

## RESEARCH

# Fetal over- and undernutrition differentially program thyroid axis adaptability in adult sheep

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## Abstract

**Objective:** We aimed to test, whether fetal under- or overnutrition differentially program the thyroid axis with lasting effects on energy metabolism, and if early-life postnatal overnutrition modulates implications of prenatal programming.

**Design:** Twin-pregnant sheep ( $n=36$ ) were either adequately (NORM), under- (LOW; 50% of NORM) or overnourished (HIGH; 150% of energy and 110% of protein requirements) in the last-trimester of gestation. From 3 days-of-age to 6 months-of-age, twin lambs received a conventional (CONV) or an obesogenic, high-carbohydrate high-fat (HCHF) diet. Subgroups were slaughtered at 6-months-of-age. Remaining lambs were fed a low-fat diet until 2½ years-of-age (adulthood).

**Methods:** Serum hormone levels were determined at 6 months- and 2½ years-of-age.

At 2½ years-of-age, feed intake capacity (intake over 4-h following 72-h fasting) was determined, and an intravenous thyroxine tolerance test (iTTT) was performed, including measurements of heart rate, rectal temperature and energy expenditure (EE).

**Results:** In the iTTT, the LOW and nutritionally mismatched NORM:HCHF and HIGH:CONV sheep increased serum  $T_3$ ,  $T_3:T_4$  and  $T_3:TSH$  less than NORM:CONV, whereas TSH was decreased less in HIGH, NORM:HCHF and LOW:HCHF. Early postnatal exposure to the HCHF diet decreased basal adult EE in NORM and HIGH, but not LOW, and increased adult feed intake capacity in NORM and LOW, but not HIGH.

**Conclusions:** The iTTT revealed a differential programming of central and peripheral HPT axis function in response to late fetal malnutrition and an early postnatal obesogenic diet, with long-term implications for adult HPT axis adaptability and associated consequences for adiposity risk.

## Key Words

- ▶ obesity
- ▶ thyroid
- ▶ metabolism
- ▶ development/fetal nutrition

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## Introduction

Prenatal and early postnatal malnutrition can mediate metabolic programming with life-lasting effects. This represents a risk for development of metabolic diseases later in life, such as obesity and type 2 diabetes (1, 2, 3), which are diseases of increasing global prevalence (4). It is of great interest to learn, if such metabolic diseases with

a fetal or early postnatal origin should be divided into functional subcategories depending on the nature and timing of the early-adverse exposure, since this potentially could call for differential treatments or interventions later in life. Research on programming of endocrine systems has mainly focused on the hypothalamic–pituitary–adrenal

axis function (5, 6, 7), glucose–insulin axis function (8, 9) and more recently programming of the hypothalamic–pituitary–adipose axis (10, 11, 12). However, much less attention has been paid to the hypothalamic–pituitary–thyroid (HPT) axis, which plays a fundamental role in normal development and maturation, in regulation of metabolism through stimulation of mitochondrial oxygen consumption, carbohydrate and lipid metabolism, and it affects functional development of the nervous and cardiovascular systems (13, 14). As summarized in Table 1, previous studies (15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27) have indicated that the thyroid state and energy expenditure (EE) can be programmed by fetal and early-life nutrition. The majority of these studies has been performed in rats by de Moura *et al.* Although, the overall pattern seems to be a downregulated HPT-axis function in adult rats subjected to fetal or early-life programming, the opposite has also been reported in rat offspring from protein and energy restricted lactating dams, as well as in rats subjected to fetal and early-life overnourishment. It can also be seen that far less is known about programming of this axis in precocial species, like sheep and humans that are born physiologically more mature than rodents. Table 1 also, exemplifies that no studies have, to our knowledge, included evaluation of the long-term consequences of changes in pre- and postnatal nutritional (matching vs mismatching exposures) for the phenotypic manifestation of any programming of the HPT axis. Thus, we aimed to study the effect of different combinations of prenatal and early-life nutrition on thyroid function and related

metabolic phenotypes in adulthood. We hypothesized that: (i) fetal under- and overnutrition programs the HPT axis differently with long-lasting implications for HPT axis function in adulthood, (ii) early postnatal development of adiposity will lead to differential phenotypical manifestations of HPT axis function depending on the nature of malnutrition in late fetal life. The Copenhagen sheep model (3) was used here, since sheep display fetal-growth trajectories and offspring maturity at birth, which are comparable to those of humans. Twin-pregnant sheep were either under-, over- or adequately nourished, in the last-trimester of gestation. After birth, the offspring were raised on a moderate, conventional diet or an obesogenic diet for the first 6 months (just after puberty) of life. After being fed the same moderate diet for another 2 years, EE, heart rate and body temperature were tested in the adult offspring at 2½ years-of-age before- and during-, an intravenous thyroxine (T<sub>4</sub>) tolerance test (iT<sub>4</sub>TT) and their voluntary feed intake over 4 h after a 72-h fasting period was measured as an indicator of feed intake capacity.

## Materials and methods

### Experimental animals and experimental design

Thirty-eight Texel sheep (22 ewes, 16 rams) from a larger experiment (12) were included in this study. Khanal *et al.* (12) published an extensive description of the experimental design and the Copenhagen sheep model. The Danish National Committee on animal experimentation

**Table 1** Summary of studies documenting programming of metabolic phenotype or thyroid state.

Species	Treatment or condition	Age at evaluation	Metabolic phenotype	Thyroid state	Reference
Rats	Fetal undernutrition	Adult	↓ Resting EE	Subclinical hypothyroid	(15)
Rats	Maternal nutrient restriction, midlactation	Adult	–	↓ Serum T <sub>4</sub> and TSH	(16)
Rats	Maternal nutrient restriction, late lactation	Adult	–	↓ Serum T <sub>4</sub> , T <sub>3</sub> and TSH	(17)
Rats	Maternal protein or energy restriction, throughout lactation	Adult	–	↑ T <sub>3</sub> and T <sub>4</sub> , ↓ TSH	(18)
Rats	Maternal protein restriction, throughout lactation	Adult	–	↓ Pituitary TSH response to <i>in vitro</i> TRH	(19)
Rats	Fetal + early-life overnutrition	At weaning	–	↑ T <sub>3</sub> , free T <sub>4</sub> , pro-TRH	(20)
Rats	Early-life overnutrition	Adult	–	↓ T <sub>3</sub> and T <sub>4</sub>	(21)
Zebra finches	Maternal protein restriction X catch up growth	Adult	↑ Resting EE	–	(22)
Sheep	Placental restriction	Lamb	–	↑ T <sub>3</sub> , ↑ T <sub>3</sub> :T <sub>4</sub> ratio	(23)
Sheep	Fetal undernutrition (late gestation)	Adult	–	↑ T <sub>3</sub> and T <sub>4</sub>	(24)
Human	Small for gestational age	Adult	–	↑ TG and TPO antibodies	(25)
Human	Small for gestational age	Adult	↓ Fasting EE	–	(26)
Human	Large for gestational age	Children	↑ Resting EE	–	(27)

