine. Heat production (HE) was calculated from O_2 consumption, CO_2 production and urinary N (UN) according to the formula by Brouwer (1965):

$$\text{HE (kJ)} = 16{\cdot}18 \times O_2\text{ (litres)} + 5{\cdot}02 \times CO_2\text{ (litres)} - 5{\cdot}99 \times \text{UN (g)}.$$

Retained energy was calculated as ME – HE for pregnant dams. For lactating dams no such calculation was done because energy output in milk was not measured.

Quantitative oxidation values for protein (OXP), fat (OXF) and CHO (OXCHO) were calculated based on gas exchange measurements and UN as described and validated for pigs by Chwalibog *et al.* (1992)

$$\text{OXP (kJ)} = \text{UN (g)} \times 6{\cdot}25 \times 18{\cdot}42;$$

$$\text{OXF (kJ)} = (1{\cdot}719 \times O_2\text{ (litres)} - 1{\cdot}719 \times CO_2\text{ (litres)} - 1{\cdot}963 \times \text{UN (g)}) \times 39{\cdot}76;$$

$$\text{OXCHO (kJ)} = (-2{\cdot}968 \times O_2\text{ (litres)} + 4{\cdot}174 \times CO_2\text{ (litres)} - 2{\cdot}446 \times \text{UN (g)}) \times 17{\cdot}58.$$

Although balance and respiration experiments were performed in consecutive 1-week periods, the results are reported in relation to time of parturition. Hence, the interval between each respiration experiment and parturition was calculated. The results are reported for periods corresponding to before implantation (BEFIMP; ≥ 33 d prepartum), about the time of implantation (IMP; 27–32 d prepartum, no hormones and metabolites), during the first third of true gestation (GEST1/3; 18–26 d prepartum), during the second third of true gestation (GEST2/3; 9–17 d prepartum), during the last third of true gestation (GEST3/3; ≤ 8 d prepartum) and during the first (LACTW1), second (LACTW2), third (LACTW3) or fourth (LACTW4) week of lactation.

During the lactation period, respiration experiments were performed with the dams and their litters. When relating the results to metabolic weight ($kg^{0{\cdot}75}$), the metabolic weight of the dam and the mean kit metabolic weight multiplied by the number of kits in the litter made up the total metabolic weight of dam and litter.

Statistical analyses

The statistical analyses were performed according to the MIXED procedure in SAS® (Littell *et al.* 1996). The material was analysed regarding the fixed effect of physiological stage as the repeated measure (BEFIMP, IMP, GEST1/3, GEST2/3 or GEST3/3, LACTW1, LACTW2, LACTW3 or LACTW4). The autoregressive order 1 (AR1) covariance structure was fitted. Pearson correlation coefficients were calculated for selected traits (SAS Institute, Inc., 1990).

Results

Reproductive performance and kit weight gain

All dams entering the experiment turned out to be pregnant and gave birth after a gestation period of on average 46·8 (SD 3·27) d. Average total litter size at birth was 7·7 (SD 2·69) kits of which 7·0 were liveborn. Stillborn kits hence accounted for 8·7 % of the total born which is a normal level for the litter size. Total litter size ranged from three to twelve kits, and the number of stillborn kits ranged from zero to two. The number of kits per litter during the suckling period (after cross-fostering) was 5·7 (SD 1·12). Kit birth weight was normal and amounted to 9·1 (SD 1·77) g. From then on kits gained weight at a normal rate and reached live weights (LW) of 89·5 (SD 18·37) g in LACTW3 (day 17 (SD 1·98)

postpartum) and then at the end of the experiment in the fifth week of lactation (day 31 (SD 2·09) postpartum) 192·1 (SD 21·22) g.

Intake of metabolisable energy and dam live weights

The intake of ME (kJ/kg$^{0·75}$) was high during BEFIMP, IMP and GEST1/3 and not significantly different between any of these stages. From then on it declined during GEST2/3 and further to a very low level during GEST3/3, the period of the most extensive fetal growth; differences between the previous stages ($P<0·05$) and between the two latter stages ($P<0·001$) being significant (Table 2). During LACTW1, ME intake had increased to the same level as during BEFIMP, IMP and GEST1/3. During each of the following weeks intake increased significantly ($P<0·05$), the amount consumed during LACTW4 being approximately twice that consumed during LACTW1 (Table 2).

