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Original article

LH release in mink (*Mustela vison*). Pattern of the LH surge and effect of metabolic status

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Abstract — The mink is a seasonal breeder with induced ovulation and delayed implantation. Reproductive processes are strongly influenced by energy supply and body condition. Items for which there is paucity or complete lack of data were the main objectives of this study: the temporal relationship between copulation and the pre-ovulatory LH surge and the influence of energy supply on LH release. A total of 30 yearling female mink with a well defined metabolic status was used. Twelve females kept in the laboratory were measured in six consecutive one-week balance periods each including the measurement of heat production by means of indirect calorimetry, and 18 females were kept under conventional farm conditions. The animals were fed so as to maintain energy balance (CON), flush fed by 2 weeks food restriction followed by 2 weeks refeeding (FLUSH), or kept in a negative energy balance (NEG). Plasma concentrations of the thyroid hormones, IGF-1 and insulin were determined weekly (n = 12), or 1 week after change in energy supply to the FLUSH group (n = 18). On the day of mating, blood samples for LH and oestradiol-17β (E_2) were taken before and immediately after mating and then 4, 8, 12, 24, 30 and 48 h thereafter. Frequent blood samplings, each lasting 60 min, were taken during the LH surge from two other females surgically fitted with venous access ports. Peak concentrations of LH were recorded on the first sampling, an average 16 min after mating. The concentrations remained elevated for 12 h, but almost decreased to basal values 24 h after mating. Plasma E_2 was high before mating and peak values were attained 4 h after mating after which it decreased. Energy supply had no significant influence on LH and E_2, but there was a tendency for a more sluggish LH release in NEG animals. The lack of response in FLUSH animals was explained by these animals having a lower intake of metabolisable energy than CON animals, the total intake not being significantly different from the NEG group. Plasma concentrations of thyroid

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hormones, IGF-1 and insulin were not significantly affected by the treatment, but in FLUSH animals the values mirrored energy supply, and in the NEG group, the values tended to decrease during the course of the experiment. It was concluded that the pre-ovulatory LH surge is an immediate response to mating, and that reproductive activity in the mink is maintained over a wide range of energy supply and body condition.

mink / energy supply / body condition / luteinizing hormone / oestradiol / metabolic hormones / heat production / substrate oxidation

Résumé — Décharge de LH chez le vison (*Mustela vison*). Effet de l'état métabolique. Le vison est un animal à ovulation induite et implantation différée dont la reproduction saisonnière est fortement influencée par l'apport énergétique et la condition corporelle. Parmi les points mal connus qui font l'objet de cette étude, il y a les relations temporelles entre la copulation et la décharge préovulatoire de LH et l'influence de l'apport énergétique sur sa sécrétion. Les 30 femelles utilisées sont âgées de un an environ et connues pour leur état métabolique. Douze d'entre elles, entretenues au laboratoire, ont été suivies pendant 6 semaines successives avec une mesure hebdomadaire de production de chaleur par calorimétrie indirecte. Les 18 autres ont été gardées dans des conditions habituelles d'élevage. Les animaux, répartis en trois lots, ont été nourris de façon à maintenir leur équilibre énergétique (lot CON), ils ont subi une restriction alimentaire pendant 2 semaines suivies par deux semaines de ré-alimentation (lot FLUSH) où ils ont été gardés en équilibre énergétique négatif (lot NEG). Des prélèvements sanguins hebdomadaires ou une semaine après le changement de régime dans le lot FLUSH ont été faits pour détermination les hormones thyroïdiennes, IGF-1 et l'insuline. Des échantillons sanguins pour LH et l'œstradiol 17β (E_2) ont été pris avant la saillie puis rapidement ensuite et 4, 8, 12, 24, 30 et 48 h plus tard. Des prélèvements plus fréquents ont enfin été pris sur deux femelles ayant un cathéter placé lors d'une intervention chirurgicale. Le pic de LH a été observé en moyenne 16 min après la saillie. Les concentrations de LH sont restées élevées pendant 12 h puis elles sont retournées au niveau de base 24 h après la saillie. Les concentrations plasmatiques de E_2 hautes avant saillie sont les plus élevées 4 h après, puis elles ont diminué. L'apport énergétique n'a pas eu d'influence significative sur LH et E_2 malgré une tendance à ce que la sécrétion de LH soit plus lente dans le groupe NEG. L'absence de réponse des animaux du lot FLUSH peut être expliquée parce qu'ils ont eu une consommation en énergie métabolisable plus faible que celle des animaux témoins (lot CON) mais une consommation totale équivalente à celle des animaux du lot NEG. Les concentrations en hormones thyroïdiennes, en IGF-1 et en insuline n'ont pas été affectées par les traitements mais dans le lot FLUSH elles ont reflété l'apport en énergie et dans le lot NEG, elles ont eu tendance à décroître au cours de l'expérience. En conclusion, la décharge ovulante est une réponse immédiate qui suit la saillie et l'activité reproductrice du vison femelle est maintenue sur une large gamme d'apport énergétique et de condition corporelle.

vison / apport énergétique / condition corporelle / hormone lutéinisante / œstradiol 17β / hormones métaboliques / production de chaleur / oxidation de substrat

1. INTRODUCTION

The metabolic status of an animal can exert a profound influence on reproductive performance, but the regulatory processes are not known in detail. Among substances involved in signalling the animal's metabolic status to the brain are glucose, insulin, thyroid hormones and IGF-1, and, as recently discovered, leptin. Besides a direct effect on the hypothalamus, at least glucose, insulin and IGF-1 can modify the reproductive response at various sites on the hypothalamus-pituitary-gonadal axis. Since LH release is very sensitive to changes in metabolic status, this hormone is often used to monitor effects of energy and nutrient supply on the reproductive axis. It has been shown in several species that severely restricted feeding will lead to anoestrus

caused by cessation of the pulsatile release of LH. LH pulsing will, however, resume rapidly in response to refeeding (for review see [23]).

The mink is a strict carnivore and a seasonal breeder with one annual breeding season extending from early to late March in the Northern hemisphere. Ovulation is induced by mating, and does not start earlier than 28 h [21], and usually occurs 36 to 72 h after mating [50]. The pre-ovulatory LH surge is poorly described: after GnRH injection, an elevated plasma LH concentration was recorded within 15 min with peak concentrations recorded after 45 min, and the duration of the peak ranging from 60 to 90 min [29]. In response to natural mating, and based on a low frequency sampling programme, elevated LH within a few hours after mating were reported, lasting at least 24 h [42]. Pulsatile LH release has only been described for males in various stages of reproductive activity or quiescence [24].