approved all experimental animal procedures. Overall, the experimental sheep had been exposed to different levels of nutrition during the last 6 weeks prior to parturition. Twin-pregnant mothers were fed diets fulfilling 50% of energy and protein requirements (LOW), 100% of energy and protein requirements (NORM) or 150% of energy and 110% of protein requirements (HIGH), according to recommendations by American National Research Council (2007) for sheep in the last-trimester of gestation. The twin lambs suckled their dams during the first 3 days after birth, whereafter the dam was removed, and the twin lambs were then assigned to each their postnatal diet from 3 days till 6 months (after puberty) of age: a conventional moderate hay-based diet (CONV) supplemented by milk replacer during the first 8 weeks of life (provided in a suckling bucket) or a high starch, high-fat diet (HCHF), consisting of a liquid mix of milk replacer and dairy cream (1:1 ratio; provided in a suckling bucket) and rolled maize, which were fed until a maximum daily allowance of 2.5L and 1 kg *ad libitum*, respectively. By the end of the differential feeding period, blood samples from overnight fasted animals, were drawn by venipuncture from the jugular vein for later analysis. Thereafter all sheep were raised in two gender divided flocks and all sheep received the same low-fat diet consisting of artificially-dried green hay fed supplemented with rolled barley *ad libitum* (28) until 2½ years-of-age (adulthood). There were thus six experimental groups: NORM:CONV ( $n=5$ , 4f, 1m), NORM:HCHF ( $n=4$ , 2f, 2m), HIGH:CONV ( $n=6$ , 4f, 2m), HIGH:HCHF ( $n=7$ , 4f, 3m), LOW:CONV ( $n=8$ , 4f, 4m) and LOW:HCHF ( $n=8$ , 4f, 4m). NORM:CONV, HIGH:CONV and LOW:CONV lambs had an average weight of  $33.2\pm 4.5$ ,  $36.4\pm 3.4$  and  $34.5\pm 3.3$  kg, respectively, at 6 months-of-age. NORM:HCHF, HIGH:HCHF and LOW:HCHF lambs had an average weight of  $38.5\pm 9.6$ ,  $37.8\pm 8.6$  and  $40.6\pm 7.2$  kg, respectively, at 6 months-of-age. NORM:CONV, HIGH:CONV and LOW:CONV sheep had an average weight of  $92.8\pm 5.1$ ,  $98.8\pm 11.8$  and  $91.2\pm 6.1$  kg, respectively, at 2.5 years-of-age. NORM:HCHF, HIGH:HCHF and LOW:HCHF sheep had an average weight of  $92.1\pm 6.5$ ,  $93.6\pm 9.9$  and  $96.9\pm 6.8$  kg, respectively at 2.5 years-of-age.

A measure of voluntary feed intake capacity was assessed in the adult sheep by determining the weight of ingested hay offered over a 4 h period following a 72 h period of fasting *ad libitum*.

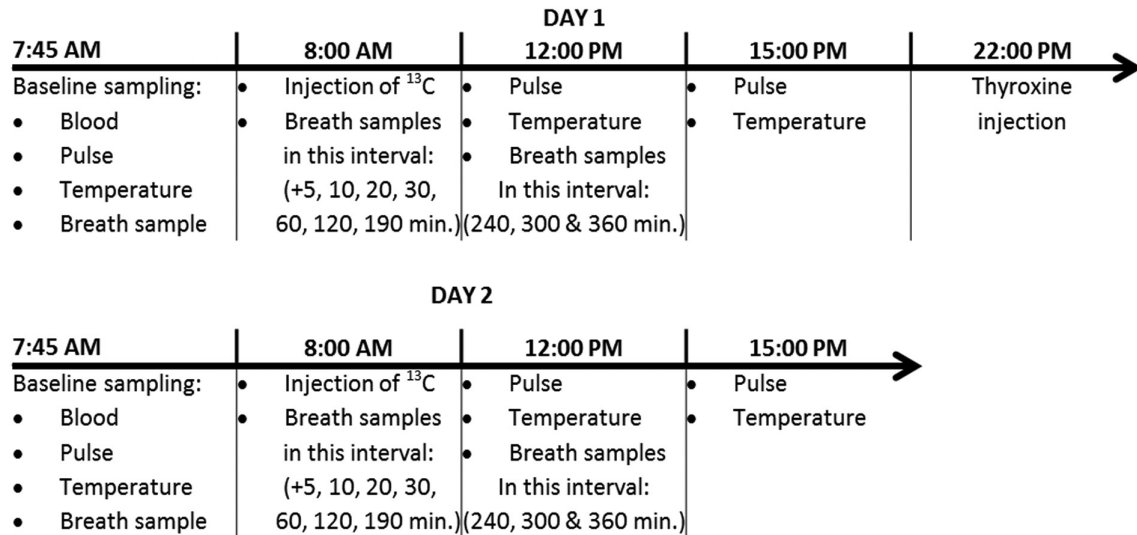
### Conduct of iTTT, metabolic measurements and sample analyses

An iTTT was conducted over 2 days in the adult sheep at 2½ years-of-age to study changes in the HPT axis sensitivity

and to obtain indicators of whole body gross metabolism. Sheep were transferred to individual sawdust-bedded pens placed within the barn, where they were normally housed. The pens allowed for limited physical contact with neighboring sheep through the bars of the pen, and the sheep were given a day to adjust to the pens before conducting the test. Sheep were fasted overnight before onset of the tolerance test and throughout the 2 day period of measurements and samplings, but had access to water *ad libitum*. The timing of intravenous thyroxine injection and the measurements and samplings performed over 2 days, preceding and following the injection, are shown in Fig. 1. On day 1, baseline blood and breath samples were collected, and pulse and temperature measured at time point 07:45 h, whereafter each sheep received a single bolus injection into the jugular vein of a 4 mg/kg metabolic body weight (MBW) dose of a 50 mg/ml  $^{13}\text{C}$ -bicarbonate (Sigma-Aldrich, product no. 372382) solution in sterile 0.9% saline (time point 08:00 h). Exhaled breath was collected in 1 L TECOBAGs (Tesseraux, Burstad, Germany) using an anesthetic mask (large 271435 or X-large 271436 from KRUSE A/S, Demark) with a mounted two-way non-rebreathing valve system (Hans Rudolph Inc., Kansas City, MO, USA), at 5, 10, 20, 30, 60, 120 and 190 min after the  $^{13}\text{C}$ -bicarbonate injection. Temperature and pulse were measured again 4 and 7 h after the injection (time points 12:00 h and 15:00 h), and breath samples were collected again 240, 300 and 360 min post-injection. The sheep were then left undisturbed until 22:00 h, where they received an intravenous bolus injection of 0.1 mg  $\text{T}_4$ /kg body weight (BW). The  $\text{T}_4$  solution was prepared by dissolving L-Thyroxine sodium pentahydrate (T2501, Sigma-Aldrich) in methanol (4 mg/ml) under sterile conditions and the solution was filtered through a 0.22  $\mu\text{m}$  polyethersulfone membrane filter immediately prior to use.

On day 2 of the tolerance test, all steps taken on day 1 were repeated at the same time points.

Whole body EE was established by the  $^{13}\text{C}$ -bicarbonate tracer technique, which has been validated in several species and can be used for free ranging animals (29, 30, 31, 32). The breath samples were used for measurement of the  $^{13}\text{C}:^{12}\text{C}$  isotope ratios ( $\delta^{13}\text{C}$ , ‰) using an IRIS (infrared isotope analyzer system,  $^{13}\text{C}$  Wagner Analysen Technik GmbH, Bremen, Germany). Heart rates were assessed using a stethoscope and a stop-watch, counting the heart-beats for 60 s, and using the average value obtained from three repeated determinations. Rectal temperatures were measured using an electronic standard rectal thermometer. All blood samples were collected in serum tubes by venipuncture of a jugular vein.



**Figure 1**

Schematic presentation of the experiment, where baseline blood samples, temperature, heart rate and energy expenditure was measured on day 1 and L-thyroxine (0.1 mg/kg LW) was injected into the jugular vein of sheep at 22:00 h. 12 h later, on day 2, all parameters were tested again, when the thyroxin response was assumed appropriately elevated.

The serum samples were left at room temperature for ~20 min and then centrifuged (1800 × G<sub>av</sub>, 15 min at 4°C) to separate serum, which was then stored in cryotubes at -20°C until analyzed. Concentrations of total T<sub>4</sub>, T<sub>3</sub> and TSH in serum were measured at the laboratory of Dr Blache, University of Western Australia, Australia, using a double-antibody radioimmunoassay as described previously (33, 34). The sensitivity was 0.12 nM for T<sub>4</sub> and 0.02 ng/ml for T<sub>3</sub> and TSH. The intra-assay variation for T<sub>4</sub>, T<sub>3</sub> and TSH was 3.0–5.1, 3.3–5.6 and 5.3–7.6% and the inter-assay variation was 5.6–6.4, 6.2–7.9 and 7.2–8.1%, respectively.