Dam LW increased from BEFIMP until GEST2/3 ($P<0·001$), but there was a non-significant ($P=0·15$) decline from GEST2/3 to GEST3/3 (Table 2), suggesting that part of the fetal growth was supported by maternal body reserves. During LACTW1, LW was below the prepartum weight ($P<0·01$), and LW decreased throughout LACTW1 to LACTW4, the decline being close to significant ($P=0·08$) (Table 2), indicating that the dams continued to mobilise energy.

Energy metabolism

HE of pregnant dams ranged from 625 to 710 kJ/kg$^{0·75}$, the differences between stages being non-significant, but there was a tendency for an increase from GEST1/3 to GEST2/3, simultaneous with declining ME intake. When relating HE to ME intake, HE made up 88 % (BEFIMP), 76 % (IMP), 82 % (GEST1/3), 101 % (GEST2/3) and 163 % (GEST3/3), indicating an increased HE in late gestation. For lactating dams with their litters, HE, when calculated in relation to metabolic body weight of the dam, increased as lactation progressed. Another approach, in which HE was calculated in relation to metabolic weight of both dam and litter, revealed HE values ranging from 534 to 604 kJ/kg$^{0·75}$, differences between lactation weeks being non-significant (Table 2). Because kit HE values could not be separated from those of the dam, it was not possible to deduce whether the dam's contribution increased as lactation progressed and food intake and milk yield increased. In pregnant dams, retained energy was positive until GEST1/3, slightly negative (-6 kJ/kg$^{0·75}$) during GEST2/3 and strongly negative (-262 kJ/kg$^{0·75}$) during GEST3/3. Because energy retained as protein was positive throughout gestation, the proportion of energy retained as fat was clearly negative already during GEST2/3, thus indicating mobilisation of body fat during GEST2/3 (Table 2).

Substrate oxidation

The oxidation of protein as a percentage of HE (OXP/HE) reflected the intake of ME, with the highest values being recorded during IMP and GEST1/3 when ME intake was

Table 2. Energy metabolism in pregnant and lactating mink dams (*n* 9), measured before and at about the time of implantation, during the first, second and last thirds of true gestation and during the first 4 weeks of lactation*

(Mean values and standard errors of the mean)

	Period of gestation										Week of lactation								
	Implantation				True gestation														
	Before		Around		First third		Second third		Last third		1		2		3		4		*P* value (effect of stage)
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
No. of observations	12		8		13		13		12		10		9		8		11		
Time before/after partus (d)	36·4	0·73	29·1	0·82	21·9	0·60	12·9	0·60	4·7	0·60	4·4	0·67	10·6	0·77	18·3	0·73	26·6	0·67	
Live weight of dam (g)	1057	28	1102	26	1155	24	1226	24	1176	24	1064	25	1045	25	1013	28	960	53	<0·001
Live weight of kits (g)											22·0	4·69	49·5	5·09	94·0	5·20	156·8	5·32	<0·001
ME per dam (kJ/kg$^{0·75}$)	778	34·0	828	43·1	768	33·1	643	33·1	435	34·6	767	38·3	1038	40·6	1296	42·7	1532	86·2	<0·001
HE per dam (kJ/kg$^{0·75}$)	685	45·6	631	41·7	626	38·4	649	38·4	711	38·6	698	39·0	917	39·3	1124	44·2	1328	38·6	<0·001
RE per dam (kJ/kg$^{0·75}$)	93	47·6	197	48·4	144	41·7	−6	41·7	−262	42·6									<0·001
RP per dam (kJ/kg$^{0·75}$)	74	9·4	66	11·3	59	8·9	64	8·9	27	9·3									<0·001
RF per dam (kJ/kg$^{0·75}$)	19	45·8	131	44·6	85	39·8	−69	39·8	−290	40·3									<0·001
ME dam + kits (kJ/kg$^{0·75}$)											571	359	656	179	678	179	695	179	0·50
HE dam + kits (kJ/kg$^{0·75}$)											534	16·0	578	16·1	557	18·1	604	34·1	0·17

ME, metabolisable energy; HE, heat production; RE, retained energy; RP, energy retained in protein; RF, energy retained in fat.