Implantation in the mink is delayed. During the embryonic diapause, the corpus luteum (CL) remains inactive, secreting very little progesterone [28]. Implantation is triggered by increasing daylength. The CLs are then activated under the influence of prolactin which (together with an unknown factor [31]) is luteotrophic in the mink [33], and induces the CLs to secrete large amounts of progesterone [30]. The length of true gestation is 30 ± 3 days, and the average total length of gestation is 52 days [44].

The mink has clear annual cycles of weight change with live weights increasing during the autumn and then decreasing throughout the winter and spring [20]. Food intake varies accordingly, and it seems that endocrine mechanisms regulating food intake are closely related to the reproductive status of the animals, and include melatonin, prolactin and IGF-1 [47]. Energy supply and body condition influence the reproductive processes to a great extent [41], and by a flush feeding regimen comprising a slight reduction in energy supply for two weeks followed by ad libitum refeeding from 4–5 day before the initiation of the breeding season and lasting until mating was completed, reproductive performance may be substantially improved, especially in yearling females [38, 39]. Hence, flush feeding has been shown to improve ovulation rate [38, 40, 45], influence progesterone profiles [45], plasma oestradiol concentrations [40] and enhance early embryonic development [43]. Moreover, limited data have indicated that flush feeding may result in a more synchronised LH release and in more distinct peaks [42], but here, sampling frequency was too limited to allow for an un-ambiguous description of the LH release pattern. Also metabolic traits such as plasma concentrations of insulin and thyroid hormones [17] as well as IGF-1 [18] have been shown to be affected by energy supply prior to the breeding season, but the response has not been related to reproductive endocrinology.

Because of the limited knowledge on the LH release pattern and connections between reproduction and quantitative energy metabolism in the mink, the present study had three main objectives. The first was to describe the pattern of the pre-ovulatory LH surge after natural mating. Secondly, because mink fitted with permanent catheters usually cannot move around freely and thereby cannot be naturally mated, we evaluated whether vascular access ports were a feasible tool to allow for normal living conditions, including natural mating, and frequent blood sampling. If so, we intended to evaluate if any fluctuations in LH secretion during the surge could be recorded. The third objective was to evaluate the influence of different planes of energy supply prior to the breeding season on the pattern of the LH release in animals with a clearly described metabolic status. In order to define the metabolic status of the animals, balance and respiration experiments, as well as blood sampling for some hormones and metabolites were included in the experimental design.

2. MATERIALS AND METHODS

2.1. Animals and housing

A total of 30 one-year-old mink females of the standard black (Scanblack) colour type [32] were used in the main experiment. They were weighed and allocated to a control group (CON; kept in energy balance), a flushed group (FLUSH) and a third group kept in a negative energy balance (NEG) in late January. Twelve of the 30 females were transferred from our experimental farm to the laboratory where they were kept under natural daylight conditions (55 °N, 12 °E) in individual metabolism cages designed as described in [25] and with devices for feeding and drinking water and equipped for quantitative collection of food residue, faeces and urine. After a one-week adaptation period, six consecutive one-week balance periods followed (Period 1–6), lasting from 6 February until 20 March, and each including a 22 h respiration experiment by means of indirect calorimetry in an open-air circulation system. The remaining 18 females were kept under conventional farm conditions in cages with nest-boxes in a non-insulated 4-row shed. Another two females were used for sequential blood sampling (see *Vascular access ports as a tool for frequent blood sampling in mink*).

2.2. Diet and daily food supply

The food was purchased from a commercial mink food kitchen (Stårup fodercentral, Højby Sjælland) on a single occasion and weighed out into daily portions in plastic bags and frozen immediately. Food was taken out of the freezer the day before use and was thawed overnight. The analysed chemical composition of the diet was dry matter (DM) 307 $g \cdot kg^{-1}$, ash 111 $g \cdot kg^{-1}$ DM, crude protein 567 $g \cdot kg^{-1}$ DM, fat 157 $g \cdot kg^{-1}$ DM and gross energy 22.6 MJ $g \cdot kg^{-1}$ DM, and the carbohydrates calculated by the difference was 165 $g \cdot kg^{-1}$ DM. The daily food supply and the calculated supply of metabolisable energy (ME) achieved by the different food supplies are presented in Table I. The energy supply to the CON group was chosen in order to guarantee that the females could be kept in energy balance, and the supplies of 450 kJ $ME \cdot d^{-1}$ (FLUSH during restriction) and 525 kJ $ME \cdot d^{-1}$ (NEG) were supposed to be clearly (85% of) respectively slightly below the energy requirement for maintenance [12]. Food residues were collected from individual animals once daily and the daily food consumption was calculated.

2.3. Balance and respiration experiments

Quantitative collection of food residues and excreta were carried out once a day between 08.30 and 12.00 h, and the total amount from each period was stored at –18 °C until analysis. 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, nitrogen (N) was determined by the micro-Kjeldahl technique using the Tecator-Kjeltec system 1030 (Tecator AB, Höganäs, Sweden), crude protein (CP) was calculated as $N \times 6.25$, fat was determined after HCl hydrolysis and gross energy (GE) by use of an adiabatic bomb calorimeter. Carbohydrates (CHO) were calculated by the difference. Urine was analysed for GE and N. ME was calculated from the difference as ME = GE – energy in the faeces (FE) – energy in the urine (UE).

Each balance period included a 22 h respiration experiment by means of indirect calorimetry in an open-air circulation system. The respiration chamber (760 L) was designed so as to permit direct insertion of the metabolism cage into the chamber. In the respiration chamber, temperature and relative humidity were kept at 15 to 18 °C and 0.65 to 0.75, respectively. Heat production (HE) was calculated from

Table I. Daily food supply and calculated daily supply of metabolisable energy (ME) of female mink fed in order to maintain an energy balance (CON), flush fed (FLUSH) or kept in a negative energy balance (NEG) prior to the breeding season.

	CON	FLUSH	NEG
No. of animals			
In the laboratory	4	4	4
Conventional farm conditions	6	6	6
Food supply, g·day $^{-1}$			
Period 1	200	200	125
Periods 2–3	200	100	125
Periods 4–5	200	300	125
Period 6	200	200	125
Calculated ME supply, kJ·day $^{-1}$			
Period 1	850	850	525
Periods 2–3	850	425	525
Periods 4–5	850	1 275	525
Period 6	850	850	525

O_2 consumption, CO_2 production and urinary nitrogen (UN) according to [9]:

$$\text{HE, kJ} = (16.18 \times O_2, \text{L}) + (5.02 \times CO_2, \text{L}) - (5.99 \times \text{UN, g}) \quad (1)$$

Retained energy (RE) was calculated as ME–HE.