### Data handling

The <sup>13</sup>C:<sup>12</sup>C isotope ratios (δ<sup>13</sup>C, ‰) in expired air were used to compute the atom percentage excess of <sup>13</sup>C in expired air at the different time points relative to the <sup>13</sup>C isotope injection as previously described (30). The atom percentage excess depicted over time forms an exponential degrading curve. The area under this curve was used to calculate the CO<sub>2</sub> production, RCO<sub>2</sub> (mol/min) as in (Eq. 1):

$$RCO_2 = \left( \frac{D}{AUC} \right) \times RF \quad (1)$$

where D is the dose of bicarbonate and RF is the fractional <sup>13</sup>C recovery in expired air. Under basal conditions,

RF varies between approximately 0.6–0.8 across species and a value of 0.7 was assumed for the first day (32, 35, 36). For experimental day 2, RF was set to be 1, based on the assumption that <sup>13</sup>C recovery increases to almost 100% after the increase in metabolism post-T<sub>4</sub> injection under fasting condition, equivalent to what has been seen during exercise (37). EE was then calculated by a modification of the Brouwer equation (38) (Eq. 2):

$$EE \left( \frac{kJ}{day} \right) = 5.16 \times RCO_2 \left( \frac{L}{day} \right) + 16.18 \times \frac{RCO_2}{RQ} - 5.9 \times N_u \quad (2)$$

RQ is the respiratory coefficient (ratio between eliminated CO<sub>2</sub> over consumed O<sub>2</sub>), and a value of 0.91 was assumed, as we have previously found for equally sized adult sheep (39). Urinary nitrogen N<sub>u</sub> and methane emission CH<sub>4</sub> have been found in previous studies, to be insignificant contributions to non-dietary induced changes in EE, even in species as rats and dogs with higher protein intakes than sheep (30, 40). They were therefore not assessed, and hence ignored in the calculations.

### Statistics

All statistical models were multifactorial models:

*Basal serum* =  $\mu + PRE * POST * SEX * AGE + birthweight + MBW + \kappa_m + \varepsilon_{PRE,POST,SEX,AGE}$

*Challenged serum* =  $\mu + PRE * POST * TIME + sex + birthweight + MBW + basal\ serum + \kappa_m + \varepsilon_{PRE,POST,TIME}$

*FeedIntake* =  $\mu + PRE * POST + sex + birthweight + MBW + \kappa_m + \varepsilon_{PRE,POST}$

*Temp/HR* =  $\mu + PRE * POST * TIME * DAY + sex + birthweight + MBW + \kappa_m + \varepsilon_{PRE,POST,TIME,DAY}$

*EE* =  $\mu + PRE * POST * DAY + sex + birthweight + MBW + \kappa_m + \varepsilon_{PRE,POST,DAY}$

In all models, the specific factor to be analyzed was described by all the qualitative explanatory variables, the overall mean ( $\mu$ ), two- to four-way interactions of fixed effects, and covariates, including birth weight, metabolic body weight and/or sex,  $\kappa_m$  is the random effect of twins and  $\varepsilon_{ijkl}$  is the residual variation  $\sim N(0, \sigma^2)$ . The universal sample space of the qualitative explanatory variables are:  $i = \{1, \dots, 3\}$ ,  $j = \{1, 2\}$ ,  $k = \{1, 2\}$  and  $l = \{1, 2\}$ . Sex was included as a fixed effect in calculations of basal serum values in the same sheep as lambs and adults, but not in the observations conducted in adults only (all remaining factors), where there was a confounding of gender and treatments due to an uneven distribution of males and females in treatment groups. All models were tested in R 2.10.1 (R Development Core Team, 2010) utilizing the packages nlme, anova and LSmeans for fitting, model reductions and multiple comparisons, respectively. Graphic model control (Plot) was carried out to find possible outliers and second, normality assumptions were evaluated by scatterplot, qqnorm and boxcox. Following this, the model was reduced by testing significance of any interactions by two-way anova and insignificant variables were eliminated from the model. Estimates and significance of the remaining factors were calculated by the function LSmeans and presented as least square means  $\pm$  s.e.m. and considered significant when  $P < 0.05$  and a tendency was declared when  $P < 0.10$ .

## Results

Overall, there were no effects of gender, pre- or postnatal nutrition treatments or their interactions unless specifically stated in the following. When interpreting the gender results or lack thereof, it must be borne in mind, that there were only one or two males in three of the groups (NORM:CONV, NORM:HCHF and HIGH:CONV).

## Serum T<sub>4</sub>, T<sub>3</sub>, TSH and their ratios

### Basal serum levels at 6 months and 2½-years-of-age

Serum concentrations of all three hormones increased with age ( $P < 0.01$ ), although the quantitative increases were small for T<sub>3</sub> (<8%). Males had lower concentrations of T<sub>4</sub> and T<sub>3</sub> than females ( $P = 0.01$  and  $P = 0.009$ , respectively) at both 6 months and 2½ years-of-age, but equal TSH concentrations (Table 2). LOW:CONV sheep had the lowest T<sub>3</sub>:T<sub>4</sub> ratios of all groups ( $P = 0.05$ ) due to numerically lowest levels of T<sub>3</sub> at both ages. The T<sub>3</sub>:TSH and T<sub>4</sub>:TSH ratios were increased by the HCHF diet at 6 months-of-age, but by 2½ years-of-age, all CONV sheep had increased their ratios to equal that of HCHF sheep ( $P = 0.001$  and  $0.01$ , respectively, for the postnatal diet and age interaction).

### Hormonal responses during the iTTT at 2½-years-of-age

The statistical analyses revealed a negative correlation between birth weight and responses to thyroxine of T<sub>4</sub>, T<sub>3</sub> and TSH ( $P = 0.0002$ ,  $P < 0.0001$ ,  $P = 0.004$ , respectively), whereas positive correlations were found for T<sub>3</sub> and TSH responses and basal hormonal levels ( $P < 0.0001$  and  $P = 0.01$ , respectively).

Serum thyroxine (Fig. 2A and B): The intravenous thyroxine injection induced a ten fold or more increase in serum T<sub>4</sub> (baseline levels around 350 nM (Table 2)) in all treatment groups between 12 and 19 h post-injection. Higher levels were reached in males compared to females ( $4238 \pm 181$  and  $3819 \pm 163$  nM, respectively,  $P = 0.001$ ). In the fetally overnourished HIGH sheep, serum thyroxine had a longer half-life of 70.2 h compared to 57.8 h in NORM and 51.0 h in LOW ( $P = 0.002$ ).

Serum T<sub>3</sub> (Fig. 2C), TSH (Fig. 2D) and ratios between hormones (Fig. 3): The dramatic increase in T<sub>4</sub> caused an approximately two-fold increase in serum T<sub>3</sub> and a 23% reduction in TSH compared to the basal levels of approximately 1.6 and 0.16 ng/ml, respectively (Table 2). T<sub>3</sub> and TSH levels remained stable (results not shown) over the 12–19 h sampling period post-injection, where levels of T<sub>4</sub> continued to be supra-physiological. Responses for all parameters depended on the specific combination of nutrition exposures in pre- and early postnatal life.