* For details of procedures, see pp. 412 and 414.

high. There was also a clear reflection of the protein requirement for retention in fetal tissues, resulting in OXP/HE decreasing significantly to GEST2/3 ($P=0{\cdot}001$) and then further to GEST3/3 ($P=0{\cdot}002$), when the lowest value of 25 % of HE was recorded. The oxidation of fat (OXF/HE) in pregnant dams was the reverse of OXP/HE, with high values during periods with low ME intake and low values when the dams were in positive energy balance. The oxidation of CHO in pregnant dams reflected that all dietary CHO was oxidised (Fig. 1). Among lactating dams there were no significant differences in substrate oxidation. The oxidation of protein made up slightly more than 35 % of HE, whereas OXF ranged from slightly below 35 % to slightly above 42 % while all dietary CHO were oxidised (Fig. 1).

Nitrogen metabolism

Ingested N, digested N and UN generally mirrored the intakes of ME with high values during periods of high energy intake. The retained N (RN) of pregnant dams, similarly, reflected to a large extent ME intake, with values about 0·5 g/d during all stages but GEST3/3, when it was less than one half of this. The RN data recorded for lactating dams were to a large extent a reflection of N excretion in milk because no milk production measurements were made, and it was therefore not possible to separate N excretion in milk from N balance in the body. The RN values increased from 0·75 g/d during LACTW1 to about 1·40 g/d in LACTW4 (Fig. 2). Also, the utilisation of the digested protein for retention (RN/digested N; %) was strongly affected by stage. Generally, the utilisation was lower in pregnant dams, for whom it ranged from 10 % (GEST3/3) to 18 % (GEST2/3), this difference being significant ($P=0{\cdot}01$), than in lactating dams, for whom it was about 25 % without significant differences between lactation weeks. All differences between pregnant and lactating dams but BEFIMP to LACTW3, BEFIMP to GEST3/3, GEST2/3 to LACTW1 and LACTW3, and finally, GEST2/3 to GEST3/3, were significant ($P<0{\cdot}05$).

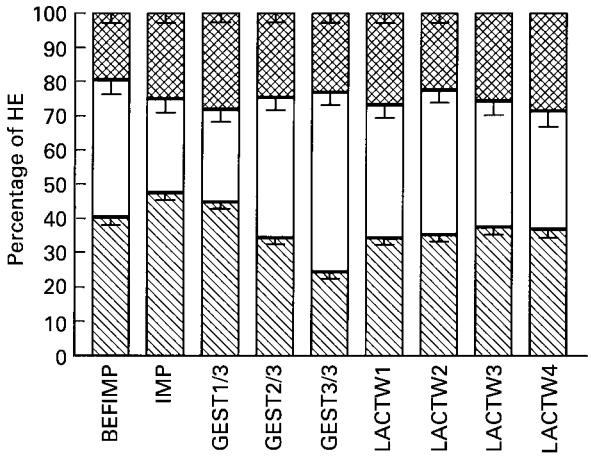

Fig. 1. Oxidation of protein (▧), fat (□) and carbohydrate (▩) in percentage of total heat production (HE) in nine pregnant or lactating mink dams measured before implantation (BEFIMP; −36·4 (SEM 0·73) d prepartum), about the time of implantation (IMP; −29·1 (SEM 0·82) d prepartum), during the first (GEST1/3; −21·9 (SEM 0·60) d prepartum), second (GEST2/3; −12·9 (SEM 0·60) d prepartum) and last third (GEST3/3; −4·7 (SEM 0·60) d prepartum) of true gestation and during weeks 1 (LACTW1; 4·4 (SEM 0·67) d postpartum), 2 (LACTW2; 10·6 (SEM 0·77) d postpartum), 3 (LACTW3; 18·3 (SEM 0·73) d postpartum) and 4 (LACTW4; 26·6 (SEM 0·67) d postpartum) of lactation. Mean values are shown, with standard errors of the mean represented by vertical bars.