Quantitative oxidation of protein (OXP), fat (OXF) and carbohydrate (OXCHO) was calculated based on gas exchange measurements and UN as described and validated for pigs by [13]:

$$\text{OXP, kJ} = \text{UN, g} \times 6.25 \times 18.42 \quad (2)$$

$$\text{OXF, kJ} = (1.719 \times O_2, \text{L} - 1.719 \times CO_2, \text{L} - 1.963 \times \text{UN, g}) \times 39.76 \quad (3)$$

$$\text{OXCHO, kJ} = (-2.968 \times O_2, \text{L} + 4.174 \times CO_2, \text{L} - 2.446 \times \text{UN, g}) \times 17.58. \quad (4)$$

2.4. Weighing and blood sampling for metabolic hormones

The animals (n = 30) were weighed at weekly intervals starting when grouped on 25 January and continuing until one week after the termination of the balance and respiration experiments (27 March). For animals kept in the laboratory (n = 12) blood samples were taken at the end of each balance period but for the animals kept under normal farm conditions (n = 18), blood sampling was only performed at the start of the experiment and at the ends of Periods 1, 2 and 4, the two latter occasions corresponding to 1 week after changes in the food supply of the flushing group took place. During sampling the animals were affixed in a conventional mink trap and blood was withdrawn by puncture of Vena cephalica antebrachii [6]. 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 h to 11.30 h. Blood was collected in heparinised tubes, and the separated plasma was stored in plastic tubes at –18 °C until the assays.

2.5. Mating and blood sampling for LH and oestradiol-17β, reproductive performance

The animals kept under normal farm conditions (n = 18) were tried for mating on

14 March, and the animals used in the balance and respiration experiments (n = 12) after these experiments were concluded on 20 March. The females were presented to a male at approximately 09.30 h (14 March; n = 18) or at approximately 09.00 h (20 March; n = 12). Exact time was recorded, and then time when mating started and ended, respectively. Females who did not accept mating within a few minutes were transferred to another male. Females not accepting mating on the designated date were not retried for mating later. Hence, all results are derived from females mated once on a day they were previously allocated to.

Blood sampling as described above was performed immediately before the female was presented to the male, then as soon after completed mating as possible, and from then on 4, 8, 12, 24, 30 and 48 h after mating. At each sampling occasion the sample volume was kept at maximum of 1.5 mL in order to avoid unnecessary loss of blood. For simplicity the sampling times will be designated –1, 1, 4, 8, 12, 24, 30 and 48 h.

At parturition the date and number of live and stillborn kits were recorded. Eight females that had not given birth on 22 May were considered as barren and were killed. The uteri were examined for implantation sites, and the occurrence of corpora lutea (CL) in the ovaries was estimated with the naked eye.

2.6. Vascular access ports as a tool for frequent blood sampling in mink

For this part of the study, two females (No. 123 and No. 131) were used. They had previously been kept under conventional farm conditions and fed according to common farm routines. They were surgically equipped with vascular access ports on 15 March according to the following procedure: Short-term anaesthesia was induced by intramuscular injection of ketamine (Ketaminol Vet®, 50 mg·kg live weight $(LW)^{-1}$) and midazolam (Dormicum®, 2 mg·kg LW^{-1}), the fur on the dorso-lateral and ventral surfaces of the neck was clipped and the region was prepared for surgery as previously described [54]. A sterile vascular access port (Preclinical Mini-Port™, Pharmacia Delta Inc. St.-Paul, Minnesota, USA) was implanted subcutaneously in the dorsolateral region of the neck and fixed with 3–0 silk sutures. The catheter was filled with isotonic saline (9 g·L^{-1}) containing heparine (100 IU·mL^{-1}) and tunneled into sulcus jugularis using a sterilised trochar and inserted into the ipsilateral carotid artery. The wounds were covered with chloramine dusting powder and on recovery the animals were subcutaneously given an analgetic injection of buprenorphine (Anorfin® GEA, Copenhagen) (20 µg·kg LW^{-1}). The animals were kept in metabolism cages in the laboratory after surgery and recovered quickly, ate normally within 2 days and no adverse effects were observed. Wound healing occurred within a few days.

On 20 March the two females were presented to males at ca 11.45 h. Blood sampling by use of a huber-point needle was carried out immediately before the females were exposed to the males and when matings were completed. Sequential blood sampling started 215 (No. 131) and 330 (No. 123) min after the end of mating. The animals were affixed in a conventional mink trap and a huber-point needle was used to penetrate the membrane of the vascular port. After each sampling, the catheter was flushed with isotonic heparine-saline in an amount corresponding to the withdrawn blood volume. Samplings continued during 1 h, and were first performed at 15, 10 and 5 min intervals, and during a 10 min period at 1 min intervals. The separated plasma was analysed for LH and albumin (see below).

2.7. 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 [4].

2.8. Hormone and metabolite assays

LH: The LH assay used an antiserum (100 µL) against ovine LH (oLH) (GDN No. 15) at a final dilution of 1:60 000, 100 µL radioiodinated oLH tracer (AFP-5551B), and 100 µL canine LH (cLH) as standard (LER1685-1) or sample. The standards were diluted in PBS buffer (0.1% BSA). The standards and samples were pre-incubated with antibody for 2 h before adding the tracer. Following overnight incubation at room temperature, 1 mL of a second antibody suspension (Decanting Suspension 3, Pharmacia/Upjohn, Uppsala, Sweden) was added to each tube. After an additional 30 min at room temperature the tubes were centrifuged at 1500 × *g* and radioactivity was quantified in a gamma counter (Searle Analytic Inc., IL, USA). The iodination (carrier-free ^{125}I, Amersham International plc, Buckinghamshire, England) was performed by the IODO-GEN method with some modifications [11] using pre-coated IODO-GEN tubes (Pierce, Rockford, IL, USA) and 10 µg of protein and an exposure time of 9 min. The antiserum bound 29% of the ^{125}I-oLH, and the cLH standard curve ranged from 0.25 to 64 µg·L^{-1}. Dilutions of mink plasma with high concentrations of LH were parallel to the cLH standard. The intra-assay coefficients of variation, calculated from three control samples, were 18% (1.5 µg·L^{-1}), 6% (6.9 µg·L^{-1}) and 2% (15.0 µg·L^{-1}). The corresponding inter-assay coefficients of variation were 15%, 8% and 9%.

