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Impact of Gestational Over- or Undernutrition and Early-life Overnutrition on Programming of the Hypothalamic-Pituitary-Thyroidal Axis in Sheep

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Impact of Gestational Over- or Undernutrition on Fetal Programming of the Hypothalamic-pituitary-thyroidal axis

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Preface

Present thesis is the final result to obtain the degree of philosophiae doctor, PhD. The work described in this thesis was carried out at Department of Large Animal Science, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark, over a period stretching from March 2012 – September 2016. My studies were funded through the SHARE scholarship, equally distributed between the, now former, Faculty of Life sciences, the Faculty of Health and Medical Sciences, University of Copenhagen, Denmark, and The Danish Council for Strategic Research , through the Research School for Animal Nutrition and Physiology, University of Copenhagen. The research was financially supported by the Danish Council for Strategic Research and the Centre for Fetal Programming (CFP). This study was further made possible through grants from the Novo Nordisk Foundation and the Lundbeck foundation.

I was supervised by Professor Mette Olaf Nielsen and Professor Bjørn Quistorff from the University of Copenhagen, Denmark, and Principal Scientist Kirsten Raun from Novo Nordisk A/S, Denmark.

The thesis is based on two experimental studies in sheep to investigate the life-long effects of prenatal and early-life nutrition on central and peripheral thyroid function.

As a part of this study I had to design an experiment to assess thyroid hormone impacts on the metabolic phenotype, which included a pilot study to determine dosage of thyroxin in a tolerance test and timing of the associated changes in selected metabolic indicators. I also had to design and apply a protocol for evaluation of thyroid histology using image analysis software (Visiopharm A/S, Denmark). This was done in collaboration with Cell Biologist and Center leader Michael Hansen at the Center for Advanced Bioimaging, Faculty of Science and Physiologist, DVM Sina Safayi.

The primary objective was to examine if prenatal and early-life nutrition asserted programming on the hypothalamic-pituitary-thyroidal axis and to determine if an obesogenic postnatal diet had an independent or correlated impact on thyroid function and to what extent adverse health effects due to unhealthy postnatal nutrition were reversible.

Hopefully this research may contribute to identify new targets for consideration in intervention strategies against development of fetal derived disorders and adult metabolic syndrome

Acknowledgements

In writing this thesis and during these last four years, I have had the tremendous pleasure to get to know so many inspiring and helpful persons, without whom I could not have come up with this end-product and whom I hope will also feel some pride and ownership of this thesis.

I would like to thank my supervisor Mette Olaf Nielsen for letting me join her initial sheep experiment back in my bachelor days, and for mentoring me through all the following years. She let me take ownership in my own little area of a larger experimental study and invited me into a very special science group. Mette Olaf Nielsen is passionate and creative and has been my biggest inspiration.

A very special thanks goes to "my" master student Nette Brinck Lyckegaard, who was brave enough to work with me on my pilot study and following experiment. She wrestled sheep and collected samples like a true champion! This could not have been done without her hard work and bright spirits.

I am very grateful to Dr. Sina Safayi for his patient guiding in making a protocol for histological evaluation of my thyroid slides and Cell biologist Michael Hansen for helping to understand specialist image analysis software.

I also very much appreciate that my co-supervisors Professor Bjørn Quistorff and Scientist Kirsten Raun took on this assignment and recommended the investigation of metabolic phenotype, that eventually became the second paper of this thesis.

Of course I also have to extend my appreciation to all involved in the experiments included in this thesis; Dennis S Jensen, laboratory technicians Vibeke G. Christensen, Semra Gündüz, Zaida R Rasmussen, Anna H. Kongsted, Sanne Husted, Marina Kjærgaard, Prabhat Khanal, Anne-Marie D. Axel. And thank you to all my wonderful colleagues at the Section for Animal Physiology and Nutrition, at the Department of Large Animal Sciences.

Dragana, sweetheart, thank you for bringing me food, bearing gifts and proof-reading.

To my family and friends, you are my world.

Copenhagen, September 2016

Lærke Johnsen

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List of publications

Present PhD thesis is based on Paper I-III, included in the end of the thesis. The papers are in the text referred to by their roman numerals.

Paper I Johnsen L., Kongsted AH and Nielsen MO: Prenatal undernutrition and postnatal overnutrition alter thyroid hormone axis function in sheep. *Journal of Endocrinology*. 2013. Vol. 216, pp. 389–402.

