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Metabolic adaptation to different protein supply in mink (Neovison vison)

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Abstract

Background: Strict carnivores such as the mink are considered to have limited ability to adapt protein metabolism to varied protein provision. However, some metabolic adaptation has previously been observed in lactating minks. This was further investigated in this study.

Methods: quantitative metabolism (balance and respiration experiments), kit growth, and plasma amino acids concentrations and liver mass in lactating dams, as well as relative mRNA abundance of some key hepatic enzymes (qPCR) after weaning of the kits, were studied. Lactating mink dams were assigned to one of three diets [high- (HP: 60% of metabolisable energy (ME)), medium- (MP: 45% of ME), or low- (LP: 30% of ME) protein], from parturition until ten weeks *post partum*. Tissue samples were collected 2, 3, 4 and 10 weeks *post partum*.

Results: Diet did not affect ($P > 0.05$) heat production. The protein provision was clearly reflected in the protein oxidation, and dams fed the HP diet had a higher N excretion, larger liver mass, and tended to have higher weight loss and lower estimated milk production through the four first lactation weeks. Plasma amino acid profiles and liver weights 10 weeks *post partum* were generally unaffected by dietary protein provision. Besides, the diets did not affect relative abundance of the studied mRNAs in hepatic tissue.

Conclusion: Metabolic adaptation in lactating mink dams seems limited mainly to adjustment of liver mass, but that the relative abundance of mRNA for key gluconeogenic enzymes is unaffected by diet indicates that these animals' capacity to regulate enzyme activity is limited.

Key words: Metabolic adaptation, protein metabolism, plasma amino acids, enzyme expression, liver mass.

Introduction

Strict carnivores such as the mink (*Neovison vison*) are usually fed diets high in protein and low in carbohydrates. This suggests that much of the digested amino acids are used as an energy source, and that the glucose homeostasis is largely supported by gluconeogenesis. In most species, maintenance of nitrogen (N) balance when protein intake varies is achieved by a variety of adaptive mechanisms, including changes in protein oxidation and protein turnover[1]. This metabolic regulation, and the mechanisms promoting nitrogen conservation when low-protein diets are fed, and preventing adverse effects arising from toxic concentrations of certain non-protein amino acids, when protein consumption exceeds the requirements, is essential. However, a generally accepted view has

been that strictly carnivorous animals with high protein requirements have a very limited capacity to adapt to a varied dietary nutrient supply, since they constantly have a high activity of hepatic gluconeogenic enzymes, and a high rate of hepatic gluconeogenesis[2]. Previous studies have though demonstrated some metabolic flexibility in a few strict carnivores, and that cats[3,4] and mink[5,6,7] are able to adapt to different protein supplies by regulating the protein oxidation rate. Cats have been suggested to be able to adapt their protein oxidation to the level of dietary protein supply if the protein requirement is met, but not if the protein supply is below this requirement[4]. Results from studies with mink, however, indicate that rates of decarboxylation and oxidation of amino acids may be regulated according to the protein supply, although the dietary protein is low or even below the requirement[5,7,8]. Also, improved performance (i.e. improved milk yield and reduced weight loss) in the lactating dam during the first four weeks of lactation has been demonstrated when protein supply was reduced below currently recommended levels, and replaced with readily available carbohydrates[5,6]. Restricted protein supply has been associated with reduced liver mass in rats[9] and mink[7], and also with reduced gene expression of fructose-1,6-biphosphatase (Fru-1,6-P_2ase) and pyruvate kinase (PKM_2) mRNA, in foetal hepatic mink tissue[(7)]. However, the mink's ability to adapt to varying protein supply is still not well understood, and one of the objectives of the present study was to investigate if the metabolic adaptation indicated in lactating minks fed high- (HP), medium- (MP), or low-protein (LP) diets was driven by changes in liver mass during the suckling period. Another objective was to reveal if possible changes in liver mass remained and also could be detected at the transcriptional level of key hepatic enzymes in dams after weaning of their kits. Our working hypothesis was that liver mass adapts to the level of dietary protein provision, and that this is the main mechanism with which mink adapts to level of protein supply.

Material and methods

The experimental procedures complied with Danish national legislation and the guidelines approved by the Member States of the Council of Europe for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes[10].

Experiment 1

Animals and diets

Twelve two-year-old mink dams of the standard brown genotype[11] were divided into three dietary treatment groups and fed ad libitum, from parturition until ten weeks *post partum*, with diets high (HP: 60% of metabolisable energy; ME), medium (MP: 45% of ME), or low (LP: 30% of ME) in protein content. Each litter was standardized to seven kits (eighty-four kits in total) by cross-fostering within 48 h of birth. The feed mixtures for the diets were prepared on a single occasion, weighed out into plastic bags, and immediately frozen. The feed was taken out of the freezer the day before use and thawed over night. Feed samples

Table 1. Ingredient and chemical composition of diets fed to lactating mink dams, providing a high- (HP), medium-(MP) or low protein (LP) supply.

	Dietary treatment		
	HP	**MP**	**LP**
Planned protein:fat: carbohydrate ratio (% of ME)	**60:35:5**	**45:40:15**	**30:45:25**
Ingredient composition, g/kg			
Cod offal	500	350	250
Cod, whole	100	100	100
Fish meal	80	60	0
Chicken, whole[1]	200	250	300
Barley and wheat (heat treated) (1:1)	20	40	80
Steamed rolled oats	0	20	40
Potato mash powder	5	25	40
Rape-seed oil	0	10	20
Vitamin/mineral mixture[2]	2.5	2.5	2.5
Water	92.5	142.5	167.5
Chemical composition			
Dry matter (DM), g/kg	292	312	335
Ash, g/kg DM	174	130	103
Crude protein, g/kg DM	605	513	325
Fat, g/kg DM	125	159	171
Carbohydrates, g/kg DM	96	198	401
Gross energy (GE), MJ/kg DM	20.8	21.8	21.9
Digestibility of nutrients			
Protein; fat; carbohydrates	80; 96; 86	81; 97; 81	78; 96; 84
Metabolisable energy (ME)			
ME, MJ/kg DM[3]	15.1	16.6	17.1
Protein:fat: carbohydrate ratio, (% of ME)	59:31:10	46:37:17	27:38:35

[1]Chicken prepared for human consumption, i.e. without head, feet, feather, or entrails.
[2]Containing, in mg/kg: α-tocopherol 21840, thiamine 10000, riboflavin 4800, pyridoxine 3200, D-pantothenic acid 3200, nicotinic acid 8000, betain anhydrous 33600, folic acid 240, biotin 80, cyanocobalamin 16, para-aminobenzoic acid 800, Fe 19712, Zn 12560, Mn 6237, and Cu 1025; in i.u. g: retinol 2800 and cholecalciferol 280.
[3]Calculated using individual coefficients of digestibility for the diets, the amount of digestible nutrient/kg diet, and metabolisable energy coefficients (18.4 kJ, 39.8 kJ, and 17.6 kJ/g digested protein, fat, and carbohydrate).

were collected daily, pooled for each week, and stored at –18°C until analysis. The ingredient and chemical compositions of the diets are presented in Table 1, and the amino acid compositions are shown in Table 2. The animals had free access to drinking water. Dams and kits were weighed at parturition, and thereafter at weekly intervals.