### NORM sheep

NORM:CONV sheep had the largest increments (similar to some other groups; see below) in serum levels of T<sub>3</sub>, and ratios of T<sub>3</sub>:T<sub>4</sub>, T<sub>3</sub>:TSH after thyroxine administration, and the largest reductions in TSH levels. NORM sheep exposed



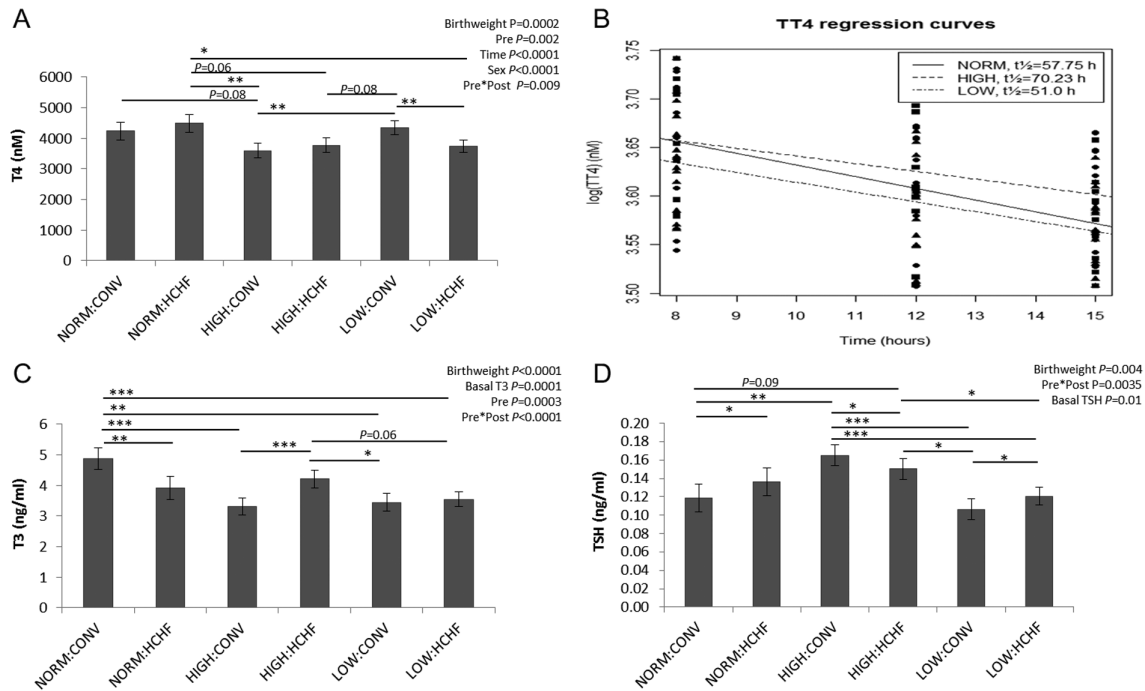
**Table 2** Mean basal serum levels of T<sub>4</sub> (nM), T<sub>3</sub> (ng/ml) and TSH (ng/ml) and T<sub>3</sub>:T<sub>4</sub>, T<sub>3</sub>:TSH and T<sub>4</sub>:TSH ratios, for female (n = 22) and male (n = 16) sheep at 6 months and 2½ years-of-age.

Parameter	Treatment groups based on combination of pre- and postnatal nutrition						Sex		P values								
	NORM:CONV	NORM:HCHF	HIGH:CONV	HIGH:HCHF	LOW:CONV	LOW:HCHF	Male	Female	Pre	Post	Pre*Post	Post*Age	Pre*Age	Sex	Age	MBW	Birthweight
6 months-of-age																	
T <sub>4</sub> (nM)	276±34	249±36	266±30	306±31	263±30	266±26	234±22 <sup>a</sup>	309±28 <sup>b</sup>	0.44	0.76	0.39	0.30	0.54	0.005	-	0.10	0.93
T <sub>3</sub> (ng/ml)	1.45±0.22	1.64±0.24	1.59±0.20	1.55±0.21	1.26±0.20	1.61±0.17	1.34±0.14 <sup>b</sup>	1.69±0.19 <sup>b</sup>	0.85	0.23	0.41	0.14	0.14	0.01	-	0.40	0.03
TSH (ng/ml)	0.11±0.02	0.12±0.02	0.13±0.02	0.12±0.02	0.12±0.02	0.11±0.01	0.12±0.01	0.12±0.02	0.79	0.74	0.65	0.17	0.42	0.98	-	0.68	0.63
T <sub>3</sub> :T <sub>4</sub>	0.0051±0.0007	0.0062±0.0008 <sup>b</sup>	0.0057±0.0006 <sup>b</sup>	0.0050±0.0006	0.0045±0.0006 <sup>a</sup>	0.0059±0.0005 <sup>b</sup>	0.0055±0.0005	0.0053±0.0006	0.86	0.14	0.05	0.36	0.55	0.60	-	0.46	0.02
T <sub>3</sub> :TSH	7.6±2.6 <sup>a</sup>	13.8±2.6 <sup>b,d</sup>	11.2±2.4 <sup>a,d,e</sup>	17.4±2.3 <sup>b</sup>	9.1±2.1 <sup>d,a,c</sup>	15.3±2.0 <sup>b,e</sup>	11.0±1.4	13.5±1.2	0.89	0.10	0.69	0.001	0.07	0.07	-	0.65	0.29
T <sub>4</sub> :TSH	1686±461 <sup>a,c</sup>	2460±458 <sup>b,d</sup>	1825±425 <sup>a,d,e</sup>	2599±406 <sup>b,c,f</sup>	1870±400 <sup>a,d,f</sup>	2644±388 <sup>b,c,e</sup>	1868±315 <sup>a</sup>	2494±394 <sup>b</sup>	0.96	0.82	0.32	0.01	0.17	0.03	-	0.79	0.97
2½-years-of-age																	
T <sub>4</sub> (nM)	365±34	339±36	355±34	395±30	353±28	355±33	323±31 <sup>a</sup>	398±21 <sup>b</sup>	0.44	0.76	0.39	0.30	0.54	0.005	<0.0001	0.10	0.93
T <sub>3</sub> (ng/ml)	1.53±0.22	1.73±0.23	1.68±0.22	1.64±0.19	1.35±0.19	1.69±0.22	1.42±0.20 <sup>b</sup>	1.78±0.14 <sup>b</sup>	0.85	0.23	0.41	0.14	0.14	0.01	-	0.40	0.03
TSH (ng/ml)	0.15±0.02	0.16±0.02	0.16±0.02	0.16±0.02	0.16±0.02	0.15±0.02	0.16±0.02	0.16±0.01	0.79	0.74	0.65	0.17	0.42	0.98	-	0.68	0.63
T <sub>3</sub> :T <sub>4</sub>	0.0046±0.0007	0.0057±0.0007 <sup>b</sup>	0.0052±0.0007 <sup>b</sup>	0.0045±0.0006	0.0040±0.0006 <sup>a</sup>	0.0054±0.0007 <sup>b</sup>	0.0050±0.0006	0.0049±0.0004	0.86	0.14	0.05	0.36	0.55	0.60	-	0.46	0.02
T <sub>3</sub> :TSH	14.1±2.5	12.7±2.6	11.4±2.4	10.0±2.4	13.5±2.2	12.0±2.3	11.0±1.4	13.6±1.2	0.89	0.10	0.69	0.001	0.07	0.07	-	0.87	0.29
T <sub>4</sub> :TSH	2942±439	2401±453	3081±428	2540±429	3126±420	2584±427	2466±425 <sup>a</sup>	3092±299 <sup>b</sup>	0.96	0.82	0.32	0.01	0.17	0.03	-	0.79	0.97

Data are presented as least square means±s.e.m. Effects of prenatal nutrition, postnatal nutrition or gender were significant or tended to be significant ( $P < 0.05$  or  $P < 0.1$ ) if the data within a row and within the respective columns are marked by different superscripts. There was no three-way interaction between treatments and age. Pre, postnatal diet; Post, postnatal diet; MBW, metabolic body weight. The last 6 weeks of pregnancy, the ewes received either a HIGH (150% required digestible energy and 110% required protein) or LOW (50% required energy and protein) or NORM diet (100% required energy and protein). Postpartum twin lambs were assigned to a HCHF diet (High-Carbohydrate High-Fat diet of cream-milk replacer mix and rolled maize supplement) or CONV diet (conventional diet with milk replacer and hay until 8 weeks followed by hay only, adjusted for moderate growth rates of approx. 225 g/day). Twins were assigned postnatal treatment groups ensuring as uniform a distribution of gender (first priority) and birth weight (second priority) as possible. The six treatment groups in present experiment was: NORM:CONV (n = 5, 4f, 1m), NORM:HCHF (n = 4, 2f, 2m), HIGH:CONV (n = 6, 4f, 2m), HIGH:HCHF (n = 7, 4f, 3m), LOW:CONV (n = 8, 4f, 4m) and LOW:HCHF (n = 8, 4f, 4m).