Hormones and metabolites

Leptin and insulin. Plasma concentrations of leptin were significantly affected by measurement period ($P=0{\cdot}02$). They decreased non-significantly from BEFIMP to GEST1/3, but in GEST2/3 a more than doubled concentration was recorded (difference between the two periods; $P=0{\cdot}008$), and this high level also remained during GEST3/3. Immediately after parturition (LACTW1), concentrations returned to the same level as during GEST1/3, and they remained in the same order throughout the measurement period (Fig. 3 (a)). During the gestation period there was a significant negative correlation between intake of ME/kg$^{0{\cdot}75}$ and leptin (r −0·41; $P=0{\cdot}003$), but during lactation there was no such correlation. Similarly, leptin and retained energy were negatively correlated (r −0·29; $P=0{\cdot}04$) in pregnant dams. No significant correlation between HE and leptin was recorded for either pregnant or lactating dams.

For insulin, strongly significant effects of the measurement period were also recorded ($P<0{\cdot}001$), but the pattern did not follow that of leptin. Instead, concentrations decreased from BEFIMP until GEST3/3 ($P<0{\cdot}001$). Insulin remained low during lactation, and there were no significant differences between lactation weeks. Only in LACTW3 was a concentration significantly higher than during GEST3/3 ($P=0{\cdot}05$) recorded (Fig. 3 (a)). There was no significant correlation between insulin and leptin for either pregnant or lactating dams.

Thyroid hormones. Among the thyroid hormones the patterns of TT_4 and FT_4 mirrored each other to a large

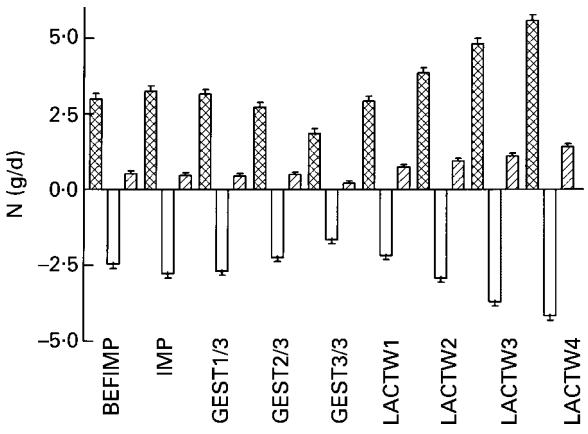

Fig. 2. Nitrogen metabolism of nine pregnant or lactating mink dams measured in balance periods before implantation (BEFIMP), about the time of implantation (IMP), during the first (GEST1/3), second (GEST2/3) and last third (GEST3/3) of true gestation and during weeks 1 (LACTW1), 2 (LACTW2), 3 (LACTW3) and 4 (LACTW4) of lactation. Data presented are digested N (▩), urinary N (□) and retained nitrogen (▨). Mean values are shown, with standard errors of the mean represented by vertical bars.