Oestradiol-17β: Oestradiol-17β was determined using a RIA previously validated for use in bovine plasma [36], with the following modifications: the standard curve was prepared with standards supplied with the radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, CA, USA) and extraction was performed with diethylether on 100 µL of mink plasma. Serially diluted plasma from mink containing high concentrations of oestradiol-17β produced displacement curves parallel to the standard curve. The intra-assay coefficients of variation calculated from the precision profile of two assays were 22% at 5.6 pmol·L^{-1}, 14% at 11.3 pmol·L^{-1} and below 10% between 22.5 and 180 pmol·L^{-1}. The inter-assay coefficients of variation for two control samples were 6% (42 pmol·L^{-1}) and 7% (77 pmol·L^{-1}). The lowest detectable amount of oestradiol-17β (defined as the intercept of maximal binding – 2 SD) was 3 pmol·L^{-1}.

Thyroid hormones: Plasma concentrations of total triiodythyronine (TT_3), total and free thyroxine (TT_4 and FT_4) were analysed by 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 coefficients of variation for TT_3 were below 10% for all concentrations, and the corresponding inter-assay coefficients of variation were 21.8% for samples with low concentration (mean = 1.1 nmol·L^{-1}) and 10.2% (mean = 3.0 nmol·L$^{-1)}$ and 8.0% (mean = 6.1 nmol·L^{-1}) for samples with medium and high concentrations, respectively. For TT_4 the intra-assay coefficients of variation were 14.3% for samples with low concentration (mean = 6.0 nmol·L^{-1}), 6.0% (mean = 20.6 nmol·L^{-1}) for medium concentrations and 4.1% (mean = 73.4 nmol·L^{-1}) for samples with high concentration, and the inter-assay coefficients of variation were 26.1% for low, 3.6% for medium and 5.0% for high concentrations, respectively. For FT_4, finally, intra-assay coefficients of variation were below 10% for all concentrations, and the corresponding inter-assay coefficients of variation were 18.8% for samples with low concentration (mean = 12.2 pmol·L^{-1}) and below 10% for samples with medium (mean = 21.9 pmol·L^{-1}) and high (mean = 41.9 pmol·L^{-1}) concentrations, respectively.

IGF-1: Plasma concentration of IGF-1 was determined by radio immunoassay

according to the manufacturers recommendations (IGF-1, cat. No. 53065, Incstar Corporation, Stillwater, MI, USA). Plasma was extracted with ODS-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 coefficient of variation, calculated from the precision profiles of five assays, was below 12% for IGF-1 concentrations between 2.9–82.5 nmol·L^{-1}. The inter-assay coefficients of variation for two control samples were 19% (mean = 10 nmol·L^{-1}) and 21% (mean = 28 nmol·L^{-1}). The minimum detectable level of IGF-1 was set to 2 nmol·L^{-1} (average 10% fall from '0'-binding of five assays).

Insulin: Plasma concentrations of insulin were determined by radio immunoassay (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 coefficients of variation for quality control samples were 5.4% (mean = 12 μU·L^{-1}), 5.3% (mean = 42 μU·L^{-1}) and 5.3% (mean = 117 μU·L^{-1}). The corresponding inter-assay coefficients of variation were 7.8, 2.2 and 6.7%.

Albumin: Albumin was measured in a computerised multichannel spectrophotometer (Cobas Mira, Hoffman-La Roche & Co, Switzerland) according to the BCG method (MPR 3 albumin, Boehringer Mannheim, Germany). The intra- and inter-assay coefficients of variation were 1.3% and 2.5 %, respectively.

2.9. Statistical analyses

Statistical analyses on animal live weights were carried out according to SAS GLM-procedure for repeated measures with the Greenhouse-Geisser correction [34]. Energy metabolism data and plasma concentrations of LH and oestradiol-17β during the pre-ovulatory surge, thyroid hormones, IGF-1 and insulin in the main experiment were evaluated with the MIXED procedure in SAS [27]. The fixed effects of treatment group and period (energy metabolism data, period-wise hormone concentrations) / sampling time (LH and oestradiol during the LH surge) and the related interaction effects were evaluated. Period / sampling time were used as repeated measures and the autoregressive order 1 covariance structure was fitted. The results are presented as least square means (LS-means) achieved according to the described models, and the significance of differences between LS-means was determined by testing with a comparison-wise error rate of 5%. When evaluating LH data from sequential blood sampling, base-line values were determined by calculating the mean and standard deviation for each set of samples. Values deviating more than two standard deviations from the mean were removed, and the base-line was defined as the mean of the remaining values. Values exceeding the base-line value with more than two standard deviations were considered significantly elevated.

3. RESULTS

3.1. Animal live weights

Animal live weights remained stable among CON animals, but decreased steadily in the NEG group until 13 March. The values were significantly below those of the CON group from 20 February onwards ($P < 0.01$). In the FLUSH group, LW reflected the varied energy supply with LW decreasing almost to the level in the NEG group during the 2-week restriction period and then returned to the level of the CON group during refeeding. During restriction, the values were significantly below those of the CON group ($P < 0.05$), but not significantly different from the NEG group (Fig. 1).

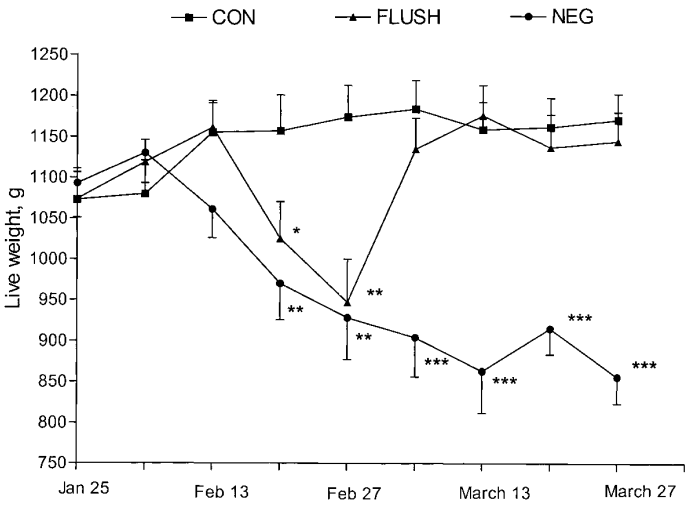

Figure 1. Animal live weights, g (mean and SEM) for female mink (n = 30) fed in order to maintain an energy balance (CON), flush fed by a two week period of moderate food restriction followed by ad libitum refeeding for two weeks (FLUSH), or kept in a negative energy balance (NEG). The animals were kept in the laboratory (n = 12) and under normal farm conditions (n = 18). Weighings were performed at weekly intervals from grouping on January 25 until one week after the end of balance period 6 (27 March). Significant differences between CON and FLUSH or NEG animals, respectively, are denoted by * ($P < 0.05$), ** ($P < 0.01$) or *** ($P < 0.001$).