Balance and respiration experiments

Two days after parturition, the animals were transferred from the experimental farm to an intensive care unit where they were kept under natural daylight conditions (May, 55°N 12°E). The animals were placed in individual metabolic cages, equipped with devices for feeding and drinking water supply, quantitative

Table 2. **Table 2: Amino acid (g/kg dry matter) composition of the experimental diets; (high protein (HP), medium protein (MP), and low protein (LP)).**

	Dietary treatment		
	HP	MP	LP
Essential amino acids			
Lysine	40.7	32.5	21.2
Phenylalanine	22.0	18.2	12.7
Methionine	16.9	13.1	8.5
Histidine	16.2	14.2	10.4
Valine	30.2	24.9	16.7
Isoleucine	24.6	20.3	13.9
Leucine	40.4	33.5	23.0
Threonine	24.7	19.9	13.2
Arginine	40.1	31.8	21.5
Tryptophan	5.8	4.9	3.3
All essential	**255.8**	**207.4**	**141.1**
Non-essential amino acids			
Cystine	5.2	4.7	3.8
Glycine	57.4	42.1	27.3
Aspartate	53.8	43.7	29.2
Alanine	40.0	31.1	20.4
Tyrosine	18.9	15.5	10.8
Glutamate	79.1	66.6	49.0
Proline	33.9	25.7	19.7
Serine	29.5	23.1	15.7
All non-essential	**317.8**	**252.5**	**175.9**
Sum of amino acids	**573.6**	**459.9**	**317.8**

>> Strict carnivores such as the mink are considered to have limited ability to adapt protein metabolism to varied protein provision.

collection of feed residues, faeces and urine[12], and with nest boxes containing bedding of wood shavings. Balance and respiration experiments were performed in weeks 1, 2, 3, and 4 *post partum*. After the fourth week of lactation, the animals were housed under conventional farm conditions, the kits being allowed to supplement their milk intake with the feed given to the dams. Seven weeks *post partum* the kits were weaned, and thereafter the dams were housed individually.

Quantitative collection of feed residues and excreta[13] took place once daily between 08.30 and 12.00 a.m., and the pooled amounts collected each week were stored at -18ºC until analysis. Each balance period included a 22-h respiration experiment, using indirect calorimetry in an open-air circulation system[14].

The dams were anaesthetized ten weeks *post partum*, using 0.4 mL of Narcoxyl® Vet. (20 mg/mL Xylazin; Intervet Scandinavia AS, Skovlunde, Denmark) and 0.8 mL of Ketaminol® Vet. (50 mg/mL Ketamin; Intervet International BV, Boxmeer, The Netherlands). Blood was sampled by means of heart puncture, whereupon the dams were killed. The liver, kidneys and intestines were quickly removed and their weights were recorded, as well as the length of the intestines. The livers were flash frozen in liquid nitrogen. The blood samples were centrifuged for 15 min at 3300 *g* to separate the plasma, and the plasma samples were stored at -18˚C until the analysis of amino acid profiles.

Analytical procedures

Chemical analyses of diets and excreta: Diets, feed residues, and faeces were

analysed for dry matter (DM) by evaporation to constant weight at 105˚C, and ash by combustion at 525˚C for six hours. Nitrogen (N) content was determined by means of the micro-Kjeldahl technique using the Tecator-Kjeltec system 1030 (Tecator AB, Höganäs, Sweden). Crude protein (CP) was calculated as N x 6.25. Fat content was determined by means of petroleum ether extraction after HCl hydrolysis, and gross energy (GE) was determined by use of an adiabatic bomb calorimeter (IKA C5000, IKA Labortechnik, Janke & Kunkel GMBH, Staufen, Germany). Carbohydrates (CHO) were calculated by difference as follows: CHO = DM – ash – CP – fat. Amino acid content of diets were determined by oxidation with a performic acid/phenol mixture and hydrolysed with HCl (6 N) for 23 hours, and the hydrolysate adjusted to pH 2.20. The amino acids were separated by means of ion exchange chromatography and determined by reaction with ninhydrin using photometric detection at 570nm (440nm for proline). Energy in urine (UE) was estimated from the N content in urine (UN) as described elsewhere[7].

Amino acids in plasma: Free amino acids in mink plasma were analysed by means of micellar electro-kinetic capillary chromatography (MECC)[(15)] of the 2.4-dinitrobenzene (2.4-DNB) derivatives of the amino acids. Plasma samples (500 μL) were mixed and shaken for 10 min with acetonitrile (1.5 mL; Sigma-Aldrich, Steinheim, Germany) and internal standard (30 μL N-Val, 4.60 μmol x μL^{-1}), after which they were centrifuged at 15000 g for 10 min. The supernatant (1 mL) was then reacted with 75 μL of 70 μM 2.4-dinitroflurobenzene (DNFB), or Sangers reagent (13 mg per mL ethanol; Sigma-Aldrich), 100 μL of ethanol, and 100 μL of 100 mM aqueous $Na_2B_4O_7$, 10 H_2O (Sigma-Aldrich). After reaction for 40 min at 50˚C, evaporation to dryness and dissolution in 200 μL of 20% methanol in water, a 2-mL sample of this solution was used for MECC in a mixture of 60 mM tetradecyl-trimethyl-ammonium bromide (TTAB), 20 mM Na_2HPO_4, $2H_2O$, 18 mM $Na_2B_4O_7$, and 13% 1-propanol (pH 9.6). All chemicals and solvents were at least of analytical quality. Reference amino acids came from the laboratory collection[16], and individual amino acids determinations were based on spiking.