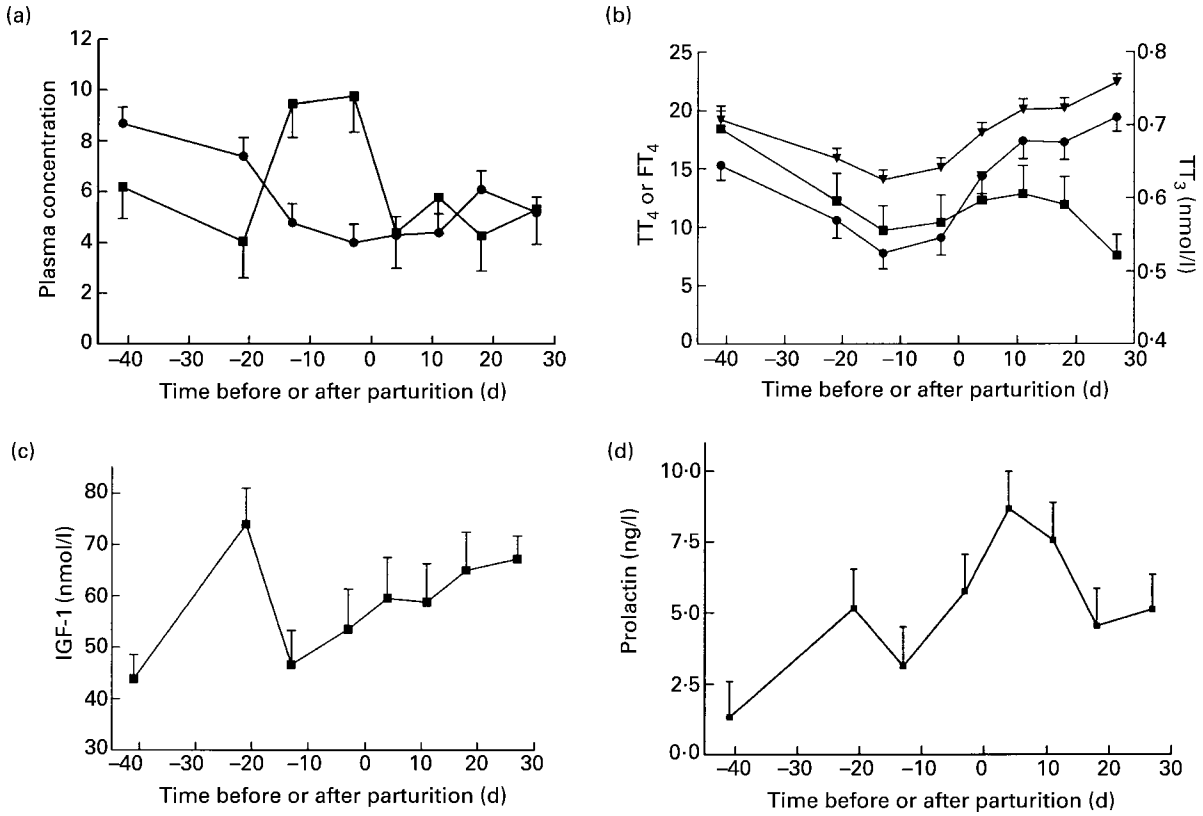

Fig. 3. Plasma concentrations of (a) leptin (■; ng/ml) and insulin (●; μU/l); (b) total thyroxine (TT_4; ●; nmol/l), free thyroxine (FT_4; ▼; pmol/l) and total triiodothyronine (■; TT_3); (c) insulin-like growth factor 1 (IGF-1); (d) prolactin in nine mink dams blood-sampled before implantation (BEFIMP; −40·7 (SEM 0·66) d prepartum), during the first (GEST1/3; −21·2 (SEM 0·78) d prepartum), second (GEST2/3; −12·8 (SEM 0·78) d prepartum) and last third (GEST3/3; −3·4 (SEM 0·82) d prepartum) of true gestation and during weeks 1 (LACTW1; 3·8 (SEM 0·92) d postpartum), 2 (LACTW2; 10·7 (SEM 0·99) d postpartum), 3 (LACTW3; 18·1 (SEM 0·92) d postpartum) and 4 (LACTW4; 27·4 (SEM 0·60) d postpartum) of lactation. Mean values are shown, with standard errors of the mean represented by vertical bars.

extent. Concentrations declined from BEFIMP to true gestation, differences being significant (TT_4, $P<0·05$; FT_4, $P<0·01$), and by LACTW1 they had returned to the same level as BEFIMP. For both hormones, concentrations continued to increase throughout the four lactation weeks, the difference between LACTW1 and LACTW4 being significant (TT_4, $P=0·01$; FT_4, $P<0·001$) (Fig. 3 (b)). The relationship between FT_4 and TT_4 did, however, vary with stage ($P<0·001$); it increased from 0·125% FT_4 during BEFIMP to 0·18% during GEST2/3. During lactation there was a slight decline from 0·126% FT_4 in LACTW1 to 0·115% in LACTW2, this level remaining constant during the following weeks. There were weak negative correlations between plasma concentrations of thyroxine (T_4) and leptin in pregnant and lactating dams (TT_4, r −0·22, $P=0·02$; FT_4, r −0·20, $P=0·04$). For TT_3 a different pattern was recorded; it showed a decline similar to that of TT_4 and FT_4 from BEFIMP to GEST3/3 ($P=0·01$), but then it remained on the same level throughout the four lactation weeks (Fig. 3 (b)). In pregnant dams TT_3 increased progressively in relation to TT_4 from 13·6% in BEFIMP to 21·5% in GEST3/3. In lactating dams the relationship between TT_3 and TT_4 remained stable, with TT_3 making up about 12% of the TT_4 concentration. There was no correlation between TT_3 and leptin but between TT_3 and insulin (r 0·25; $P=0·01$) when pregnant and lactating dams were considered together.