3.2. Energy metabolism data

Intake of ME was significantly higher in the CON group than in the FLUSH and NEG groups which had a similar ME intake. FLUSH animals increased their ME intake substantially during the first week of refeeding, but during the second week their intake was below the average intake of the NEG group. Heat production was not significantly affected by treatment, but there was a tendency for NEG animals to have the highest HE. Energy retention was positive among CON animals, almost zero in the FLUSH group and clearly negative in the NEG group, with treatment effects being significant (Tab. II). The FLUSH group had a positive energy balance in Period 1 and in the first period of refeeding (Period 4), but in all other periods RE was negative (Tab. II).

The relative contribution to the total HE by oxidation of protein (OXP/HE) and fat (OXF/HE) were clearly affected by the experimental treatment, with low values (around 35%) for OXP/HE in the NEG group and during restriction in FLUSH animals. For CON animals and FLUSH animals in positive energy balance OXP/HE was between 47 and 62%. Fat oxidation was reverse to that of protein with the lowest recorded value of 12% of HE in the CON group during the Period 1, and an average of 25% of HE in CON animals. In FLUSH animals it ranged from 18% during the first week of refeeding to 45–50% in Periods 2–3 and 5–6. In NEG animals OXF made up about 50% of HE. The contribution from OXCHO to HE ranged from 11 to 26%, and was hence quantitatively of less importance than OXP and OXF (Tab. II).

3.3. Metabolic hormones

The experimental treatment had no significant effects on the concentrations of metabolic hormones in the animals kept in

Table II. Intake of metabolisable energy (ME), heat production (HE) and retained energy (RE), oxidation of protein (OXP), fat (OXF) and carbohydrate (OXCHO) in percent of HE in female mink (n = 12) fed to maintain an energy balance (CON), flush fed (FLUSH) or fed restrictedly (NEG) prior to the breeding season. Data are presented for the total experiments (Periods 1–6) and period-wise.

	Period							Pooled	P-value; effect of		
	1–6	1	2	3	4	5	6	SEM	T*	P*	T × P
ME, kJ·kg$^{-0.75}$								21.1	0.002	< 0.001	< 0.001
CON	804^{A}	934^{a}	910^{a}	910^{a}	751^{b}	748^{b}	574^{c}				
FLUSH	685^{B}	953^{a}	465^{b}	484^{b}	1020^{a}	595^{c}	593^{c}				
NEG	650^{B}	608	637	641	649	691	674				
HE, kJ·kg$^{-0.75}$								64.2	0.14	0.19	0.02
CON	658	654	672	675	696	650	602				
FLUSH	710	727^{abd}	614^{cd}	647^{d}	746^{ab}	719^{a}	808^{b}				
NEG	853	830	825	890	856	872	845				
RE, kJ·kg$^{-0.75}$								70.0	0.02	< 0.001	< 0.001
CON	157^{A}	301^{a}	258^{a}	253^{a}	71^{bc}	114^{b}	–54^{c}				
FLUSH	–10^{A}	246^{a}	–137^{b}	–151^{b}	297^{a}	–111^{b}	–205^{b}				
NEG	–190^{B}	–213	–174	–234	–193	–168	–157				
OXP/HE, %								3.21	0.008	< 0.001	< 0.001
CON	52.5^{A}	62.4^{a}	58.8^{ac}	51.6^{b}	46.8^{b}	48.5^{bc}	47.0^{bc}				
FLUSH	42.3^{A}	53.9^{a}	36.4^{b}	35.9^{b}	56.9^{a}	37.7^{b}	33.0^{b}				
NEG	33.6^{B}	33.5	34.1	33.1	34.6	32.1	34.5				
OXF/HE, %								4.09	0.007	< 0.001	< 0.001
CON	25.2^{A}	11.5^{c}	17.3^{bc}	21.7^{bc}	24.6^{bc}	30.3^{b}	45.9^{a}				
FLUSH	39.4^{B}	27.2^{b}	47.4^{a}	45.2^{a}	17.5^{b}	49.7^{a}	49.6^{a}				
NEG	49.9^{B}	53.7	50.0	50.7	47.1	50.5	47.1				
OXCHO/HE, %								1.56	0.04	0.02	0.37
CON	21.4^{A}	22.9^{ab}	20.8^{ab}	24.1^{ab}	26.2^{a}	18.6^{b}	15.8^{b}				
FLUSH	16.2^{B}	16.1^{ab}	14.3^{b}	17.1^{ab}	22.7^{a}	10.7^{b}	16.4^{ab}				
NEG	14.8^{B}	11.0	14.3	14.5	16.5	15.7	16.7				

* T: treatment group; P: period; T × P: interaction between treatment group and period.
$^{A, B}$ Values within trait in the column for Periods 1–6 that share no common superscript differ significantly (P < 0.05) between treatments.
$^{a, b, c, d}$ Values within a row that share no common superscript differ significantly (P < 0.05) between periods.

Table III. Plasma concentrations of total triiodothyronine (TT_3), total and free thyroxine (TT_4 and FT_4), IGF-1 and insulin in female mink in the laboratory (n = 12) fed to maintain an energy balance (CON), flush fed (FLUSH) or fed restrictedly (NEG) prior to the breeding season. Data are presented for the total experiment (Periods 1–6) and period-wise. Samplings were performed at the end of each period.