Assessment of gene expression: Total RNA was isolated from homogenized liver samples using the RNeasy Mini Kit (Qiagen NV, Venlo, the Netherlands) and transcribed into cDNA in 8.6 μL of reverse transcriptase (RT) mix containing 5 μL of RT buffer (Promega GmbH, Mannheim, Germany), 0.5 of μM dNTPs, 32 U RNasin® ribonuclease inhibitor (Promega), 200 U of M-MLV RT (Promega), and 1 μg of random hexamer primers (Amersham Biosciences, Copenhagen, Denmark). PCR primer oligonucleotides were designed from canine mRNA sequences for phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-biphosphatase (Fru-1,6-P_2ase), pyruvate kinase (PKM_2), and glucose-6-phosphatase (G-6-Pase), and tested by means of conventional PCR on mink and canine liver cDNA samples,

Table 3. Sequence of gene specific q PCR primers.

Gene		Primer sequence	Product size (bp)	Genebankaccession No.
G-6-Pase	F*	5'-primer: CCG AAT CTA CCT TGC TGC TC	207	NM1002993
	R**	5'-primer: AGT GTC CAC AGG AGG TCC AC AC		
Fru-1.6-Pase	F	5'-primer: TAC CTG GAA GGC ACT TTG CT	176	XM547066
	R	5'-primer: GAT GCC TCC TCC TCA CTC TG		
PKM_2	F	5'-primer: AAGATCACCCTGGACAATGC	247	XM535531
	R	5'-primer: GGAAGTCAGCACCTTTCTGC		
PEPCK	F	5'-primer: GAT GTT CAA TCG CAT CAA CG	245	XM_543068
	R	5'-primer: GGC TGA TTC TTT GCT TCA GG		
18S rRNA	F	5'-primer: GAT ACC GCA GCT AGG AAT	450	AY265350
	R	5'-primer: ATC TGT CAA TCC TGT CCG		

*F: forward primer **R: reverse primer

using canine genomic DNA samples as the control. 18S rRNA was chosen as the reference gene (Table 3). The PCR conditions for each primer pair were optimized by determining the $MgCl_2$ concentration and annealing temperature at which only the specific product was found. Furthermore, PCR products generated from mink liver cDNA were sequenced to confirm product identity. The relative quantification of enzyme mRNA was done by means of q PCR, using SYBR Green I detection and the LightCycler 480 Real-Time PCR System (Roche Diagnostics A/S, Copenhagen, Denmark). Reactions were carried out in 20-μL volumes consisting of FastStart Master SYBR Green I Mix, 3 mM $MgCl_2$, and 0.5 μM gene specific primer. Each run consisted of serial dilutions of a pool of liver cDNA to generate a standard curve (5 x). Dilutions of liver cDNA generated, using the same RNA extraction method, were chosen as standards to ensure that diluted (10 x) cDNA were amplified. The amplification program consisted of pre-incubation for FastStart polymerase activation at 95°C for 10 min, followed by 45 amplification cycles, as follows: 95°C at 5 sec (20°C/sec), 60-69°C for 10 sec (20°C/sec), and 72°C for 4-18 sec (20°C/sec). SYBR Green fluorescence was acquired at 72°C in each amplification cycle. After the last cycle, the melting curve was generated by starting the fluorescence acquisition at 65°C, and making measurements every 0.1 sec until 95°C was reached. A calibrator comprising a diluted (10 x) pool of liver cDNA was measured in triplicate and included in every run. Furthermore, the PCR efficiency was calculated for both the target and reference gene by determining the fitting coefficients of a relative standard curve. The final relative quantification was efficiency corrected.

Calculations

Metabolisable energy (ME) was calculated as follows: GE – energy in faeces

(FE) – UE. Heat production (HE) was calculated from O_2 consumption, CO_2 production, and nitrogen in urine (UN), using the following formula: HE, kJ = 16.18 * O_2, L + 5.02 * CO_2, L – 5.99 * UN, g [17]. Retained energy (RE) was calculated as: RE, kJ = ME – HE. The respiratory quotient (RQ) was calculated as: RQ = CO_2, L /O_2, L. Quantitative oxidation of protein (OXP), fat (OXF), and carbohydrate (OXCHO) were calculated from measurements of gas exchange and excretion of N in urine[18] as follows:

OXP, kJ = UN, g * 6.25 * 18.42
OXF, kJ = (1.719 * O_2, L – 1.719 * CO_2, L – 1.963 * UN, g) * 39.76
OXCHO, kJ = (-2.968 * O_2, L + 4.174 * CO_2, L – 2.446 * UN, g) * 17.58

>> Protein provision was clearly reflected in the protein oxidation, and dams fed the high protein diet had a higher nitrogen excretion, a larger liver mass, and a tendency towards higher weight loss and lower estimated milk production during the first four weeks of lactation.

Provided the RQnp value is between 0.707 and 1.00, the above method can be used to calculate the oxidation of the various nutrients[18]. However, some animals had RQnp values below 0.707. In such cases, OXCHO = 0 and OXF includes both the net oxidation and cost of fat mobilization; OXF was then calculated as oxidation of fat plus the value of the CHO oxidation[14].

Milk production (g/day) was calculated by using estimates for milk intake in relation to gained live weight (3.5 g, 4.2 g, 5.0 g, and 5.3 g milk/g gained weight in week 1, 2, 3, and 4, respectively[19]. Energy output (LE) and output of nutrients in the milk were calculated, using the chemical composition of mink milk[5].

Experiment 2

Animals and diets

A total of 27 two-year-old female mink of the standard brown genotype, each nursing six kits, were used together with 3 kits per dam. The dams were allocated to three dietary treatments, HP, MP and LP at parturition and fed *ad libitum* until euthanasia. The diets had the same ingredient composition as diets in Experiment 1, and differences in chemical composition as compared to the values in Table 1 were small. These dams were part of an experiment into amino-acid requirements of lactating mink dams, and results on quantitative metabolism traits, milk yield, and estimated amino- acid requirement have been reported by Fink et al. (2006). These particular dams and kits were intended for hepatic enzyme activity analyses and protein turnover studies, but the reporting here is limited to organ weights.

Tissue collection

Three dams from each dietary treatment, and three kits from each dam, were euthanized in lactation weeks 2, 3, and 4 (at each occasion 9 dams and 27 kits). Procedures for dam euthanasia were as described above, and kits were killed by decapitation after anaesthesia. Livers were quickly excised, weighed, flash frozen in liquid nitrogen, and stored at -80 °C pending further analyses.