Paper II Johnsen L., Lyckegaard NB, Quistorff B, Raun K and Nielsen MO: Late gestation over or- overnutrition and early postnatal overnutrition affect thyroid function and metabolic phenotype in adult sheep. Submitted to *Thyroid*. August 2016.

Paper III Johnsen L and Nielsen MO: Effects of late gestation under- or overnutrition and early postnatal overnutrition on the hypothalamic-pituitary-thyroidal axis in sheep. In preparation.

Abbreviations

CONV	Hay based diet, moderate in carbohydrates and fat
CVD	Cardiovascular disease
DIT	Diiodotyrosine
HCHF	High carbohydrate, high fat diet
HIGH	Diet providing 150%/110% recommended energy/protein
HPT	Hypothalamic-pituitary-thyroidal (axis)
LGA	Large-for-gestational-age
LOW	Diet providing 50% recommended energy and protein
MetS	Metabolic syndrome
MIT	Monoiodotyrosine
NORM	Diet providing 100% recommended energy and protein
SGA	Small-for-gestational-age
TG	Thyroxine-binding globulin
TH	Thyroid hormones
TR	Thyroid receptor
TRH	Thyrotropin-releasing hormone
TSH	Thyroid stimulating hormone
T2D	Type 2 diabetes
T3	Triiodothyroxine
T4	Thyroxine

Summary

Obesity and correlated co-morbidities, in concert termed metabolic syndrome, are globally increasing at epidemic-like haste. Babies born small for gestational age and babies born large for gestational age, common in developing and industrialized countries, respectively, have an increased risk of developing metabolic syndrome in adulthood. Early-life overnutrition, through diets high in fat and carbohydrates, is believed to be a both independent and additive risk factor for adult metabolic syndrome. The hypothalamic-pituitary-thyroidal axis is the core regulator of overall maintenance of metabolic homeostasis. Thyroid disease is one of the most prevalent endocrine dysfunctions worldwide and there is evidence that thyroid dysfunction is an independent risk factor for metabolic syndrome and that hypothalamic-pituitary-thyroidal function may be a target for fetal and early-life metabolic programming.

This thesis aimed to investigate age-related biological and phenotypical changes in the hypothalamic-pituitary-thyroidal axis, caused by prenatal over- or undernutrition alone, or in combination with early-life postnatal overnutrition and if adverse impacts of an early-life obesogenic diet were reversible. Experiments were carried out in a translational sheep model where fetal programming was induced through late gestation maternal malnutrition, in two different studies. In Experiment 1, twin-bearing sheep were fed to either fulfill energy and protein requirements or fed 50% of this, to essentially give rise to lambs born small for gestational age. These lambs were raised on a conventional or high-carbohydrate-high-fat diet until 6 months-of-age. At 6 months, thyroid hormone response to fasting was tested and half the lambs were slaughtered and autopsied. Remaining sheep were raised on a moderate diet until 2-years of age, to assess 1) if there were lasting effects of the obesogenic diet after 1½ year diet correction and 2) if there were lasting effects of prenatal undernutrition, not present in 6 month-old lambs. At 2 years-of-age, adult thyroid hormone response to fasting was tested again and all sheep were slaughtered and autopsied. Experiment 2, was an elaboration on Experiment 1, with the addition of prenatal overnourishment, but with similar postnatal treatments and challenges. Exceptions were that sheep from Experiment 2 were more severely overnourished, lived until 2½ years-of-age, were subjected to a thyroxine tolerance test in adulthood, and more tissues for sampling were excised in Experiment 2 compared to 1.

The overall findings from Experiment 1 were that fetal undernutrition caused adult hyperthyroidism and increased thyroid hormone receptor expression in liver, cardiac and longisimus dorsi muscles, but decreased receptor expression in visceral and subcutaneous adipose tissues. The postnatal obesogenic diet increased thyroid hormone levels in adolescent lambs, but this was

reversed after diet correction, and not evident in adult sheep. Prenatal undernutrition programmed thyroid function at the secretory level and thyroid response differentially in target tissues, which was increasingly manifested with age. The differential impact on TH signaling in adipose versus other tissues could be part of a mechanism whereby fetal malnutrition can predispose for obesity and other metabolic disorders.