	Period							Pooled	*P*-value; effect of		
	1–6	1	2	3	4	5	6	SEM	T*	P*	T × P*
TT_3, nmol·L^{-1}								0.04	0.08	0.05	0.20
CON	0.57	0.53	0.58	0.64	0.65	0.51	0.73				
FLUSH	0.69	0.73^{ac}	0.51^{b}	0.55^{bc}	0.77^{a}	0.78^{ac}	0.81^{a}				
NEG	0.55	0.58^{ab}	0.48^{ab}	0.59^{ab}	0.64^{a}	0.40^{b}	0.57^{ab}				
TT_4, nmol·L^{-1}								0.76	0.10	0.001	0.06
CON	14.2	13.1	13.7	14.4	16.8	13.6	16.7				
FLUSH	16.7	18.4^{a}	13.9^{bc}	13.4^{b}	20.0^{a}	17.3^{ac}	17.4^{ab}				
NEG	16.2	15.2^{a}	12.2^{b}	16.0^{a}	16.4^{a}	16.7^{a}	19.5^{a}				
FT_4, nmol·L^{-1}								0.63	0.19	< 0.001	0.01
CON	21.0	20.7^{ab}	21.3^{ab}	21.3^{ab}	22.7^{a}	20.4^{b}	21.5^{ab}				
FLUSH	22.8	23.9^{a}	22.5^{ab}	21.1^{b}	24.4^{a}	22.1^{b}	22.4^{ab}				
NEG	21.7	21.1^{bc}	19.7^{b}	21.5^{ac}	22.1^{ac}	21.8^{b}	23.6^{a}				
IGF-1, nmol·L^{-1}								3.36	0.31	0.002	0.35
CON	35.9	24.8^{b}	34.6^{ab}	35.5^{ab}	53.2^{a}	39.6^{ab}	35.4^{ab}				
FLUSH	43.0	44.4^{b}	28.8^{b}	34.5^{b}	77.7^{a}	45.9^{b}	31.0^{b}				
NEG	36.8	36.0	39.2	41.0	43.9	32.1	28.1				
Insulin, μU·L^{-1}								1.50	0.86	0.53	0.36
CON	7.6	8.7	9.8	9.5	7.0	6.3	5.4				
FLUSH	8.6	11.2	7.4	7.1	8.6	7.0	7.2				
NEG	8.6	8.5	10.1	7.8	8.5	8.7	6.5				

* T: treatment group; P: period; T × P: interaction between treatment group and period.
[a, b, c] Values within a row that share no common superscript differ significantly ($P < 0.05$) between periods.

the laboratory (n = 12), but there were significant period effects for TT_3, TT_4, FT_4 and IGF-1 as well as significant interactions between treatment and period for TT_4 and FT_4. Concentrations of TT_3, TT_4 and IGF-1 clearly reflected the food restriction and refeeding periods among FLUSH animals (Tab. III).

Similarly, when evaluated for all females (n = 30) the concentrations of metabolic hormones were not significantly affected by treatment, but there were significant interactions between treatment and day of sampling ($P < 0.05$) for TT_3, TT_4, IGF-1 and insulin. Total triiodo-thyronine, TT_4 and insulin were significantly affected by the day of sampling (P = 0.03, $P < 0.001$ and P = 0.002, respectively); their concentrations decreased during restriction and increased during refeeding among FLUSH animals. In the NEG group, hormone concentrations decreased during the course of the experiment, whereas they remained almost stable in the CON group (data not shown).

3.4. Mating

Among females tried for mating on 14 March, one female belonging to the NEG group did not mate. Another female (FLUSH) had a very short mating, lasting only 10 min, and since LH remained on basal concentrations throughout the sampling period this female was excluded from the calculations. On 20 March, two females, one from both the FLUSH and NEG groups, failed to mate. Hence, the results are based on 10, 8 and 8 females in the CON, FLUSH and NEG groups, respectively. The time between when the female was first exposed to the male and when mating commenced ranged from 6 to 50 min, with NEG females being significantly (P = 0.02) more reluctant to start mating (mean 23 min, SEM 4.9) than the CON (mean 11 min, SEM 1.4) and FLUSH females (mean 14 min, SEM 1.7). Matings lasted an average 33 (SEM 6.2), 36 (SEM 8.2) and 46 (SEM 5.3) min in the CON, FLUSH and NEG groups, respectively; the differences between the treatments were non-significant.

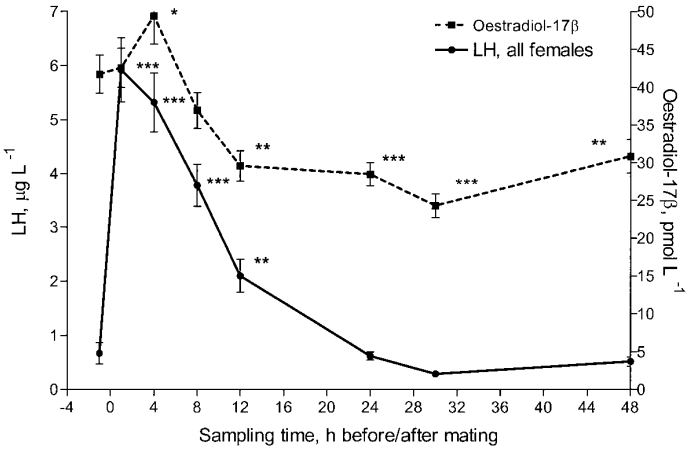

Figure 2. Plasma concentrations of luteinising hormone (LH) and oestradiol-17β (mean and SEM) of female mink (n = 30) immediately before and after mating and 4, 8, 12, 24, 30 and 48 h after mating. The animals were kept in the laboratory (n = 12) and under normal farm conditions (n = 18). Values differing significantly from those found immediately before mating are denoted by * ($P < 0.05$), ** ($P < 0.01$) or *** ($P < 0.001$).

3.5. Pre-ovulatory LH surge

Date of mating had no significant effect on LH concentrations, and therefore data from animals sampled 14 and 20 March are reported together.

All females: On the blood sampling preceding mating concentrations of LH were basal (below 0.1 $\mu g \cdot L^{-1}$). The next sampling was performed an average 16 min after mating was completed, and LH concentrations peaked at this sampling. Concentrations remained high on the sampling 4 h after mating and then declined, but still 12 h after mating LH was significantly above ($P = 0.005$) the pre-mating concentrations. LH had almost returned to basal concentrations 24 h after the end of mating and it remained at this low level during the following 24 h (Fig. 2).

Treatment effects: The food supply did not affect plasma LH concentrations significantly ($P = 0.15$), but there was a tendency for the peak to occur later in the NEG group, and for the decline to start earlier in the FLUSH group. All animals had reached basal concentrations 24 h after the end of mating (Fig. 3). One female in the NEG group with a LW of only 587 g did not, despite a mating lasting 54 min, exhibit any rise in LH over basal concentration.

3.6. Oestradiol-17β

Neither treatment nor mating date had significant effects on plasma oestradiol-17β concentrations. Oestradiol data are therefore only reported in relation to sampling time which had a highly significant ($P < 0.001$) effect on concentrations. Concentrations were rather high on the two first samplings (before and immediately after mating), and then increased to reach a peak 4 h after mating. The peak was not very pronounced, but peak concentrations differed significantly ($P < 0.05$) from all other sampling occasions. After a post-mating decline, concentrations had risen slightly on the sampling 48 h post mating ($P = 0.03$) (Fig. 2).