\*Thyroid weights, total and in % of bodyweight have been included, these results were previously presented in Khanal et al. (12).





**Figure 2**

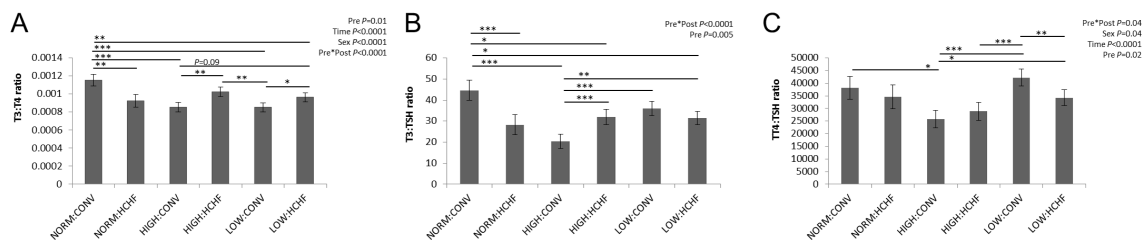
Group LSmeans of serum total  $T_4$  (nM) (A),  $T_3$  (ng/mL) (C) and TSH (ng/mL) measured 12, 16 and 19 h after injection of 0.1 mg thyroxine/kg LW, in 2.5-year-old sheep, male and female (m: male, f: female), with a history of NORM, HIGH or LOW in late gestation, combined with a CONV or a HCHF diet for the first 6 months postnatal, yielding NORM:CONV ( $n=5$ , 4f, 1m), NORM:HCHF ( $n=4$ , 2f, 2m), HIGH:CONV ( $n=6$ , 4f, 2m), HIGH:HCHF ( $n=7$ , 4f, 3m), LOW:CONV ( $n=8$ , 4f, 4m) and LOW:HCHF ( $n=8$ , 4f, 4m). (B) Regression curves of total  $T_4$  (log(conc)) (not presented as LSmeans), in 2.5-year-old sheep, male and female, where prenatal treatments NORM (■), HIGH (●) and LOW (▲) significantly impacted  $T_4$  half-life upon injection ( $P=0.01$ ). Experimental design and dietary treatments have been fully described in the legend to Table 2. Significant differences are  $*P\leq 0.05$ ,  $**P\leq 0.01$ , or  $***P\leq 0.001$ . CONV, conventional; HCHF, High-Carbohydrate-High-Fat; HIGH, overnutrition; LOW, undernutrition; NORM, adequate nutrition.

to the early postnatal obesogenic HCHF diet had reduced responses in  $T_3$  ( $P=0.0002$ ), TSH ( $P=0.02$ ),  $T_3:T_4$  ( $P=0.001$ ), and  $T_3:TSH$  ( $P<0.0001$ ) compared to NORM:CONV sheep.

**HIGH sheep**

Had similar increases in  $T_3$  as CONV sheep after thyroxine administration, but reduced their TSH levels less (Fig. 2D),

resulting in lower  $T_3:TSH$  (Fig. 3B) and  $T_4:TSH$  levels (Fig. 3C). Interestingly, HIGH and NORM sheep had opposite responses to the early postnatal HCHF diet for all parameters ( $T_3$   $P<0.0001$ ; TSH  $P=0.003$ ;  $T_3:T_4$   $P<0.0001$  and  $T_3:TSH$   $P<0.0001$ ), except  $T_4:TSH$ , which was unaffected by postnatal diet in both NORM and HIGH). Thus, in HIGH:HCHF sheep  $T_3$ ,  $T_3:T_4$ ,  $T_3:TSH$  levels were comparable to those reached in NORM:CONV



**Figure 3**

LSmeans of serum total  $T_3:T_4$  (A),  $T_3:TSH$  (B) and  $T_4:TSH$  (C) ratios, 12, 16 and 19 h post-injection of 0.1 mg thyroxine/kg LW, in 2.5 year-old-sheep, male and female, with a history of NORM, HIGH or LOW in late gestation, combined with a CONV or a HCHF diet for the first 6 months postnatal, yielding NORM:CONV ( $n=5$ , 4f, 1m), NORM:HCHF ( $n=4$ , 2f, 2m), HIGH:CONV ( $n=6$ , 4f, 2m), HIGH:HCHF ( $n=7$ , 4f, 3m), LOW:CONV ( $n=8$ , 4f, 4m) and LOW:HCHF ( $n=8$ , 4f, 4m). Experimental design and dietary treatments have been fully described in the legend to Table 2. Significant differences are  $*P\leq 0.05$ ,  $**P\leq 0.01$ , or  $***P\leq 0.001$ . CONV, conventional; HCHF, High-Carbohydrate-High-Fat; HIGH, overnutrition; LOW, undernutrition; NORM, adequate nutrition.

post-injection, and levels in HIGH:CONV were as low as those observed in NORM:HCHF. Furthermore, HIGH sheep exposed to the early postnatal CONV were less efficient in reducing TSH levels in response to thyroxine as compared to HIGH:HCHF sheep ( $P < 0.05$ ; Fig. 2D).

**LOW sheep**

LOW:CONV sheep had the smallest increases in  $T_3$  and hence the lowest  $T_3:T_4$  ( $P = 0.004$  and  $P = 0.001$ ) after thyroxine administration, similar to values observed in the nutritionally mismatched NORM:HCHF and HIGH:CONV sheep, whereas LOW:CONV had the largest reductions in TSH. In LOW sheep exposed to the early postnatal HCHF diet, the suppressive effect of thyroxine on TSH was reduced ( $P < 0.05$ ), resulting in lower  $T_4:TSH$  ( $P < 0.01$ ), but higher  $T_3:T_4$ . In LOW sheep, levels of  $T_3$  and  $T_3:TSH$  ratio were unaffected by the early postnatal diet (in contrast to NORM and HIGH sheep).

In general, response patterns in the nutritionally mismatched HIGH:CONV and NORM:HCHF sheep thus resembled each other, and the same was true for the nutritionally matched groups: NORM:CONV and HIGH:HCHF. Response patterns in LOW sheep did not resemble any of the NORM or HIGH groups in a consistent way and was less consistently affected by the postnatal diet than NORM and HIGH sheep.