Statistical analyses

Experiment 1: Statistical analyses of dam live weights, feed intake, energy and

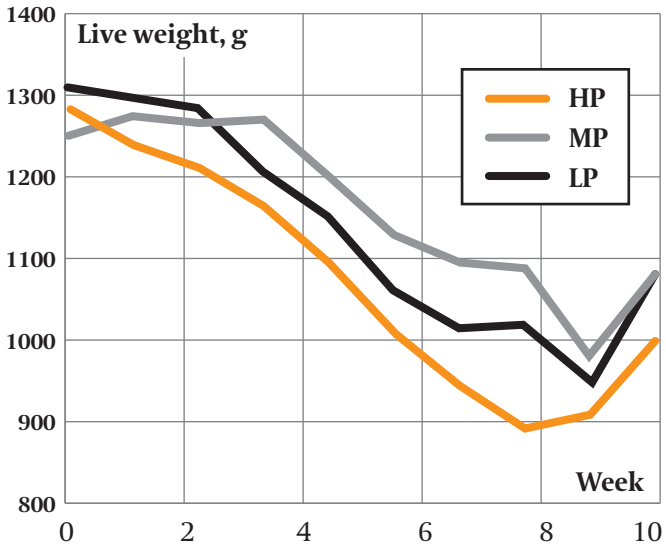
nitrogen metabolism, liver and kidney masses, intestinal mass and length, and q-PCR data were carried out using the GLM procedure in SAS[20] according to a model comprising the fixed effects of treatment group and period, and their interactions. The model was reduced for non-significant interaction effects. Plasma amino-acid profiles and kit live weights were analysed as repeated measures analyses according to the MIXED procedure in SAS[(21)], according to a model comprising the fixed effects of treatment group, sampling week, and their interactions, with sampling week as repeated measure. For kit live weights, sex was included as fixed effect, and the three way interaction between treatment group, week, and sex was included in the analysis. Results are presented as least square means (LS-means), and the root mean square error (GLM-procedure) and square root of residuals (MIXED procedure) were used as measures of variance. Effects were considered significant if $P < 0.05$, and as a tendency if $0.05 < P < 0.10$.

Experiment 2: Data were analysed by the GLM procedure in SAS[20] according to a model comprising the fixed effects of treatment group and period, and their interactions. Results are presented as least square means (LS-means), and the root mean square error was used as a measure of variance. Effects were considered significant if $P < 0.05$, and as a tendency if $0.05 < P < 0.10$.

Results

Experiment 1

Figure 1. Body weight of mink



Body weight of mink dams fed high (HP), medium (MP) or low (LP) protein diets through weeks 1 – 10 post partum.

Live weights

The live weights tended to differ ($P = 0.08$) between treatment groups among dams through week 1 to 4 *post partum*, where dams fed the HP diet had a lower live weight than dams fed the MP diet. In all dams live weights decreased ($P < 0.01$) with progressive stage of lactation (Table 4). Ten weeks *post partum*, three weeks after the kits were weaned there were no differences ($P > 0.05$) in live weights between

Table 4. **Live weights and feed intake in twelve mink dams through week 1 – 4 post partum, and the effect of dietary treatment [high- (HP), medium- (MP) and low protein (LP)], and period of lactation (period 1 = 1 – 2 weeks post partum, and period 2 = 3 -4 weeks post partum), on daily metabolisable energy (ME) intake, heat production (HE), calculated energy output in milk (LE), retained energy (RE) and respiratory quotient (RQ) in relation to metabolic body size (LW0.75), and oxidation of protein (OXP), fat (OXF) and carbohydrate (OXCHO) in relation to total HE.**

	Dietary treatment			Lactation period		RMSE*	P-value; effect of	
	HP	MP	LP	1 (1-2 weeks)	2 (3-4 weeks)		Diet	Period
Live weights, kg								
	1200	1255	1237	1263[a]	1197[b]	56.4	0.08	< 0.01
Feed intake, g/day								
	294[a]	273[ab]	240[b]	223[a]	315[b]	54.1	0.03	< 0.001
Energy metabolism, kJ/kg$^{0.75}$/day								
ME	1021	1203	1305	950[a]	1402[b]	291	0.08	< 0.001
HE	638	628	607	612	637	58.3	NS**	NS
LE	666	863	736	486[a]	1025[b]	203	0.08	< 0.001
RE	-281	-287	-43	-148	-260	291	0.06	NS
RQ	0.756[a]	0.796[b]	0.783[b]	0.777	0.780	0.03	0.03	NS
Substrate oxidation, % of HE								
OXP	40[a]	33[b]	19[c]	32	30	3.9	< 0.001	0.06
OXF	55[ab]	48[a]	61[b]	55	55	11.5	0.03	NS
OXCHO	5[a]	19[b]	24[b]	15	17	9.5	< 0.001	NS

*RMSE; Root mean square error
**NS; No significance or tendency (P > 0.1)
[abc] Values within row that share no common superscript differ significantly (P < 0.05)

dams (Figure 1).

Kit live weights from birth until 9 weeks of age were significantly affected by dietary treatment of their dams (P=0.01), and there was a strong three-way interaction between diet, kit age, and sex (P<0.001). During the first four weeks of life, kit live weights were not affected ($P > 0.05$) by dietary treatment of the dams, although LP kits were the heaviest. However, after the kits started to consume solid feed in addition to milk, and until nine weeks of age, growth rates were higher in HP and MP kits, resulting in kits (LSmean for male and female kits) fed the MP diet having the highest ($P < 0.01$) live weights (809.1 g) at the end of the experiment. The kits fed the HP diet (742.6 g), and those fed the LP diet (730.0 g), had the same live weights at an age of nine weeks (Table 5).