Insulin-like growth factor-1. Plasma concentrations of IGF-1 were low during BEFIMP and then increased significantly ($P<0·001$) in GEST1/3, declining to BEFIMP levels during GEST2/3 and GEST3/3, when food intake was low. Although there was a tendency for an increase in IGF-1 concentrations during lactation there were no differences between lactation weeks and concentrations were not significantly different from those during GEST3/3 (Fig. 3 (c)).

Prolactin. The BEFIMP period was characterised by very low prolactin concentrations, but they increased significantly to GEST1/3 ($P<0·05$). After a slight decrease during GEST2/3, concentrations increased again in GEST3/3. The highest concentration was recorded during LACTW1, and then lower concentrations were recorded during LACTW3 ($P<0·05$) and LACTW4 (NS) (Fig. 3 (d)).

Glucose. Plasma glucose showed a pattern similar to that of insulin, with declining concentrations from BEFIMP to late true gestation ($P<0·05$). Indeed, during LACTW1 concentrations even lower than during GEST3/3 were recorded ($P<0·05$), but then concentrations increased and had reached BEFIMP levels by LACTW4 although insulin concentrations remained low (data not shown). Correlations between glucose and leptin and insulin, respectively, were not significant.

Fructosamine. Plasma concentrations of fructosamine were not significantly affected by stage of gestation or

lactation, and they showed no general conclusive pattern (data not shown).

Discussion

The present data have clearly demonstrated the occurrence of elevated plasma concentrations of leptin during GEST2/3 in mink. The magnitude of the increase was slightly higher than that reported for human subjects (Hardie *et al.* 1997; Highman *et al.* 1998; Schubring *et al.* 1998; Sivan *et al.* 1998), about the same as in rats (Kawai *et al.* 1997; Amico *et al.* 1998) but considerably lower than in mice (Gavrilova *et al.* 1997; Tomimatsu *et al.* 1997). The pattern in mink does not concur completely with findings in sheep, in which high leptin concentrations have been recorded in mid- (Ehrhardt *et al.* 2001) but not in late pregnancy (Ehrhardt *et al.* 2001; M Tygesen, A-H Tauson, MO Nielsen, D Blache, T Jensen, K Kortegaard, AB Jensen, P Nørgaard and H Raring, unpublished results).

High leptin concentrations could be caused by a high food intake and increasing adiposity as well as by high insulin concentrations as would be expected in non-pregnant animals (mink, Tauson & Forsberg, 2002; other species, Baile *et al.* 2000; Havel, 2000; Schwartz *et al.* 2000; Frübeck, 2001). This was certainly not the case in the present study, because the animals, despite free access to food, decreased their food intake during GEST2/3, resulting in a negative energy balance, fat mobilisation and decreasing LW during the late part of gestation. This is also contrary to findings in other species, in which gestational hyperleptinaemia is not accompanied by a decrease in food intake and mobilisation of body fat (human subjects, Butte *et al.* 1997; Highman *et al.* 1998; Schubring *et al.* 1998). Gestational hyperleptinaemia may be caused partly by the secretion of placenta-derived leptin (human subjects, Masuzaki *et al.* 1997; Señarís *et al.* 1997; baboons, Henson *et al.* 1999; rats, Amico *et al.* 1998; García *et al.* 2000), a possibility that cannot be excluded in the present experiment. Also, a mechanism similar to that in mice, where the gestational hyperleptinaemia is attributed to the binding of leptin to the soluble form of the leptin receptor secreted from the placenta (Gavrilova *et al.* 1997), could be possible in the mink. Another cause of high leptin concentrations during pregnancy could be the secretion of hormones that may stimulate leptin secretion. Among these are oestrogens and insulin. We previously demonstrated a high correlation between plasma insulin and leptin in non-pregnant mink dams and during GEST1/3 (r 0·75, $P<0·001$; Tauson & Forsberg, 2002). However, because insulin concentrations declined in late gestation when food intake was low, which is in accordance with previous findings in mink demonstrating a close relationship between food intake and plasma insulin (Fink & Tauson, 1998; Fink *et al.* 1998; Tauson *et al.* 2000), the hyperleptinaemia found in the present study could not be explained by the stimulation by high insulin concentrations. Oestrogen seems more likely to have exerted stimulation because high concentrations of oestradiol-17β occur during the late part of gestation in mink (Tauson, 1991).