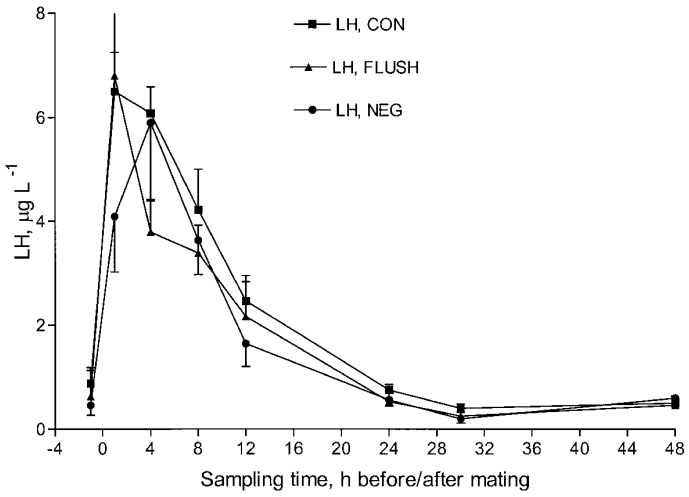

Figure 3. Plasma concentrations of luteinising hormone (LH) (mean and SEM) of female mink ($n = 30$) fed in order to maintain an energy balance (CON), flush fed by a two week period of moderate food restriction followed by ad libitum refeeding for two weeks (FLUSH) or kept in a negative energy balance (NEG). The animals were kept in the laboratory ($n = 12$) and under normal farm conditions ($n = 18$) and were blood sampled immediately before and after mating and 4, 8, 12, 24, 30 and 48 h after mating.

3.7. Frequent blood sampling during the pre-ovulatory LH surge

Of the two females fitted with vascular access ports, one (female 131) accepted intromission about 20 min after she was exposed to the male, whereas the other one (female 123) started mating 90 min after she was first presented to a male. Intromission lasted 60 min for female 131, and at sampling after it was completed, LH was 6.40 $\mu g \cdot L^{-1}$. Female 123 had a shorter mating of about 30 min, and on sampling after mating her plasma LH concentration was 4.9 $\mu g \cdot L^{-1}$ (Fig. 4).

All plasma samples were analysed for albumin and LH in order to estimate whether the frequent sampling had resulted in a decline in plasma albumin which would suggest a dilution of plasma and concomitantly underestimated LH concentrations. Since all plasma albumin values were higher than two standard deviations below the mean for each sampling occasion, it was not considered necessary to normalise LH values in relation to albumin.

Sequential sampling started 215 min after completed mating in female 131, and the first recorded value of 0.50 $\mu g \cdot L^{-1}$ was close to the pre-mating concentration. The following three samplings were performed at 10 and 15 min intervals with values increasing to between 3.80 and 4.50 $\mu g \cdot L^{-1}$. On the fifth sampling, performed one minute after the fourth, a clear peak value of 8.0 $\mu g \cdot L^{-1}$ was recorded; on the following sampling one minute later LH was only 3.2 $\mu g \cdot L^{-1}$. The remaining measurements, lasting until 280 min after completed mating showed very little fluctuation, with

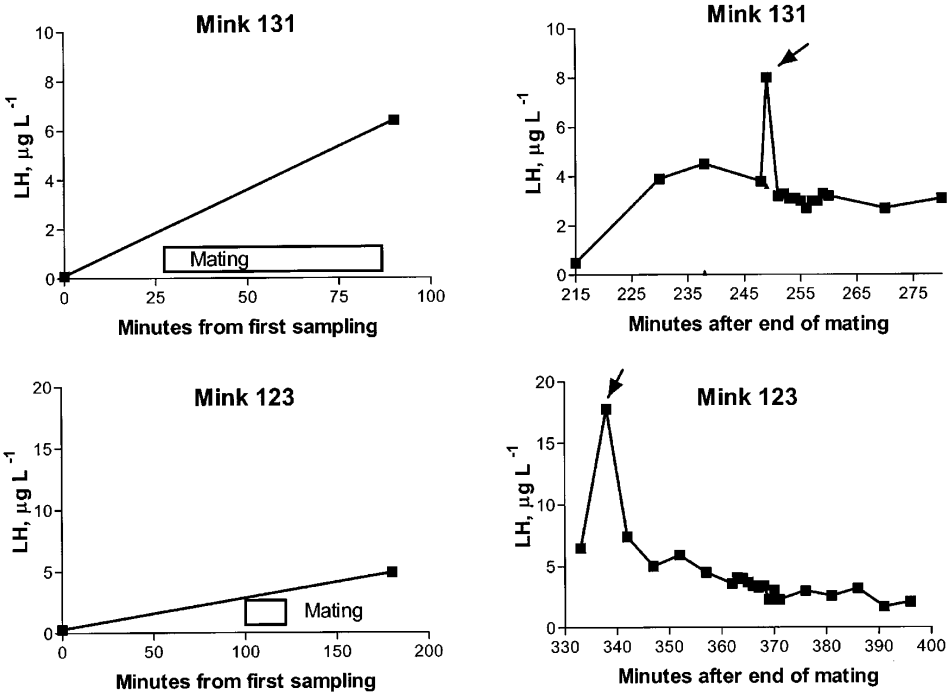

Figure 4. Plasma concentrations of luteinising hormone (LH) of two individual female mink (no. 131 and no. 123) before and after mating (left panel), and frequent blood sampling for 60 min during the LH surge (right panel). Arrows indicate LH values that are significantly above baseline.

concentrations remaining between 2.7 and 3.3 $\mu g \cdot L^{-1}$ (Fig. 4, top panel).

Sequential samplings on female 123 started about 330 min after completed mating with a first plasma LH concentration of 6.50 $\mu g \cdot L^{-1}$. Five min later LH peaked at 17.80 $\mu g \cdot L^{-1}$, and on the third sampling four min later, it had decreased to 7.40 $\mu g \cdot L^{-1}$. During the rest of the 60 min sampling period, LH concentrations ranged between 5.90 and 1.70 $\mu g \cdot L^{-1}$ with the lowest concentrations being recorded towards the end of the sampling period (Fig. 4, lower panel).

3.8. Reproductive performance

In the CON group, 8 out of 10 mated females gave birth, and average litter size was 6.4 kits. In the FLUSH group, 6 out of 9 mated females (only 8 had LH surges) gave birth with average litter sizes of 5.7 kits. In the NEG group only 2 out of 8 mated females whelped, and litter size was 5.0. Eight barren females (1 CON, 3 FLUSH and 4 NEG) were examined for CL and implantation sites. All females had several CL, or as in 2 NEG females without implantation sites, possibly luteinised follicles. Five females (3 FLUSH and 2 NEG) showed obvious signs of implantation.