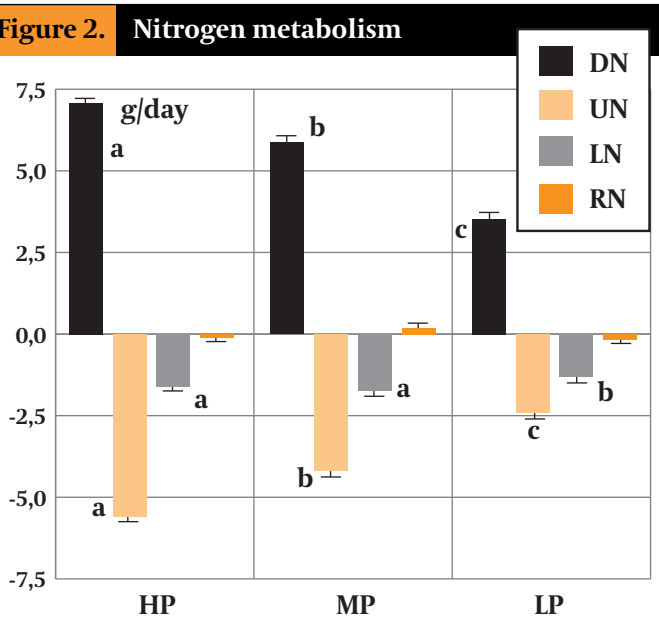
Table 5. **Live weights in mink kits (n=84) at birth, 2 and 4 weeks of age, nursed by dams fed high- (HP), medium- (MP), or low protein (LP) diets from parturition, and at seven and nine weeks of age (n=72) where the offspring continued with the same diet as the dam after weaning**

Sex	Male kits			Female kits			
Dietary treatment	HP	MP	LP	HP	MP	LP	Square root of residuals
Kit age, weeks							
Birth	12.3	11.2	13.0	10.5	11.6	12.5	50.99
2	80.5	73.9	81.5	67.1	67.9	76.0	
4	167.4	168.6	178.4	135.3	154.8	170.3	
7	494.3	536.3	498.3	399.5[b]	461.0[a]	431.4[b]	
9	844.3	880.1	820.5	640.8[b]	738.2[a]	639.5[b]	

[a,b] Values that share no common superscript differ significantly P<0.05.

Statistical significance; effect of	
Diet	0.01
Week	<0.001
Sex	<0.001
Diet * Week	0.07
Diet * Week * Sex	<0.001

Figure 2. **Nitrogen metabolism**



Nitrogen metabolism in lactating mink dams (n = 4 per treatment group) through week 1-4 post partum fed high (HP), medium (MP) or low (LP) protein diets. The presented data are digested nitrogen (DN), urinary nitrogen (UN), retained nitrogen (RN), nitrogen excreted in milk (LN). LS mean values are shown with standard errors of the mean represented by vertical bars. a,b,c LS mean values within measured parameter with unlike lower-case superscript letters were significantly different (P-value < 0.05) between dietary treatment.

Nutrient intake, energy metabolism, nitrogen metabolism and substrate oxidation

Dams fed the HP diet had the highest feed intake (g/day) (Table 4). Intake reflected dietary composition and contents of amino acids, resulting in higher intake of essential and non-essential amino acids in dams fed the HP diet than in dams fed the LP diet (Table 6). However, the metabolisable energy (ME) of the diets ranged from 15.1 MJ/kg DM in the HP diet to 17.1 MJ/kg DM in the LP diet (Table 1), and the intake of ME per kg metabolic body size was significantly ($P <$

Table 6.

Intake of amino acids (g/day) in lactating mink dams (n = 4 per treatment group), fed high- (HP), medium- (MP), or low protein (LP) diets, during period 1 (1 - 2 weeks post partum), and period 2 (3 – 4 weeks post partum), calculated in relation to metabolic live weights (kg0.75).

	Essential amino acids, (EEA)										SUM of EAA	EAANon-essential amino acids, (NEAA)									SUM of NEAA
Mink Dams (n=12)	Lys	Phe	Met	His	Val	Ile	Leu	Thr	Arg	Trp		Cys	Gly	Asp	Ala	Tyr	Glu	Pro	Ser		
Period 1																					
HP	2.00	1.08	0.83	0.80	1.49	1.21	1.99	1.21	1.97	0.29	**12.9**	0.26	2.82	2.65	1.97	0.93	3.89	1.67	1.45	**15.6**	
MP	1.59	0.89	0.64	0.70	1.22	1.00	1.64	0.98	1.56	0.24	**10.5**	0.23	2.06	2.14	1.53	0.76	3.27	1.26	1.13	**12.4**	
LP	0.92	0.55	0.37	0.45	0.72	0.60	1.00	0.57	0.93	0.14	**6.3**	0.16	1.18	1.26	0.88	0.47	2.12	0.85	0.68	**7.6**	
Period 2																					
HP	3.06	1.65	1.27	1.22	2.27	1.85	3.03	1.85	3.01	0.44	**19.6**	0.39	4.31	4.04	3.00	1.42	5.94	2.55	2.22	**23.9**	
MP	2.32	1.30	0.94	1.01	1.78	1.45	2.39	1.42	2.27	0.35	**15.2**	0.34	3.01	3.12	2.22	1.11	4.76	1.84	1.65	**18.1**	
LP	1.50	0.90	0.60	0.73	1.18	0.98	1.63	0.93	1.52	0.23	**10.2**	0.27	1.93	2.06	1.44	0.76	3.46	1.39	1.11	**12.4**	

0.05) higher in dams fed the LP diet than dams fed the HP diet, and was increasing ($P < 0.001$) in all dams with progressive stage of lactation (Table 4). Dams fed the HP diet had higher ($P < 0.001$) N intakes, and higher ($P < 0.01$) N excretion via faeces and urine than did dams fed the MP or LP diet. The calculated N excretion in milk did not differ significantly between dams fed HP and MP diets, but was lower in dams fed the LP diet. The amount of retained N (g/day) did neither differ ($P > 0.05$) between the treatment groups, nor between the two periods of lactation (Figure 2).

Dietary treatment did not affect ($P > 0.05$) the HE, though the OXP was approximately twice as high ($P < 0.001$) in dams fed the HP diet as in dams fed the LP diet. The fat oxidation was lowest in the dams fed the MP diet and highest in dams fed the LP diet ($P = 0.03$). There was no difference between dams fed MP and LP diets in terms of OXCHO, but dams fed the HP diet had a significantly lower OXCHO ($P < 0.001$).

The LE tended to

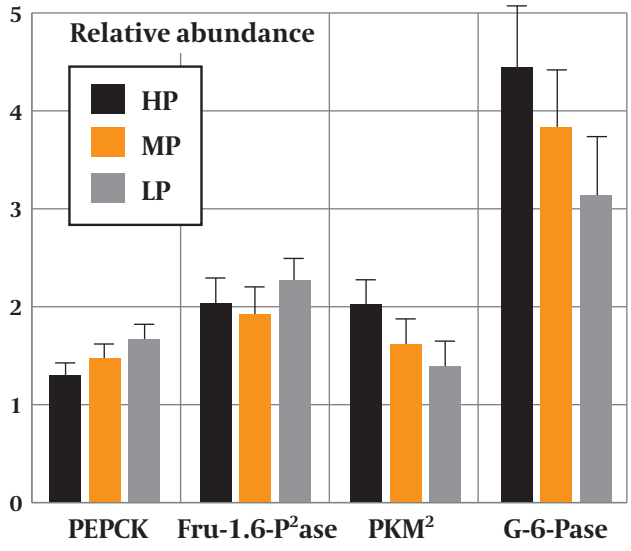
Table 7. Plasma amino acid profiles in mink dams fed a high- (HP), medium- (MP), or low-protein (LP) diet in lactation weeks 1 through 4, and week 10 post partum (μmol/ml).