Main findings from Experiment 2 were; a sexual dimorphic T4 and TSH response to prenatal and postnatal diets, confined to 6-months-old lambs. Early-life overnutrition independently caused adult central hypothyroidism and prenatal overnutrition caused adult overt hypothyroidism whereas prenatal undernourished sheep appeared euthyroid. Prenatal under- and overnutrition as well as early-life overnutrition affected adult thyroid axis responsiveness to both fasting and a thyroxine challenge. Early-life overnutrition reduced adult energy expenditure in prenatal adequately fed and overnourished offspring. Early-life overnutrition also increased adult feed intake in prenatal adequately fed and undernourished sheep, possibly relating to downregulated hypothalamic TSHr expression. There were widespread effects of treatments on gene expression levels at both 6 months- and 2½ years-of-age. Effects were discernible in programmed sheep according to postnatal nutrition. It seemed that a postnatal conventional diet was more deleterious in prenatal overnourished sheep to adult thyroid function, than the obesogenic diet.

In Experiment 1 effects of early-life overnutrition were sparse and largely reversible, contrary to the findings in Experiment 2 and attributed to the more severe postnatal overnutrition in Experiment 2. In Experiment 1, prenatal undernutrition affected thyroid function already in lambs and became increasingly manifested in adulthood. It is possible that differences in gender distribution as well as early-life protein intake contributed to the differentiated responses. The overall conclusions reached in this thesis were that late gestation maternal under- and overnutrition, as well as early-life postnatal overnutrition can solicit metabolic programming to the hypothalamic-pituitary-thyroidal axis. Where fetal and early-life programming repress thyroid function, decrease energy expenditure and increase feed intake, programmed thyroid function should be considered a contributing factor to development of metabolic syndrome in programmed individuals.

Summary in Danish

Forekomsten af fedme og associerede sygdomme, tilsammen kaldet metabolisk syndrom, stiger globalt med epidemi-lignende hast. Børn der fødes lette eller tunge for gestations alder har en øget risiko for at udvikle metabolisk syndrom i voksenlivet. Førstnævnte er ofte forbundet med fødsler i udviklings lande og sidstnævnte er en stigende tendens i industrialiserede lande. Tidlig postnatal overernæring med fedt og letfordøjelige kulhydrater er en selvstændig og additiv risikofaktor for at udvikle metabolisk syndrom i voksenlivet. Den endokrine hypothalamus-hypofyse-skjoldbruskkirtel akse regulerer og vedligeholder metabolisk homeostase og på verdensplan er sygdomme i skjoldbruskkirtelen nogle af de mest forekommende endokrine lidelser. Studier har peget på at dysfunktion i skjoldbruskkirtelens aktivitet er endnu en risiko faktor for udvikling af metabolisk syndrom og at denne hormonelle akse er et mål for føtal og tidlig postnatal metabolisk programmering.

Formålet med denne afhandling, var derfor at undersøge alders-relaterede biologiske og fænotypiske ændringer i hypothalamus-hypofyse-skjoldbruskkirtel aksen som følge af prænatal under- eller overernæring, alene, eller kombineret med tidlig postnatal overernæring og i forlængelse af dette, at undersøge om skadelige effekter af den postnatale overernæring kunne reverseres med en kost-ændring. Forsøgene blev udført i en translationel fåre-model, hvori føtal programmering blev introduceret ved at fejlernære får i det sidste trimester af deres drægtighed. I Forsøg 1, blev tvillingebærende får fodret med en diæt svarende til det anbefalede for energi og protein eller de fik halvdelen af det anbefalede (50%). Tvillingelammene blev så opdelt og enten fodret efter anbefalingerne for at sikre en moderat daglig tilvækst eller fodret med en diæt rig på fedt og kulhydrater, de første 6 måneder. Ved denne alder blev skjoldbruskkirtelens respons på faste undersøgt, hvorefter halvdelen af lammene blev slagtet og vævene blev dissekeret. De resterende får blev ad-libitum fodret med grøn-hø, som har et lavt fedt indhold, indtil de var 2 år. Dette var for at kunne vurdere 1) om der var vedvarende effekter af den tidlige overernæring og om 2) der var vedvarende eller opståede effekter af den føtale underernæring. Ved 2 års alderen blev skjoldbruskkirtelens respons på faste testet på ny og de resterende får blev slagtet og dissekeret. Forsøg 2, var en udvidelse af Forsøg 1, hvor også prænatal overernæring blev inkluderet, men med næsten identiske postnatale behandlinger og faste respons tests. Overernæringen var sværere i Forsøg 2, fårene levede til de var 2½ år gamle, voksne dyr gennemgik en T4 respons test og der blev udtaget flere væv til analyser i Forsøg 2, end i Forsøg 1.