Elevated leptin concentrations were recorded after the period of high food intake and positive energy balance at IMP and during GEST1/3, and concentrations remained high during the period with low food intake, negative energy balance and high rate of fat oxidation. Indeed, in gestation there were significant negative correlations between leptin and ME intake as well as leptin and retained energy. If the increase in leptin had been a response to normal feedback mechanisms, a decline in response to the suppression of food intake during the last 20 d of gestation would have been expected. These findings and the lack of a correlation between HE and leptin are in conflict with the normal regulation of leptin in response to food intake and adiposity, and they indicate that leptin is regulated differently in pregnant mink than in non-pregnant animals.

Thyroid hormone concentrations declined during the last 20 d of true gestation, which probably reflected the low food intake and the negative energy balance. However, there is evidence that the thyroid axis may influence leptin secretion and metabolism, but it seems that the mechanisms may differ between species. Hypothyroid rats have increased leptin concentrations, but the infusion of thyroid hormones exerts an inhibitory effect on leptin concentrations (Escobar-Morreale *et al.* 1997; Leonhardt *et al.* 1999). This effect was maintained over a wide range of thyroid hormone levels representing states from severe hypothyroidism to hyperthyroidism, and supply of both exogenous triiodothyronine (T_3) and T_4 had inhibitory effects (Escobar-Morreale *et al.* 1997). Furthermore, there is evidence that the effects of leptin and T_3 on oxidative metabolism in rats are additive and independent of thyroid status, as demonstrated by the normalisation of O_2 consumption and CO_2 production in thyroidectomised rats after treatment with T_3 or leptin and treatment with both T_3 and leptin giving an additive response (Wang *et al.* 2000). Our finding of a weak negative correlation between TT_4 and FT_4 and leptin hence concurs with the rat results, whereas the lack of a relationship between TT_3 and leptin is not in full agreement with Escobar-Morreale *et al.* (1997). On the other hand, also with a substantial increase in plasma leptin by means of peripheral infusion, plasma concentrations of TT_4 remained stable in well-fed lambs (Morrison *et al.* 2002).

The main part of fetal growth occurs very late in gestation in mink (Tauson *et al.* 1991, 1994), and therefore these results imply that a significant part of the accretion in fetal tissues was derived from nutrients mobilised from the body of the dams. N retention was positive throughout gestation, so the main contribution was made up by fat mobilisation. In other species (rats, Naismith & Morgan, 1976; Pine *et al.* 1994; sheep, McNeill *et al.* 1997) there may be a substantial accretion of labile protein reserves during pregnancy. These reserves may be used if the protein supply is insufficient in late gestation or may provide an important contribution for sustaining milk production in early lactation. Because N retention in early gestation in the present study was far higher than what could be expected for retention in fetal tissue (Tauson *et al.* 1991, 1994), these data suggest that the pregnant mink dam accretes a labile protein reserve. The oxidation of protein reflected the increasing protein requirement with progressing stage of gestation by

decreasing values; from 48 % (IMP) to the lowest level of 25 % of HE (GEST3/3). These values are considerably higher than 11–13 % of HE as reported for pregnant sows (Theil *et al.* 2002). Also, the relatively low utilisation of digested N for retention showed that the protein supply was clearly above the requirement throughout the gestation period.

Leptin has been suggested to be an important growth factor for the fetus (Mostyn *et al.* 2001), but the possible mechanisms remain unclear. Positive correlations between cord-blood leptin concentrations and the birth weight of infants have been reported in some studies (Harigaya *et al.* 1997; Koistinen *et al.* 1997), but Schubring *et al.* (1998) found no correlation between maternal leptin concentration, cord-blood concentration and birth weight. In an adolescent sheep pregnancy model there was, however, a clear negative relationship between leptin in the maternal circulation and lamb birth weight (Thomas *et al.* 2001). In this investigation the maternal hyperleptinaemia was recorded during the period of most intensive fetal growth so a growth-promoting role for the mink fetus cannot be excluded, but as the origin of the circulating leptin is not known this remains a speculation.