	Week post partum					RR[1]	P-value, effect of	
	1	2	3	4	10		Diet	Week
Essential[‡]								
Lysine	0.276	0.264	0.234	0.220	0.254	0.116	NS[**]	NS
Phenylalanine	0.072	0.088	0.088	0.071	0.065	0.040	NS	NS
Methionine	0.217^{bc}	0.255^{acd}	0.285^{a}	0.226^{bd}	0.200^{b}	0.076	<0.001	< 0.01
Histidine	0.093^{a}	0.055^{b}	0.048^{b}	0.042^{b}	0.034^{b}	0.035	NS	<0.001
Isoleucine	0.092^{ac}	0.116^{bd}	0.129^{b}	0.107^{ad}	0.077^{c}	0.036	< 0.01	<0.001
Leucine	0.104^{bc}	0.133^{ac}	0.163^{a}	0.119^{ac}	0.090^{b}	0.063	0.06	0.01
Threonine	0.424^{a}	0.318^{b}	0.289^{b}	0.265^{b}	0.324^{b}	0.109	NS	<0.001
Arginine	0.045^{a}	0.046^{a}	0.029^{b}	0.031^{b}	0.023^{b}	0.017	NS	0.001
Tryptophan	0.088^{a}	0.079^{a}	0.062^{b}	0.055^{b}	0.048^{b}	0.032	0.05	<0.001
All essential[‡]	**1.36**	**1.35**	**1.29**	**1.16**	**1.15**	**0.393**	**0.07**	**NS**
Non-essential[τ]								
Asparagine	0.093^{a}	0.094^{a}	0.080^{ab}	0.064^{b}	0.106^{a}	0.043	NS	0.03
Glutamine/Serine[*]	0.411^{a}	0.317^{b}	0.305^{b}	0.263^{c}	0.335^{b}	0.105	NS	0.002
Aspartate/Proline[*]	0.185^{b}	0.230^{a}	0.252^{a}	0.228^{a}	0.156^{b}	0.064	NS	<0.001
Glycine	0.479^{a}	0.440^{a}	0.478^{a}	0.422^{a}	0.327^{b}	0.142	NS	0.02
Glutamate	0.234	0.192	0.216	0.182	0.179	0.095	0.01	NS
Alanine	0.389^{b}	0.414^{b}	0.514^{a}	0.470^{a}	0.350^{b}	0.171	NS	0.04
Tyrosine	0.033	0.042	0.039	0.036	0.024	0.022	NS	NS
All non-essential[τ]	1.92	1.89	1.92	1.71	1.49	0.484	NS	NS
Total	3.20	3.01	3.15	2.77	2.59	0.771	NS	NS
BCAA[#]	0.196^{bc}	0.246^{ac}	0.290^{a}	0.225^{b}	0.168	0.094	0.03	0.004
LNAA[¤]	0.289^{bc}	0.381^{ac}	0.445^{a}	0.383^{ac}	0.284^{b}	0.134	0.05	0.01

[1]RR = Square root of residuals. [**]NS; No significance or tendency (P > 0.1). [a, b, c, d]Values not sharing a superscript differ significantly (P < 0.05); effect of week. [‡]Valine not quantified. [τ]Cystine not quantified. *These two amino acids most often co-elute in one peak. [#] Branch chained amino acids except valine. [¤]Large neutral amino acids except valine.

be higher ($P = 0.08$) in dams fed the MP diet, than dams on other treatments. However, LE increased ($P < 0.001$) in all dams as lactation progressed. Dams on all treatments were in negative energy balance during lactation, but dams fed the LP diet tended ($P = 0.06$) to lose less energy than dams fed the HP and MP diets (Table 4).

Figure 3. The relative abundance



The relative abundance of phosphoenol-pyruvate-carboxykinase (PEPCK), fructose-1,6-biphosphatase (Fru-1,6-P2ase), pyruvate kinase (PKM2) and glucose-6-phosphatase (G-6-Pase) mRNA normalized to 18s rRNA in the hepatic tissue of mink dams (n =4 per treatment group) 10 weeks post partum, given a high (HP), medium (MP) or low (LP) protein supply from parturition. LS mean values are shown with standard errors of the mean represented by vertical bars.

Plasma amino acid profiles

Plasma amino acid profiles in the dams were generally unaffected ($P > 0.05$) by dietary treatment, except that LP dams had lower ($P < 0.05$) methionine, isoleucine, and leucine concentrations than dams fed the HP diet, and higher ($P < 0.05$) glutamate concentration than dams on both HP and MP dietary treatments. In fact, methionine concentrations differed ($P < 0.001$) between all treatment groups, being lowest in dams fed the LP diet and highest in dams fed the HP diet. Both the branched chained amino acids (BCAA) and the large neutral amino acids (LNAA) were significantly lower ($P < 0.05$) in dams fed the LP diet than dams fed the HP diet. As the 10-weeks experimental period progressed, most of the free amino acids in plasma decreased ($P < 0.05$) in all dams (Table 7).

Liver, kidney and intestinal masses, and mRNA relative abundance

Liver masses and intestinal mass and length were similar ($P > 0.05$) among dams on different dietary treatments. Dams fed the HP diet, however, had larger ($P < 0.01$) kidney masses than dams fed MP and LP diets (Table 8). Further, there were no differences ($P > 0.05$) in the relative abundance of any of the studied mRNAs among the dams (Figure 3).