4. DISCUSSION

4.1. The pre-ovulatory LH surge and oestradiol 17-β

The release of LH in response to mating in induced ovulators is a result of cervical stimulation. In this study this response was very rapid since peak values were already attained at the first sampling after mating which occurred an average 16 min after completed mating. Indeed, even in a female sampled 4 min after completed mating, plasma LH had reached a very high concentration. This is similar to the cat, in which LH release occurs within minutes of copulation [48]. In the rabbit, elevated plasma concentrations occur within 30 min and peak concentrations are attained after 90 min [15], whereas a non-significant increase is found after 20 min and peak values are attained within 2 to 3 h in the llama [1]. The ferret exhibits a significant elevation during the first 2 h after introduction of the male and peak values 6 h later [10]. In our study, one female with a very short mating (10 min) failed to show any LH surge, indicating that the mating stimulus must have a certain duration to induce LH release and subsequently ovulation. This agrees well with the observation by [49] and [3] that the conception rate of mink females mated for less than 12 min [49] or 6 min [3] is generally very low.

The duration of the pre-ovulatory LH surge was more than 12 and less than 24 h, which is somewhat shorter than that indicated by [42], but our previous sampling programme was too in-frequent to allow for a precise determination. The duration of the LH surge in the mink can be compared with about 30 h in the pig, 12 h in the rat, 10 h in the sheep [8], 10–12 h in the cow [37], between 6 and 12 h in the llama [1], 1–3 days in the dog [14] and about 8 h in the cat [48], 12 h in the ferret [10] and 4 h in the rabbit [15].

In induced ovulators, copulation usually occurs only when high plasma concentrations of oestradiol and, hence, large antral follicles are present. This has been demonstrated in rabbits [51], cats [35], camels [16], llamas and alpacas [2]. The high concentration of plasma oestradiol-17β found here immediately before mating fits this concept. Peak oestradiol-17β concentrations in mink on the day of mating have been previously reported [26], and [40] found a cyclic variation in oestradiol-17β temporally corresponding to follicular cycles. It was also suggested from the present data that oestradiol increases as an effect of mating as in the rabbit. In rabbits, however, the peak is

reached within minutes after mating and levels decline during the 4 h after mating [5, 22]. In the mink, high oestradiol concentrations which increase until 4 h after mating, are coherent with the role of the hormone in generating a positive feed-back to LH in the late follicular phase and stimulating the enlargement and rupture of the ovulatory follicles, although this has not been confirmed in species with induced ovulation. Declining concentrations of oestradiol following copulation have, besides in this study, been reported in cats [19, 35], in ferrets [52], and in camels at 18 h post-mating [7]. In llamas and alpacas, concentrations return to basal levels within 4 days after mating [2].

4.2. Vascular access ports as a tool for frequent blood sampling in mink

Our results showed clearly that with the use of vascular access ports, natural mating could be performed. In addition, the frequent blood sampling programme showed that samplings could be performed at intervals as short as 1 min. The sampling period in this study was too short for documentation of a pulsatile LH release; however, both females sampled during the LH surge exhibited one value significantly above the baseline concentrations, indicating that a pulsatile release may occur during the pre-ovulatory LH surge.

4.3. Effect of metabolic status on the pre-ovulatory LH surge

Metabolic status influences the reproductive processes to a great extent. Changes in metabolic status can, besides by quantitative energy metabolism measurements, be monitored by metabolic hormones and blood metabolites, out of which some are involved in turning reproduction on and off by signalling metabolic status to the brain. If a situation of negative energy balance persists over a prolonged period, body fat reserves are exhausted and it is likely that the GnRH pulse generator, and subsequently pulsatile LH release, stop and the animal enters anoestrus [23].

The flush feeding regimen (FLUSH) applied here was used in order to demonstrate an acute reproductive response, i.e. a response achieved without major changes in body weight or body composition. The animals in negative energy balance (NEG) were supposed to illustrate a chronic response, including a substantial decrease in body weight and loss of body fat, although the level of restriction used here was less severe than in studies reported to cause total suppression of the GnRH pulse generator [23, 53].

The energy metabolism measurements showed that NEG animals were in clearly negative energy balance throughout the experiment, and fat oxidation making up for 50% of the total heat production indicated that the main part of the body weight loss was caused by fat mobilisation. All but one NEG female had pre-ovulatory LH surges but there was a tendency for a more sluggish release resulting in peak values being attained 4 h after mating contrasting to CON and FLUSH animals in which peaks were attained on the first sampling after mating. Also the fact that there were no significant treatment effects on the concentrations of metabolic hormones, indicates that the level of energy restriction in the NEG group, despite declining concentrations of insulin, IGF-1 and TT_3 during the course of the experiment, was, in all animals but one, not sufficient to switch reproduction off. These results may seem to contrast with the literature (see reviews by [23, 53]), but again it must be pointed out that the level of energy restriction used here was less severe than in studies where LH release is completely abolished. The female in which no LH release was recorded was extremely lean with a body weight of only 587 g on the day of mating, and despite her

permitting mating no ovulation occurred. The poor reproductive outcome in the NEG group could therefore in only one case be conclusively attributed to failure to ovulate.

Flush feeding has previously been shown to improve reproductive outcome in mink [41]. In the present study, however, FLUSH animals consumed significantly less ME ($P < 0.001$) than did CON animals. In fact, their average ME intake was close to that of NEG animals, and they were only in positive energy balance during the first week of the experiment and the first week of refeeding, and were therefore in negative energy balance the week preceding mating. This may explain that, in contrast to the results of [42], the pattern of the LH surge was similar in CON and FLUSH animals.

The energy intake was clearly reflected in the relative contribution of protein and fat oxidation to the total heat production: OXP/HE was more than 50% when the animals were in a positive energy balance, but still made up to about 35% during restriction. Although OXP in reality denotes deamination of protein rather than true oxidation [46], it reflects the level of the nutrient which is not used in protein metabolism but as a metabolic fuel. The values found here can be compared with about 15% OXP/HE in growing pigs [13] and demonstrate the profound importance of protein as an energy source in the mink. Period effects were significant for thyroid hormones and IGF-1 and with insulin there was only a tendency for them; concentrations decreased during restriction and increased during refeeding. These results were in good agreement with [17, 18].

The results of this study suggest that normal ovulatory function in the mink can be maintained over a wide range of body weight and body fat content, but that in very lean animals, similar to other species, LH release and ovulation are abolished.

4.4. Conclusions

The present study has shown that the preovulatory LH surge in mink is an immediate response to the mating stimulus, and persisting over more than 12 but less than 24 h. Metabolic status affects the reproductive outcome, but LH release and ovulation is maintained in animals over a wide range of body weight and fat content.

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