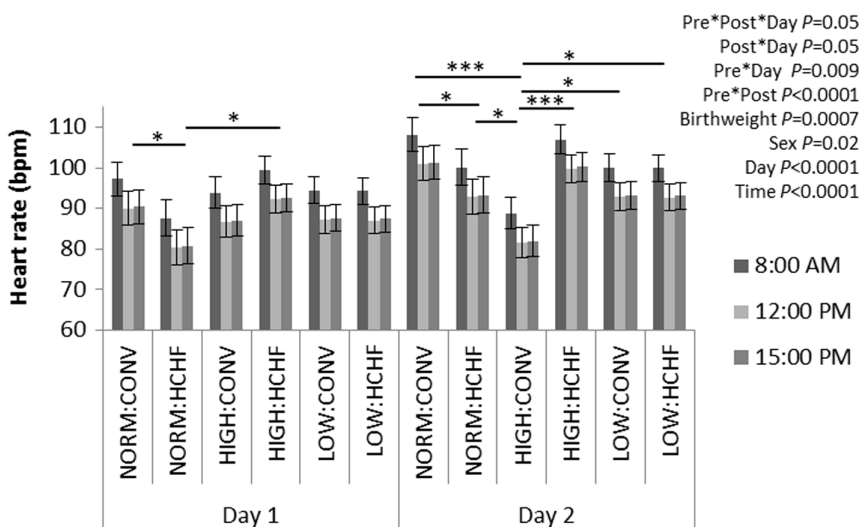
**Heart rates and rectal temperature during the ITTT**

Heart rates were higher in the morning than in the afternoon on day 1 and 2 ( $P < 0.0001$ ; Fig. 4). Males had lower heart rates (around 85.0 b.p.m) compared to females

(93.9 b.p.m) ( $P = 0.005$ ). Rectal temperatures (Fig. 5) dropped during the day on day 1, but increased on day 2 ( $P < 0.001$  for the Time\*Day interaction). The highest heart rates and rectal temperatures (day 1) were observed in the NORM:CONV and HIGH:HCHF groups, and the lowest in the nutritionally mismatched NORM:HCHF and HIGH:CONV and in all LOW sheep ( $P = 0.001$  for the pre- and postnatal nutrition interaction), similar to the response patterns after thyroxine administration for  $T_3$  (Fig. 2C) and  $T_3:T_4$  (Fig. 3A). Thyroxine increased heart rates from an average of 90 b.p.m before administration (day 1) to an average of 95 b.p.m after administration (day 2) ( $P < 0.0001$ ) (Fig. 4), and body temperatures were increased day 2 with on average 0.2°C ( $P = 0.001$ ) above the untreated level (day 1) of 38.3–39.5 (Fig. 5). Only HIGH:CONV sheep did not have a numerical increase in their heart rates after thyroxine administration. There were no differences in increments in heart rate or temperature from day 1 to day 2 between the groups.

**Energy expenditure during the ITTT**

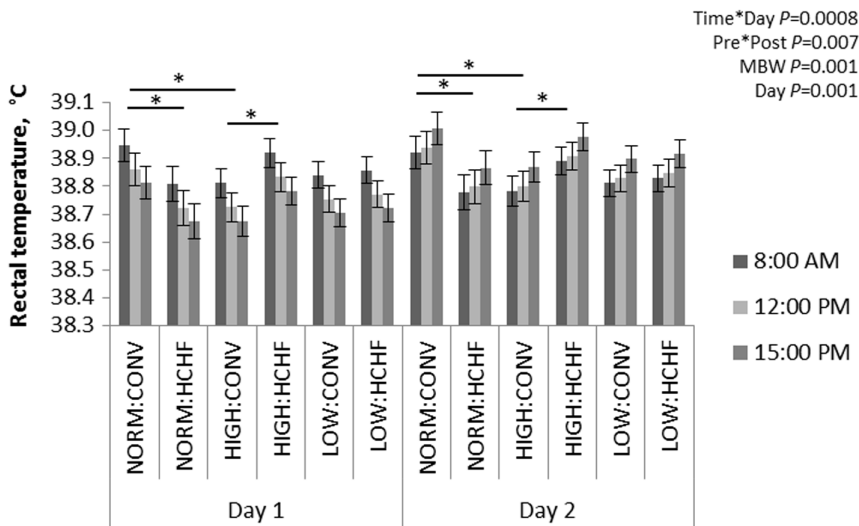
The daily EE averaged 497 kJ/MBW/day prior to thyroxine administration (day 1). NORM and HIGH sheep exposed to the early postnatal HCHF had lower EE compared to those fed the CONV diet, whereas EE of LOW sheep was unaffected by the early postnatal diet ( $P = 0.05$  for the pre\*postnatal nutrition interaction) (Fig. 6). Thyroxine administration increased EE day 2 by ~40% in HCHF compared to only ~18% in CONV sheep ( $P = 0.00001$  and  $P = 0.003$ , respectively), and this obliterated any group differences except for a higher EE in LOW:HCHF ( $P < 0.05$  and  $P = 0.06$  compared to LOW:CONV and NORM:HCHF, respectively).



**Figure 4**

LSmeans of heart rates measured at basal state (day 1) at 08:00, 12:00 and 15:00 h and after injection of 0.1 mg thyroxine/kg LW (day 2), at 12 (08:00 h), 16 (12:00 h) and 19 (15:00 h) hours post-injection, in 2.5-year-old sheep, male and female, with a history of NORM, HIGH or LOW in late gestation, combined with a CONV or a HCHF diet for the first 6 months postnatal, yielding NORM:CONV ( $n = 5, 4f, 1m$ ), NORM:HCHF ( $n = 4, 2f, 2m$ ), HIGH:CONV ( $n = 6, 4f, 2m$ ), HIGH:HCHF ( $n = 7, 4f, 3m$ ), LOW:CONV ( $n = 8, 4f, 4m$ ) and LOW:HCHF ( $n = 8, 4f, 4m$ ). Experimental design and dietary treatments have been fully described in the legend to Table 2. Significant differences are  $*P \leq 0.05$ ,  $**P \leq 0.01$ , or  $***P \leq 0.001$ . CONV, conventional; HCHF, High-Carbohydrate-High-Fat; HIGH, overnutrition; LOW, undernutrition; NORM, adequate nutrition.





**Figure 5**

LSmeans of rectal temperatures measured at basal state (day 1) at 08:00, 12:00 and 15:00 h and after injection of 0.1 mg thyroxine/kg LW, at 12 (08:00 h), 16 (12:00 h) and 19 (15:00 h) hours post-injection, in 2.5 year-old sheep, male and female, with a history of NORM, HIGH or LOW in late gestation, combined with a CONV or a HCHF diet for the first 6 months postnatal, yielding NORM:CONV ( $n=5$ , 4f, 1m), NORM:HCHF ( $n=4$ , 2f, 2m), HIGH:CONV ( $n=6$ , 4f, 2m), HIGH:HCHF ( $n=7$ , 4f, 3m), LOW:CONV ( $n=8$ , 4f, 4m) and LOW:HCHF ( $n=8$ , 4f, 4m). Experimental design and dietary treatments have been fully described in the legend to Table 2. Significant differences are  $*P\leq 0.05$ ,  $**P\leq 0.01$ , or  $***P\leq 0.001$ . CONV, conventional; HCHF, High-Carbohydrate-High-Fat; HIGH, overnutrition; LOW, undernutrition; NORM, adequate nutrition.

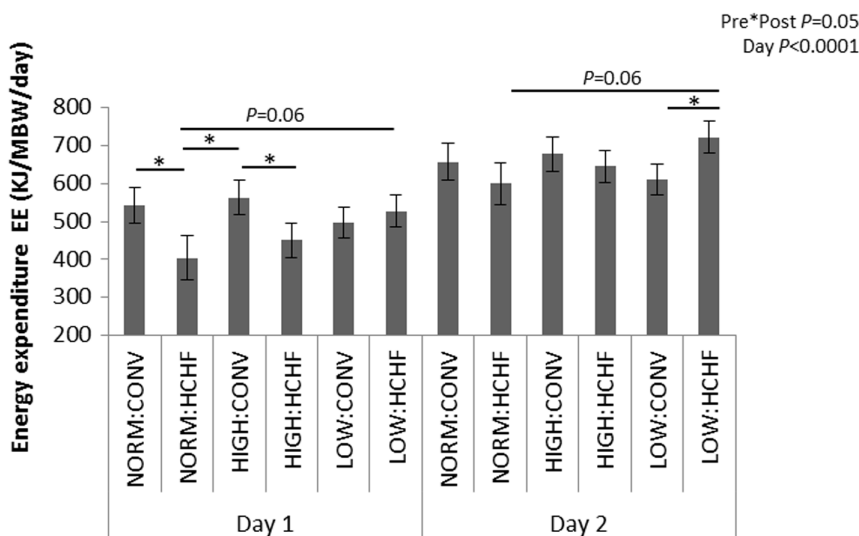
**Feed intake capacity in 2½ year-old sheep**

Feed intake capacity (feed intake over 4 h, following a 72 h fasting period) was increased in NORM and LOW, but not HIGH, sheep that had been fed the early postnatal HCHF diet ( $P=0.02$  and  $0.03$ , respectively) (Fig. 7). Adult feed intake tended to correlate positively with birth weight and correlated positively with MBW ( $P=0.08$  and  $P=0.006$ ).