De overordnede resultater fra Forsøg 1 var at føtal underernæring ledte til hyperthyroidisme (overaktiv skjoldbruskkirtel) i de voksne får, med samtidig opregulering af

relevante gener i lever, hjerte og skelet-musklen longisimus dorsi og samtidig nedregulering i viseralt og subkutant fedtvæv. Den postnatale overernæring øgede skjoldbruskkirtlens hormoner i de store lam, men dette kunne rettes op ved overgang til den fedtfattige grøn-hø, og var således ikke evident i voksne får. Det vil sige at prænatal underernæring programmerede skjoldbruskkirtlen sekretorisk og programmerede target vævs respons i forskellig retning, og at effekterne af den prænatale diæt, manifesterede sig med tiltagende alder.

De overordnede resultater fra Forsøg 2 indikerede en aldersbestemt køns-relateret dimorfisme i T4 og TSH respons på prænatal under- og overernæring og postnatal overernæring, kun observeret i lam. Ydermere, var der i Forsøg 2 en selvstændigt programmerende effekt af tidlig overernæring der ledte til central hypothyroidisme, prænatal overernæring medførte at voksne dyr blev programmeret for "overt" hypothyroidisme, hvorimod prænatalt underernærede får havde uforandret skjoldbruskkirtel funktion, når man så på cirkulerende hormoner. Både prænatal under- og overernæring, såvel som postnatal overernæring påvirkede akse respons på faste og T4 challenge. Tidlig postnatal overernæring reducerede energi omsætningen i voksne får som havde været tilstrækkeligt ernæret prænatalt og i prænatalt overernærede får, samtidig øgede den postnatale overernæring også foderindtags kapaciteten i prænatalt tilstrækkeligt fodrede får, samt prænatalt underernærede får, hvilket blev tilskrevet specifik nedregulering af TSHr i hypothalamus. Der var udbredte effekter på gen ekspression i både 6 måneder- og 2½ år gamle lam og får. Det var muligt at skelne mellem programmerings effekter ifølge interaktion mellem præ- og postnatal ernæring og deraf fremgik det at den moderate postnatale diæt muligvis var mere uhensigtsmæssig for postnatal udvikling af skjoldbruskkirtelen i prænatalt overernærede får, end den postnatale overernæring.

I Forsøg 1 var effekterne af tidlig overernæring få og ikke vedvarende, modsat Forsøg 2, formentligt på grund af den sværere overernæring i forsøg 2. I forsøg 1 var effekter af prænatal underernæring tydelige i lam og manifesterede sig yderligere med alderen, modsatrettet resultaterne fra Forsøg 2. Det er muligt at forskelle i kønssammensætning og forskelle i tidligt postnatalt protein indtag havde betydning for disse forskelle. De overordnede konklusioner var at hypothalamus-hypofyse-skjoldbruskkirtel funktion undergår metabolisk programmering som følge af sendrægtig under- og overernæring af får, såvel som tidlig postnatal overernæring. I de tilfælde at føtal og postnatal programmering nedsætter aksens funktion, nedsætter energi omsætningen og øger fødeindtaget, må man betragte programmering af denne akse, som en medvirkende faktor i udviklingen af metabolisk syndrom i programmerede individer.

Introduction

Metabolic programming

Metabolic programming asserted through either gestational over- or under-nutrition appear to be major risk factors for development of obesity and other metabolic diseases later in life, such as insulin resistance and cardiovascular disease, and reversal of the epidemic development of these diseases is globally a major public-health concern as reviewed in the following.