Similar to those in other species (human subjects, Hardie *et al.* 1997; Schubring *et al.* 1998; Sivan *et al.* 1998; rat, Kawai *et al.* 1997; Amico *et al.* 1998; Brogan *et al.* 1999; Woodside *et al.* 2000), plasma leptin concentrations declined shortly after parturition and remained low. This is in agreement with findings in sheep (Ehrhardt *et al.* 2001; M Tygesen, A-H Tauson, MO Nielsen, D Blache, T Jensen, K Kortegaard, AB Jensen, P Nørgaard and H Raring, unpublished results) and cattle (Block *et al.* 2001). The reason for the decline in leptin in lactation is probably the high energetic demands for maintaining a high milk yield. Hence, it has been demonstrated in rats (Brogan *et al.* 1999; Woodside *et al.* 2000) and cattle (Block *et al.* 2001) that if the energetic costs of lactation are eliminated, plasma leptin concentrations increase. Low plasma concentrations in early lactation may also have a beneficial effect in being permissive for lactational hyperphagia. ME intake increased by 3·5 times from GEST3/3 to LACTW4. Despite this, the dams were unable to sustain their energy requirement by food intake alone, as shown by declining LW. The use of data from Fink *et al.* (2001) on milk yield and energy output in milk in a mink dam suckling six kits confirmed that a substantial mobilisation of body reserves was necessary to nurture the kits in this investigation. Another factor that might have contributed to the low plasma leptin concentrations during lactation was the increasing concentrations of TT_4 and FT_4, which could exert an inhibitory effect on leptin (Escobar-Morreale *et al.* 1997; Leonhardt *et al.* 1999).

The patterns of food intake and energy balance were generally reflected in the plasma concentrations of IGF-1, which in turn were not clearly related to concentrations of leptin. Similarly, in periparturient dairy cows, plasma IGF-1 concentrations were depressed during periods of negative energy balance (Block *et al.* 2001). Exogenous leptin supply did not cause the modification of IGF-1 release in sheep on reduced nutrition (Nagatani *et al.* 2000) or in the well-fed state (Morrison *et al.* 2002), so collectively these data indicate that the interaction between nutritional status, leptin and IGF-1 is not clearly elucidated.

Reproduction in mink is highly dependent on photoperiod. Prolactin, which is luteotrophic in mink (Papke *et al.* 1980), starts to rise over basal concentrations about the time of the vernal equinox, and it might be permissive for the increase in food intake in the period of IMP because in mink dams that were not mated prolactin concentrations remained at basal levels, food intake was low and animals lost weight (Tauson, 1997). Conversely, if exogenous prolactin implants were given to females that were not mated, food intake approached the level in mated females (Tauson *et al.* 1999). Here the prolactin concentrations started to rise before those of leptin. In rats, prolactin may stimulate leptin synthesis (Gualillo *et al.* 1999), so it can be speculated that the photoperiod-dependent increase in plasma prolactin contributes to the induction of gestational hyperleptinaemia in the mink.

Conclusions

Hyperleptinaemia during the last 20 d of gestation in the mink has been demonstrated. This state occurred concomitantly with a low intake of ME, negative energy balance, high rate of fat oxidation and loss of body weight, suggesting that leptin may exert anorexigenic effects in pregnant mink. Decreasing thyroid hormone concentrations may have been permissive for the increase in leptin, and prolactin may also have stimulated leptin synthesis. Plasma leptin concentrations declined rapidly after parturition and remained low allowing for lactational hyperphagia. Despite a 3·5-fold increase in ME intake from the very last part of gestation until LACTW4, dams were unable to sustain their energy requirement solely by food intake and therefore mobilised body reserves, which was reflected in decreasing body weights. The suppression of food intake in late gestation, possibly caused by high circulating leptin concentrations, might be a permissive mechanism for a rapid increase in food intake after parturition. This may be a suitable strategy for an animal that gives birth to altricial young. Such an animal has a moderate nutrient and energy requirement for deposition in fetal tissues, but the lactation period imposes very high energetic demands owing to large litters of rapidly growing young which are completely dependent on their mother's milk for nourishment during the first 4 weeks of life.

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