**Discussion**

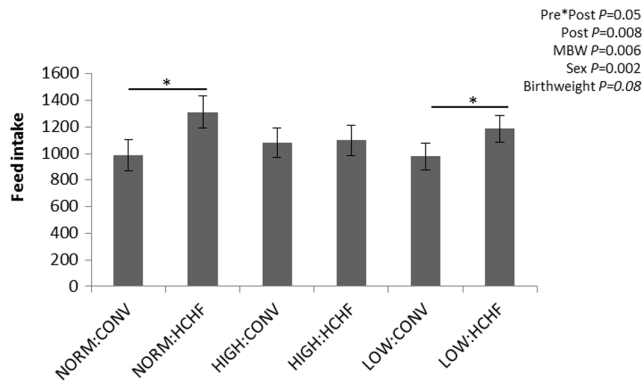
The aim of this experiment was to study the long-term consequences of prenatal over- vs undernutrition on HPT axis function and subsequent obesity development, and to relate alterations in HPT axis function to certain whole body metabolic traits in sheep. Serum levels of THs

observed during the iTTT, but not basal concentrations in the unchallenged state, revealed lasting and differential changes to the HPT axis regulatory function depending on the combination of nutrition exposures in late gestation and early postnatal life (Fig. 8). This is noteworthy, since the thyroid is an organ that develops and assumes secretory activity in early fetal life (embryonic day 42–45 in sheep) (41). It was interesting that adverse effects of the early postnatal HCHF diet appeared to be absent in the prenatally overnourished HIGH sheep. Thus, the nutritionally matched HIGH:HCHF and NORM:CONV sheep resembled each other, and perhaps even more surprising, so did the nutritionally mismatched HIGH:CONV and NORM:HCHF sheep. Prenatal undernutrition, however, induced consistent changes in LOW sheep, particularly  $T_3$  responses, which were mostly observed irrespectively of the early postnatal diet.



**Figure 6**

Group LSmeans for energy expenditure in 2.5-year-old fasted sheep, male and female, with a history of NORM, HIGH or LOW in late gestation, combined with a CONV or a HCHF diet for the first 6 months postnatally, yielding NORM:CONV ( $n=5$ , 4f, 1m), NORM:HCHF ( $n=4$ , 2f, 2m), HIGH:CONV ( $n=6$ , 4f, 2m), HIGH:HCHF ( $n=7$ , 4f, 3m), LOW:CONV ( $n=8$ , 4f, 4m) and LOW:HCHF ( $n=8$ , 4f, 4m). Day 1 represents basal energy expenditure and day 2 the sheep have been subjected 0.1 mg thyroxine/kg LW 12–19 h previously. Experimental design and dietary treatments have been fully described in the legend to Table 2. Significant differences are  $*P\leq 0.05$ ,  $**P\leq 0.01$ , or  $***P\leq 0.001$ . CONV, conventional; HCHF, High-Carbohydrate-High-Fat; HIGH, overnutrition; LOW, undernutrition; NORM, adequate nutrition.



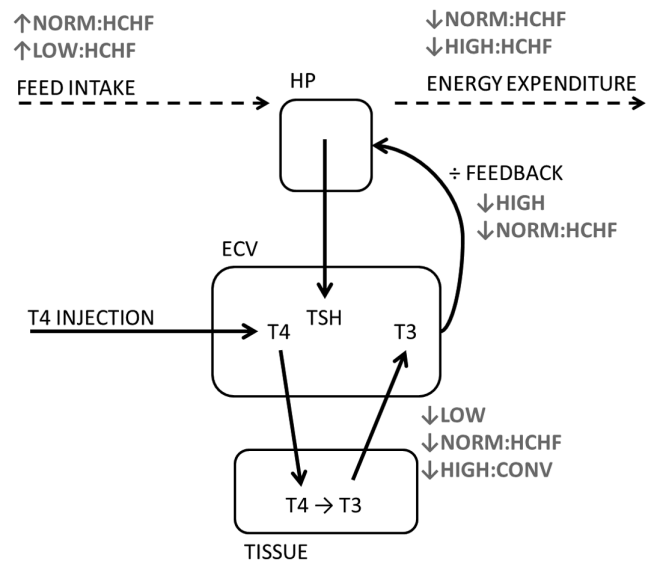
**Figure 7** Voluntary feed intake based on residues of 2 kg green hay weighed 4 h after feeding, following a 72 h fasting challenge in 2.5-year-old sheep, male and female, with a history of NORM, HIGH or LOW in late gestation, combined with a CONV or a HCHF diet for the first 6 months postnatal, yielding NORM:CONV ( $n=5$ , 4f, 1m), NORM:HCHF ( $n=4$ , 2f, 2m), HIGH:CONV ( $n=6$ , 4f, 2m), HIGH:HCHF ( $n=7$ , 4f, 3m), LOW:CONV ( $n=8$ , 4f, 4m) and LOW:HCHF ( $n=8$ , 4f, 4m). Experimental design and dietary treatments have been fully described in the legend to Table 2. Significant differences are  $*P \leq 0.05$ ,  $**P \leq 0.01$ , or  $***P \leq 0.001$ . CONV, conventional; HCHF, High-Carbohydrate-High-Fat; HIGH, overnutrition; LOW, undernutrition; NORM, adequate nutrition.

**Does exposure to overnutrition in late fetal life provide an adaptive advantage to an obesogenic diet in early postnatal life?**

It was surprising that the adult HIGH:HCHF sheep had similar response patterns to NORM:CONV sheep, and even more surprising that there were similar response patterns for NORM:HCHF and HIGH:CONV sheep, in spite of the contrasting postnatal dietary exposures.

There have been indications from this same study that HIGH sheep had a superior ability to LOW and NORM sheep to recover normo-adiposity and normal-metabolic function after development of obesity in early postnatal life, when their diet was corrected to a low-fat diet later in life (12). In mice, it has been found that pups exposed to a high-fat/high-sucrose diet in utero developed less severe phenotypic alterations at weaning, if their dams continued on this diet throughout lactation, rather than being transferred to a normal chow diet during lactation (42). As adults they had improved resistance toward development of hyperleptinemia upon exposure to a high-fat diet.

Thus, overnutrition in fetal life appear to provide an adaptive advantage, when an individual is exposed to overnutrition postnatally, and this is in contrast to what has been observed individuals born small for gestational age (43, 44).



**Figure 8** Summary of the most notable findings in relation to HPT axis responses according to 2.5-year-old sheep's prenatal and postnatal dietary intake in response to an intravenous thyroxine tolerance test (male and female). The depicted findings regarding feed intake and energy expenditure are in the basal unchallenged state. Experimental design and dietary treatments have been fully described in the legend to Table 2.

The HIGH:CONV sheep appeared to have less extensive  $T_4 \rightarrow T_3$  activation (peripheral, thyroidal or both), as reflected in their lower basal and challenged  $T_3:T_4$  ratios. This sort of peripheral programming has also been seen in Japanese macaques and female sheep, where maternal high-fat diets disrupted fetal tissue TH receptors and their downstream regulators (45, 24). This interference of peripheral TH function in tissues, quantitatively important in energy metabolism, is in line with the observation that HIGH:CONV sheep did not increase their heart rate in response to a thyroxine surge.