Paradoxically under-nutrition in developing countries coexist with increasing prevalence of overweight and obesity associated with diet-related chronic diseases including diabetes mellitus, cardiovascular disease (CVD), stroke, hypertension and certain cancers (WHO, 2006). Many developing countries are experiencing an upward shift in population dynamics associated with socio-economic development. This upward shift has been linked to a consecutive epidemiological transition with increased prevalence of CVD and type 2 diabetes (T2D), and evidence suggest that as income levels rise, obesity become more prevalent among children and young adults. The association between prenatal nutrition and postnatal health risks has a great impact in developing countries in which a very strong link between poor maternal nutritional status and low birth weight and increased infant and childhood morbidity and mortality has been established (Amuna & Zotor, 2008). Recent studies provide evidence that fetal overnutrition have similar long-term effects on offspring as fetal undernutrition (Ford & Long 2012; Grattan 2008; Muhlhausler *et al.*, 2007; Khanal *et al.*, 2016) and that effects of fetal malnutrition are expressed in a 'U' – shaped curve relating birth weight to the risk of adult obesity, with individuals born at the extremes of the birth weight spectrum having a tendency to develop similar adverse outcomes on health and disease (Dyer & Rosenfeld 2011).

Scholze *et al*. (2010) studied the epidemiological and economic burden of the metabolic syndrome (MetS) and its consequences in patients suffering from hypertension, in the three European countries: Germany, Spain and Italy. MetS is the term for medical disorders that, in concert, increase the risk of CVD and diabetes. These include fasting hyperglycemia, impaired fasting glucose homeostasis, impaired glucose tolerance or insulin resistance, systemic inflammation, hypertension, central obesity, decreased high density lipoprotein cholesterol and elevated blood triglycerides and these symptoms have often been related to prenatal programming (Fernandez-Twinn & Ozanne, 2010). These studies suggested that hypertensive patients with MetS significantly inflate national costs of illness, due to the increase in CVD risk and T2D. Mean annual costs per

patients with hypertension and MetS are two to three times higher than for those without MetS. The economic burden of this group of patients is predicted to increase as the proportion of the population over 50 years of age grows and prevalence of MetS components increase. The cost evaluations have only included healthcare costs and not costs relating to morbidity or premature mortality, which would inflate cost estimates even further, while development of new antihypertensive medications and targeted treatment recommendations to hypertensive MetS patients in the future could reduce the predicted future costs (Scholze *et al.* 2010). The human nutrition observations and global obesity trends underlines the importance of understanding the precise physiological mechanisms behind diet-related diseases, to prevent their occurrence and to be able to develop the best possible treatments.

As mentioned, malnutrition with insufficient amounts of energy, protein and essential micronutrients may give rise to babies born small-for-gestational-age (SGA) while exposure to high energy diets during pregnancy can result in babies being born large-for-gestational-age (LGA) and the two widely different nutritional insults during fetal life appear to have similar pathological outcomes later in life. The mechanistic explanations for the effects of metabolic programming can be stunned maturation/differentiation of the cells of specific organs and tissues and/or possible epigenetic modifications altering gene expression, but the field is far from fully explored. Nutritional insults may limit the number of functional structures within an organ and change cell-cell signaling pathways regulating actions of an organ. For example fetal programming has been reported to reduce nephrons in the kidney and reduce neuron density within the hypothalamus of rodents (Langley-Evans, 2007; Plagemann *et al*., 2000). Epigenetics are heritable changes in genome function, leading to alterations in gene transcription without alterations in the DNA sequence (Langley-Evans, 2007).

Animal models of rodents (Kjaergaard *et al*., 2014; Lisboa *et al*., 2010), non-human primates (Grayson *et al*., 2010a and 2010b), pigs (Myrie *et al*., 2011; Liu *et al.*, 2012) and sheep (Long *et al.*, 2012; Oliver et al 2005, Nielsen *et al.*, 2013) have been used to study the mechanisms behind fetal and early-life metabolic programming. Sheep have proven to be an interesting translational model of fetal and early-life development, due to a relatively long gestational period (~147 days) with singleton or twin fetuses, with similar fetal growth trajectories and physiological maturity at birth. The gestational period can be divided into trimesters with developmental steps equal to that of human reproduction which is not possible in species that give birth to altricial offspring. In sheep fetal undernutrition was shown to cause earlier cessation of growth, which resulted in smaller body

size and lower total energy expenditure in the adult animal and also increased the weight of thyroid glands of adult female sheep (Nielsen *et al.,* 2013).