Experiment 2

The absolute and relative liver weights of the dams were significantly ($P=0.003$ and $P=0.005$, respectively) affected by dietary treatment, dams fed the HP diet having the highest liver weights and the largest livers in relation to body weight, and dams fed the LP had the smallest both in absolute and relative terms. MP dams were intermediate and absolute liver weights did not differ from those of HP dams. Relative liver weights of MP dams did not differ from any of the other

Table 8. **Live weights (LW), body length (BL), liver, kidney and intestinal masses in percent of LW, length of intestine and length of intestine in relation to body length 10 weeks post partum in mink dams (n = 4 per treatment group), given a high (HP), medium (MP) or low (LP) protein supply from parturition.**

	Dietary treatment				P-value; effect of
	HP	MP	LP	RMSE*	Diet
LW, g	1155	1146	1121	138	NS**
BL, cm	42.8	41.9	42.5	0.94	NS
Liver, % of LW	3.88	3.35	3.68	0.57	NS
Kidney, % of LW	0.77[a]	0.65[b]	0.62[b]	0.06	< 0.01
Intestine, % of LW	3.67	3.06	3.09	0.36	0.07
Intestine, cm	175.8	160.3	168.3	3.14	0.07
Intestine:BL	4.1	3.8	4.0	0.17	NS

*RMSE; Root mean square error. **NS; No significance or tendency (P > 0.1)
[ab]Values within row that share no common superscript differ significantly (P < 0.05)

>> Metabolic adaptation in lactating mink dams seems limited mainly to adjustment of liver mass, but that the relative abundance of mRNA for key gluconeogenic enzymes is unaffected by diet indicating that these animals have a limited capacity to regulate enzyme activity.

treatment groups. In kits maternal diet affected absolute (P<0.001) but not relative (P=0.14) liver weights, but there was a tendency (P=0.06) for an interaction between maternal diet and suckling week for the absolute weights, and a significant interaction (P<0.001) for relative weights. Absolute liver weights were generally highest in kits from dams fed the LP diet and lowest in those suckled by dams on the HP diet, and differences were significant (P<0.05) in all measured suckling weeks. Absolute weights of livers from MP kits were similar to those of HP kits in suckling week 2, but similar to those of LP kits in weeks 3 and 4. Relative to body weight there was no clear pattern with the MP kits having the highest ratio between liver and body weight in suckling week 2. In suckling week 3 there was no difference in relative liver weight between treatment groups, whereas in week 4 kits suckled by HP dams had the smallest, and kits from LP dams the largest, relative liver weights. In kits suckled by HP and MP dams the relative liver weight declined significantly (P<0.01) from suckling week 2 to 4, but this was not the case in LP kits (Table 9). There was no obvious pattern of a correlation between kit body weight and relative liver weight.

Discussion

Metabolic flexibility is the capacity of the organism to adapt fuel oxidation to fuel availability. Adaptation to dietary N intake is essential, because the capacity of the body to store N is limited[22]. The mechanisms by which adaptation is achieved include changes in protein oxidation and protein turnover. Protein oxidation is a term used to describe the release of energy from the carbon skeleton of amino acids after deamination, which is affected by amino acid catabolic enzymes[23]. Most animals can up- and down-regulate those catabolic enzymes in order to

Table 9. Experiment 2. Dam and kit liver weights in lactation weeks 2 through 4

Dams	Week	Diet HP	Diet MP	Diet LP	RMSE*	P-value; effect of Diet (D)	P-value; effect of Week (W)	P-value; effect of D*W
Body weight, g		1082	1114	1099	96.2	NS**	0.02	NS
Liver, g		38.0[a]	34.1[a]	28.6[b]	4.70	0.003	0.07	NS
Liver, % of BW		3.52[a]	3.07[ab]	2.60[b]	0.50	0.005	NS	NS
Kits								
Body weight, g	2	44.8[b]	51.7[b]	74.2[a]	23.69	<0.001	<0.001	0.004
	3	63.2[b]	103.0[a]	86.5[a]				
	4	139.0	152.9	146.8				
Liver, g	2	1.72[b]	2.27[b]	2.77[a]	0.99	<0.001	<0.001	0.06
	3	2.61[b]	3.84[a]	3.20[a]				
	4	4.59[b]	5.44[a]	6.24[a]				
Liver, % of BW	2	4.11[b]	4.68[a]	3.75[c]	0.60	0.14	<0.001	<0.001
	3	3.84	3.72	3.69				
	4	3.30[b]	3.57[b]	4.23[a]				

*RMSE; Root mean square error. **NS; No significance or tendency (P > 0.1)
[a, b, c] Values that share no common superscript differ significantly (P<0.05) as an effect of dam dietary treatment group.

maintain amino acid homeostasis. This regulation may occur by changing the rate of enzyme degradation or, more generally, by changing the rate of enzyme synthesis through increasing the rate of mRNA synthesis[2,24,25]. Although it is generally accepted that the hepatic ureagenic, gluconeogenic and catabolic enzymes of strictly carnivorous animals have a very limited capacity to adapt effectively to a varied dietary protein supply, findings in mink[5, 7, 26, 27] and cat[3, 4, 24, 28, 29] have demonstrated that metabolic adaptation may be less limited in such animals than earlier suggested. In the present study we investigated whether the metabolic adaptation previously indicated in lactating minks may be reflected in changes in functional liver mass of the lactating dam, and if any such changes remain after weaning off the kits. Further, we investigated whether the post-weaning transcriptional level of key hepatic enzymes indicates ability to adapt mRNA abundance to substrate availability.

Although dams fed the HP diet had higher feed intake than LP dams, their ME intake (kJ/kg$^{0.75}$/day) was lower, and these dams lost more weight than LP and MP dams during the first four weeks of lactation. The excess protein content of the HP diet was also indicated by the high amount of excess N excreted in urine. In the present study, indirect calorimetry was used to assess substrate oxidation.

Since the protein oxidation measured by indirect calorimetry in fact is a measure of protein deamination, measurement of amino acids used in gluconeogenesis, and consequently amino acids lost from protein metabolism, is included. Similar to previous findings in mink[5,7,8], we found that the differences between the protein and carbohydrate contents of the diets were clearly reflected in the substrate oxidation, resulting in LP dams having the lowest OXP and highest OXCHO, and dams fed the HP diet having the highest OXP and lowest OXCHO as a percentage of HE. The OXP in LP dams was though higher (19 %) than the 13 % of HE seen for lactating sows fed balanced diets[30]. Since the N-balances did not reflect mobilization of lean tissue mass, this level of OXP indicates that the LP diet sustained the protein requirement of the lactating mink. Thus, these results support the suggestion that lactating mink dams have some ability to regulate its protein oxidation rate when protein supply is reduced.