The failure of HIGH:CONV sheep to suppress serum TSH levels to the same extent as in other groups, points to a less sensitive negative feedback control of TSH secretion in response to a dramatic increase (more than two fold) in  $T_3$  concentrations. This indicates additional development of central resistance to  $T_3$ . In humans, it is a common observation that obesity is associated with isolated hyperthyrotropinemia. The mechanism behind this hyperthyrotropinemia is not well understood, but it is apparently not explained by changes in peripheral activity of THs (46). Instead, it is thought that isolated elevated TSH may represent a possible hypothalamic-pituitary hormone resistance and disturbed negative feedback

signaling (46, 47). Maternal overnutrition has been shown to cause leptin resistance in rats through decreased STAT3 and SOCS3 gene signaling in the arcuate nucleus (20), and increased circulating leptin concentrations have the capability to stimulate pro-TRH formation directly and by affecting neurons of the HPA axis, which in turn can trigger pituitary TSH stimulation (48, 49). The same mechanisms may be at play in both NORM:HCHF sheep and HIGH:CONV sheep. The adult sheep in our study could not be characterized as obese, but had a certain degree of adiposity, which may explain why the hyperthyrotropinemia was only revealed during the iTTT.

Although the postnatal HCHF diet gave rise to opposite alterations in HIGH and NORM sheep for most HPT axis traits, the HCHF fed HIGH and NORM sheep had similar reductions in adult EE, although they had been fed the same moderate diet for 2 years. It has also been observed in humans that weight loss following obesity decreases EE (50), which is instinctively counter-productive to maintaining lipobody-homeostasis. Obesity-induced changes in EE have been linked to gliosis (remodeling) of the hypothalamus in mice and humans (51, 52), and this remodeling could be reversible in mice (53). The permanent changes in EE in our study could not be directly related to functional changes in the HPT axis, and the early-life programming of the HPT axis function as well as EE were clearly irreversible in our sheep. Future studies are clearly needed to resolve the differential programming of hypothalamic functions involved in regulation of energy metabolism and associated obesity risk.

### Programming of the HPT axis by late gestation undernutrition is manifested irrespective of the early postnatal nutrition

In general, the two LOW groups had very similar response patterns across all the tested parameters. This was unexpected, since fasting-tolerance tests in these sheep revealed pronounced interactions between pre- and postnatal treatments, where LOW:HCHF sheep were predisposed to develop adult hypercholesterolemia, hypercreatinemia and fasting-induced hyperuremia (12). However, both LOW:CONV and LOW:HCHF sheep had similar reductions in  $T_4 \rightarrow T_3$  activation resembling that of NORM:HCHF (and HIGH:CONV) sheep in response to the thyroxine administration. The smallest increments in  $T_3$  levels and numerically lowest TSH concentration in response to a thyroxine surge were observed in LOW, irrespective of the postnatal diet (in contrast to NORM

and HIGH sheep). Since the lowered  $T_3$  was not reflected in higher serum TSH, the hypothalamus/pituitary sensitivity to modulate TSH production in response to changes in circulating levels of  $T_3$  may have been reduced. It was solely in the LOW:CONV sheep that lower basal levels of  $T_3$  as well as TSH indicated a less effective activation of  $T_4 \rightarrow T_3$  and a reduced HPT axis sensitivity, respectively. During the iTTT, LOW:HCHF sheep increased EE more than LOW:CONV sheep, possibly relating to a greater capacity for peripheral  $T_4 \rightarrow T_3$  conversion during the tolerance test. In general, EE was not reduced in LOW:HCHF compared to LOW:CONV (in the unchallenged state), contrary to NORM and HIGH sheep exposed to the HCHF diet.

Although prenatal undernutrition did not alter the basal metabolic phenotype, early postnatal exposure to the HCHF diet was associated with an increased voluntary feed intake of all LOW sheep, and this may be a main risk factor for development of adult obesity. During fasting-, glucose- and insulin-tolerance tests, LOW:HCHF sheep were observed to have increased plasma levels of blood urea nitrogen (BUN), cholesterol, creatinine, lactate, triglyceride (TG) and non-esterified fatty acid (NEFA) in one or more of these tolerance tests, as previously reported (28). So, although functional programming of the HPT axis and the hypothalamic control of appetite seemed to be of primarily prenatal origin in LOW sheep, other peripheral endocrine systems and metabolic features under their control were indeed adversely affected by a mismatching obesogenic dietary exposure in early postnatal life.

In a different study including adult female sheep only, we found that basal TH levels were increased in adulthood in sheep with a history of late gestation undernutrition (24). This was not observed in the present study. In both experiments however, the long-lasting programming of the HPT axis among LOW sheep was of prenatal rather than postnatal origin. Several differences between the two experiments could have accounted for the different phenotypic outcomes. In the present compared to the previous experiment, dietary energy intake was higher due to a higher digestibility of the (low-fat) hay-based diet, which allowed sheep to deposit more fat and reach higher body condition scores in the present (12) compared to the previous experiment (3). In fact, all female sheep in the present experiment had  $T_3$  levels, which were almost as high as the LOW, and higher than the NORM, female sheep in the previous experiment (24). Furthermore, they were of different breeds, and we cannot rule out that this may have influenced responses to the dietary interventions as well, although both breeds were bred for meat production.

In the present study, females had higher basal  $T_4$  and  $T_3$  both at 6 months- and 2½ years-of-age, as well as increased HR. However, we did not see indications of differential long-term consequences of the pre- and postnatal nutrition histories in adult males as compared to adult females for any of the other reported parameters.

In conclusion, the findings presented in this paper point to differential prenatal programming of functions both central (hyperthyrotropinemia in all HIGH sheep and to lesser extent in NORM and LOW sheep exposed to an early postnatal obesogenic diet) and peripheral (reduced  $T_4 \rightarrow T_3$  activation in all LOW and the nutritionally mismatched NORM:HCHF and HIGH:CONV sheep) to the HPT axis. Interestingly, the nutritionally matched NORM:CONV and HIGH:HCHF sheep therefore attained similar phenotypic traits relating to HPT axis function. In adult LOW sheep, early-nutritional programming was primarily of prenatal origin and targeted peripheral  $T_4 \rightarrow T_3$  activation. The long-term implications of early nutrition for HPT axis function were not reflected in basal TH or TSH levels, but exclusively observed in response to an intravenous injection of thyroxine. In future studies, due consideration should therefore be given to the diagnostic approach for evaluation of early programming of HPT axis function. Future studies are needed to establish the mechanisms underlying the early-life nutrition programming of HPT axis plasticity, and the apparent resistance in HIGH individuals toward adverse programming of the HPT axis function.

Programming of HPT axis function did not appear to be a dominant factor explaining the observed lasting impacts of early-life nutrition on adult EE and feed intake. The surprising lack of lasting hyperphagia and an apparently normal HPT axis function in HIGH:HCHF sheep suggest a resistance in fetally overnourished individuals toward (early) obesity development, although their adult EE was also permanently reduced. Thus, we encourage a reassessment of nutritional recommendations for humans to differentiate between individuals born small- as compared to large for gestational age.

#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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#### Author contribution statement

PhD Fellow L J was in charge of the animal experimentation, laboratory procedures, statistical evaluation of data and for writing the manuscript. MSc N B L was an invaluable help in practical planning and execution of the experiment, evaluated data on EE and participated in manuscript revision. Dr P K evaluated activity data and performed manuscript revision. Prof. M O N, vice-director of the Danish Centre for Fetal Programming, Denmark, developed the Copenhagen sheep model and designed the overall sheep experiment, participated in the experiment and performed manuscript revision. Prof. B Q and Principal Scientist K R contributed with valuable inputs in the interpretation of results and manuscript revision.

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