Thyroid hormones (THs), produced and secreted from the thyroid gland, stimulate cellular metabolism by increasing oxygen consumption and heat production and are essential for normal growth and development trough stimulation of growth hormone and insulin-like growth factor 1 (Yen, 2001). Thus, the observations from the study, spurred the notion that the thyroid and thyroid function was an additional target of programming, that could perhaps explain the metabolic phenotype of the adult sheep. A second sheep study was undertaken, which included fetal overnutrition, as maternal overnutrition is common in western countries and as reviewed maternal overnutrition /fetal macrosomia is another proposed risk factor for adult metabolic disease.

It was thus the intention with this thesis, to analyze thyroidal function in two distinct experiments with sheep; Experiment I) Late gestation undernutrition in combination with recommended postnatal nutrition or early-life overnutrition, and Experiment II) Late gestation undernutrition or overnutrition in combination with postnatal nutrition or early-life overnutrition.

Programming of the hypothalamic-pituitary-thyroidal axis

There are still many unresolved questions regarding how fetal nutrition impact development, metabolic and endocrine function later in life, and how this in turn can be linked to a predisposition for adverse health outcomes later in life. There have been reports of altered thyroid function in relation to both the clustered occurrence of obesity, insulin resistance and cardiovascular disease and in relation to fetal growth restriction. For example patients with MetS have also been found to have stimulated thyroid cell proliferation and increased thyroid volume and stimulated or decreased TH production (Sari *et al.,* 2003; Pergola *et al.,* 2008; Rezzonico *et al.,* 2008; Ayturk *et al.* 2009; Roos *et al.,* 2007). The association between clinical conditions seen in MetS and thyroid dysfunction instigate the notion that the hypothalamic-pituitary-thyroidal (HPT) axis is a target of prenatal and early life programming and this section gives a brief review of the existing evidence for metabolic programming of thyroid function.

Fetal restriction and undernutrition: In a birth cohort study, Kajantie *et al.* (2006) found that a small body size at birth and during childhood increased the risk of spontaneous hypothyroidism in adult women, whereas Brix *et al.* (2000) found, in a population based twin case–control study, that low birth weight is not associated with thyroid autoimmunity or non-autoimmune thyroid disease. Thyroid gland metabolism has been shown to be downregulated in both nutrient restricted suckling

rats (Bonomo *et al.*, 2008; Lisboa *et al.*, 2010) and in fetal growth-restricted lambs (Rae *et al.*, 2002); in the rat study by Lisboa *et al.* (2010), thyroid gland metabolism remained downregulated into adulthood. Others (Dutra *et al.*, 2003, Lisboa *et al.*, 2008) have reported that neonatal protein and energy restriction in suckling rats led to adult hyperthyroidism and increased liver deiodinase activity.

Fetal overnutrition: There are still very few studies available which have looked into programming of thyroid function through prenatal overnutrition. In rats a maternal high-fat diet throughout gestation and lactation induced hyperthyroidism at weaning (Franco *et al.*, 2012), whereas overnutrition with proteins in pregnant heifers did not affect basal thyroid function of 6-month-old calves (Micke *et al.*, 2015)

Early-life overnutrition: Again findings are not consistent. Obesity in children have been shown to increase TSH and T3 levels (Stichel *et al.*, 2000; Aypak *et al.*, 2013), while others found that only TSH was raised, without effects on free and total THs, suggesting clinical euthyroidism (Lobotková *et al*., 2014; Torun *et al*., 2014). These studies cannot shed light on long term effects of childhood obesity, whether persisting into adulthood or reversed by dietary intervention and they have not taken birth weight into account, which might be relevant, if this correlates to HPT axis function later in life.

Evidence to programming of the HPT axis may be divergent and this may partly be attributed to different species as well as timing and type of nutritional insult. The sum of studies, reporting consequences of prenatal and early-life nutrition to HPT axis function, validates that this field is well worth further examination.

Hypotheses

Based on previous findings concerning possible HPT axis programming and the association between MetS and thyroid dysfunction, the following hypotheses were formulated:

- Late gestation maternal malnutrition as well as early-life nutrition programs both central and peripheral HPT axis function
- Fetal under- as well as overnutrition causes similar fetal metabolic programming of the HPT axis
- Prenatal malnutrition combined with early-life overnutrition is an additional risk-factor for long-term adverse health outcomes.
- Altered central and peripheral thyroid function explain a diverged energy metabolism of different tissues in programmed subjects and facilitate altered fat deposition patterns.