The kidney mass was greater in dams fed the HP diet than those fed the LP diet. The kidneys have two primary functions, i.e. waste excretion and water excretion regulation. The main waste compound of protein metabolism is urea, and increased protein intake is known to cause renal hypertrophy and increase the glomerular filtration rate[31]. Thus, the increased kidney size in the animals fed the HP diet reflects an adaptive response to filtration induced by increased protein intake. This is also found in rats[32] and mice[33]. As well, although not to the same extent as in the liver, gluconeogenesis also occurs in the cortex of the kidneys. It has been demonstrated that rats allocated to low carbohydrate diets have increased rates of gluconeogenesis in kidney tissue[34]. In the present study, renal enzyme activities were not investigated. However, the increased kidney mass in dams fed the HP diets may indicate more gluconeogenetic activity in these animals.

High protein diets have been demonstrated to increase liver weight in rat[35], pig[36, 37] and cat[38], and protein restriction has tended to reduce the functional liver mass in mink[7] and in suckling rats[9]. These adaptations in liver weights will, despite unchanged activity of the amino acid degrading enzymes per gram of liver, increase the rate of amino acid catabolism[39]. The present study gave diverging results since liver weights (both absolute weights and in percent of live weights) did not differ among dams exposed to different dietary treatments in Experiment 1, while a clear diet effects for dams was shown in Experiment 2 with those fed the HP diet having significantly higher liver mass than those fed the LP diet. However, the livers from the dams in Experiment 1 were collected after weaning of the kits, whereas those from Experiment 2 derived from lactating dams. This may explain the seen differences. Furthermore, dietary treatment did not affect the abundance of the studied mRNAs in dam hepatic tissue, suggesting that the level of transcript did not respond to dietary protein provision. If the level of transcript could be directly translated to enzyme activity, our results would have corroborated the view that mink, like cat, cannot regulate the rate of hepatic enzyme activity, including gluconeogenesis, but this needs to be confirmed. Mink even seems to have higher glycolytic and gluconeogenic enzyme activities

compared to the corresponding activities in rat and cat[40]. However, it may be other enzymes than those investigated in the present study that are of more importance for metabolic regulation in mink, such as the trans-aminating and de-aminating enzymes. Furthermore, tissue collection and evaluation of gene expression occurred in dams three weeks after weaning of their kits, and thus the metabolic pressure on the dams was less than during lactation. Therefore it cannot be excluded that results had been different if the dams were still lactating.

Other mechanisms of importance for adaptation to dietary N intake in humans include salvage of urea-N by the gastrointestinal microflora[41, 42]. It has been suggested that when the dietary protein intake is low, a larger proportion of the urea production is transferred to the gut, followed by hydrolysis to NH_3 that can be used to support the intestinal microbial population. These microbes can then provide a source of amino acid and nucleic acid-N to non-ruminant animals[43] and humans[41, 44]. An increased entry of urea into the gastro-intestinal tract during low dietary protein intakes will then, potentially, enable more urea-N to be retained which might be beneficial under conditions where the protein supply is low[(41)]. This salvage of urea-N has been shown to contribute to positive N-balance in rats[43]. However, high-protein-fed (70 % energy from protein) cats showed an increased urea production, but a very low rate of gut entry of urea-N, and cats fed a moderate (20 % energy from protein) protein diet had no increased urea recycling[24]. The authors suggested that carnivore species with the very short gastro-intestinal tract have a low microbial urease activity and may therefore not be able to salvage urea-N when the protein supply is low. It is though possible that adaptation of these mechanisms first come into force when the protein supply is below the requirement. In the present study, the amount of retained N did not differ among the dams. This supports the suggestion that mink, like cat, is not able to adapt to different protein levels by changes in the microbial urease activity. However, if such adaptations first occur when the protein supply is below the requirement, the result from the present study supports the assumption that the LP diet sustained the protein requirement of the lactating mink.

It has been demonstrated that the rate of protein turnover in adult cats may adapt to the dietary protein level[29]. Furthermore, by increasing the dietary content of bacterial protein meal, the protein turnover rate increased in male mink[45]. However, only a very limited impact on protein turnover has been indicated in lactating mink when feeding medium or low protein diets[46, 47]. Similar to earlier findings in lactating mink[5,6], dams fed the LP diet in Experiment 1 seemed to raise the heaviest kits over the first four weeks of lactation, but the effects were non-significant. As well, live weights among dams tended to differ during the first four weeks of lactation, where dams fed the HP diet lost more of their body weight than dams fed the LP diet. In the fourth week of lactation, dams fed the LP diet had intakes of some amino acids (leucine and cystine) which were lower than the previously estimated requirements[6]. Nevertheless, this was not reflected in the plasma amino-acid profiles. The plasma amino-acid profile is mainly the

result of the dietary protein concentration and the pattern of the protein ingested, the catabolism of amino acids, and protein turnover[48]. For the most limiting amino acids in a diet the observed concentration in plasma parallels their rank in dietary protein[49]. Ten weeks *post partum,* three weeks after weaning, live weights among dams did not differ, and we did not find any association between concentrations of amino acids supplied by the diet and the concentration of free amino acids in plasma. These findings support prior suggestions that diets with restricted protein content may be beneficial during lactation, since such diets may be more efficiently used for milk production, and also preventing weight loss. An explanation for this improved performance may be reduced energetic costs for glucose production by less amino acids being used in gluconeogenesis and as energy source. In addition, the results may also indicate that the LP diet was providing sufficient protein and energy supply to the dams in this period.

Conclusion

The dietary protein provision in the present study was clearly reflected in the rate of protein oxidation and in hepatic tissue mass during lactation, but when dams were euthanized three weeks after weaning, diet did not affect the abundance of the studied mRNAs in hepatic tissue or tissue mass. Lactating, but not dry, dams on the HP diet had larger relative liver mass than those fed LP. It may therefore be that the metabolic adaptation in lactating dams is not caused by any significant capacity to regulate the rate of gluconeogenesis, but may be caused by changes in relative liver mass. Further, protein turnover seems little affected by protein provision, and salvage of urea-N by the gastro-intestinal microflora is probably negligible. It is possible that other enzymes than those investigated in the present study are more important for N conservation in mink. To clarify the metabolic adaptation in mink further studies are needed, among those investigations to reveal if the level of transcript is mirrored by the level of the corresponding protein, and into the adaptability of ureagenic, transaminating, and deaminating enzymes.

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Author contributions

RF performed Experiment 1 as part of her post doc, and participated in Experiment 2. She drafted the first manuscript which was later rewritten and finalized by CL. The qPCR studies were supervised by PDT who critically revised the manuscript and in a later phase by CFM who likewise participated in writing up and revising the manuscript. AHT, supervisor of RF, CFM and CL, supervised the design of the studies, led the group, critically discussed and revised the manuscript. The authors declare that there are no conflicts of interest.

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