Objectives

The overall objectives was thus to test these hypotheses by conducting studies in the Copenhagen sheep model, to investigate;

- age-related biological and phenotypical change in the HPT axis, caused by prenatal over- or undernutrition in combination with early postnatal nutrition interventions
- potential gene and tissue targets of fetal programming induced by late gestation under- or over nutrition, which can lead to functional changes at different levels in the thyroid hormone axis,
- whether exposure to an obesogenic diet in early postnatal life has an independent or correlated impact on severity of the manifested fetal programming effects
- to what extent adverse impacts of an obesogenic diet in early life are reversible in sheep with different prenatal nutrition histories.

Thyroidology

The following section is meant to give an overview of the development of the functional thyroid gland and the central and peripheral function of this endocrine system, to give context to the chosen focus areas of the included papers in this thesis. As earlier mentioned, thyroid hormones stimulate cellular metabolism by increasing oxygen consumption and heat production and are essential for normal growth and development. The following will briefly present knowledge on the fetal development of the thyroid, in the ovine compared to human fetus and thyroid hormone production within the thyroid gland, as well as the regulation of thyroid hormone synthesis within the HPT axis and finally the effect of thyroid hormone stimulation in target cells. Lastly, potential gender differences and interactions between the HPT axis and other endocrine axes will be briefly covered.

Development of the thyroid gland

In the context of this thesis it is the aim to examine which important factors determining thyroid development could be influenced by late-gestation programming and the translational relevance when comparing sheep and human thyroid function.

Overall, the thyroid gland originates as an outgrowth from the developing pharyngeal floor in the early embryo and migrates to the proximal part of the trachea, caudal to the larynx, where terminal differentiation of thyroid cells and formation of the thyroid follicular architecture takes place. Human and sheep thyroid ontogenesis can be divided into three phases that roughly parallel to the trimesters of the gestation period; 1) embryogenesis, 2) the hypothalamic-pituitary quiescence period, and finally 3) synchronized HPT axis maturation and hormonal secretion (Fisher 1991), these three periods may also be referred to as the pre-colloid, colloid and follicular stages (Forhead & Fowden 2014) and a comparison of the timing of developmental stages of thyroid hormone bioavailability among human, sheep and rat fetuses is illustrated in Table 1.

Table 1 Timing of stages of thyroid function among human, sheep, and rat foetuses. The percentage of total gestation (G) are given in brackets (Forhead&Fowden, 2014).

Developmental stage	**Human** (weeks)	**Sheep** (days)	**Rat** (days)
Gestational age at term	40	145	21
Thyroid gland organogenesis			
Pre-colloid	7–13 (0.18–0.33G)		
Colloid	13–14 (0.33–0.35G)	50–55 (0.34–0.38G)	17 (0.81G)
Follicular	>14 (>0.35G)	>55 (>0.38G)	18 days–3 weeks postnatally
TRH in hypothalamus	10–12 (0.25–0.30G)	<60 (0.40G)	16 (0.76G)
TSH in anterior pituitary gland and circulation	10–12	<60	17 (0.81G)
TSH receptor in thyroid gland	10–12		15 (0.71G)
Iodide uptake in thyroid gland	10–12	50 (0.34G)	
Thyroglobulin synthesis	10–12		15
Iodinated amino acids	14 (0.35G)	70 (0.48G)	17 (0.81G)
Synthesis and secretion of thyroid hormones	16–18 (0.40–0.45G)	60–70 (0.40–0.48G)	17.5 (0.83G)
Rise in plasma T_3	30 weeks to birth	135 days to birth	Birth to 3 weeks postnatally
Gene and protein expression of thyroid hormone transporters	7–9 (0.18–0.23G) cerebral cortex		
Thyroid hormone receptor binding	10–16 (0.25–0.40G) brain, heart, liver, and lung	<50 (0.34G) brain, liver, and lung	14–16 (0.67–0.76G) brain, heart, liver, and lung

From this it can be seen that ovine ontogenesis resembles that of humans, as the developmental stages occur at similar time points in terms of percentage of total gestation length. An exception to this is the thyroid hormone dependent brain maturation. This generally occur in the before mentioned phase 3, which stretches from gestational day 70-90 to postnatal week 2 in lambs and in children this maturation continues up to 2 years-of-age (Fisher 1991). As mentioned HPT embryogenesis occurs within the first trimester, and in the beginning maternal thyroid status is key to implantation and early embryonic development and the binding proteins responsible for blood transport of the hydrophobic THs are also expressed in placental trophoblasts where they facilitate secretion of maternal TH to the fetus (Colicchia *et al.*, 2014). By the end of the first trimester however, the thyroid is able to synthesize THs in the ovine and human fetus and the approximate timing of selected important events in the thyroid system maturation of the ovine fetus can be seen in Figure 1.

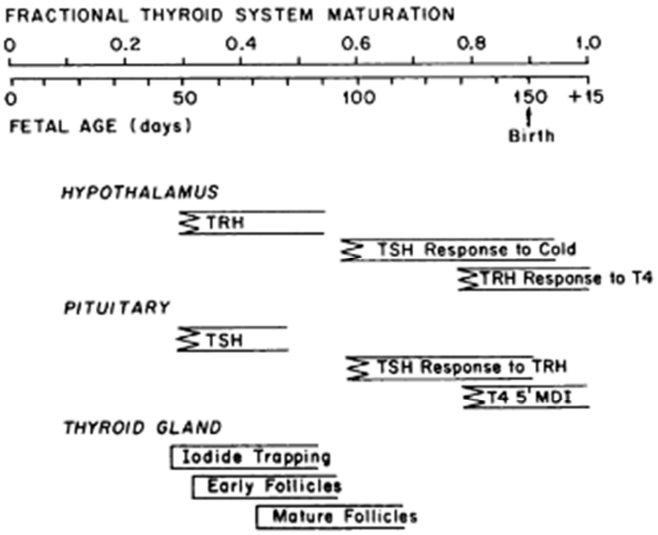


Figure 1 Approximate timing of selected important events in the thyroid system maturation in the ovine fetus/lamb (Fisher, 1991)

Hence forward the absolute volume of thyroid colloid, epithelium and stroma increase continuously throughout the second half of fetal intra-uterine life (Bocian-Sobkowska *et al.*, 1997). The sheep placenta is relatively impermeable to maternal THs ¼ into the gestation period (Fischer, 1991) and nutritional programming effects related to maternal TH status should not affect offspring thyroid levels directly. The fetal circulating THs are not only controlled by thyroid output, but also peripheral tissue metabolism. THs undergo deiodination and sulfation to render them more or less active, in peripheral tissues, the trend being that they are relatively inactive until the end of gestation, where developmental changes in peripheral deiodination are induced by a prepartum rise in cortisol. The more bioactive availability of THs at this point are thought to in turn mediate prepartum maturational effects of cortisol on pulmonary gas exchange, hepatic glucogenesis, cardiac function and thermogenesis (Forhead & Fowden 2014).

Programming of the HPT axis could theoretically occur throughout gestation either through effects central to the axis or through effects of programming to the peripheral tissues who concertedly act to control TH bioavailability. Programming early in gestation could in theory be more detrimental to TH synthesis, whereas programming after the first trimester could affect HPT axis feedback/maturation as well as thyroid growth.

Control of thyroid hormone synthesis

Secretion of THs from the thyroid gland is controlled through negative feedback to the hypothalamus and pituitary; the TH feedback system is presented in Figure 2. Low plasma concentrations of THs is a signal to the hypothalamus to synthesize and secrete thyrotropin-releasing hormone (TRH) which stimulates pituitary synthesis and secretion of thyroid stimulating

hormone (TSH) which in turn stimulates TH production and secretion from the thyroid gland. This system acts is affected by both exogenous and endogenous factors. The exogenous effects such as temperature and light will not be elaborated here. Circulating THs are relatively stable and do not show a significant diurnal rhythm, and these factors mainly determine longer-term adaptations, such as seasonal TH response. Endogenous effects are primarily the interchangeable relation between other hormones, such as somatostatin, growth hormones and cortisol; excess circulating THs exert negative feedback on hypothalamic somatostatin, which in turn decrease pituitary TSH release; growth hormones have a stimulatory effect on TH release and cortisol, typically increased in fasting, is a signal to lower TH mediated metabolism (Grant Maxie, 2007; Yen, 2001).

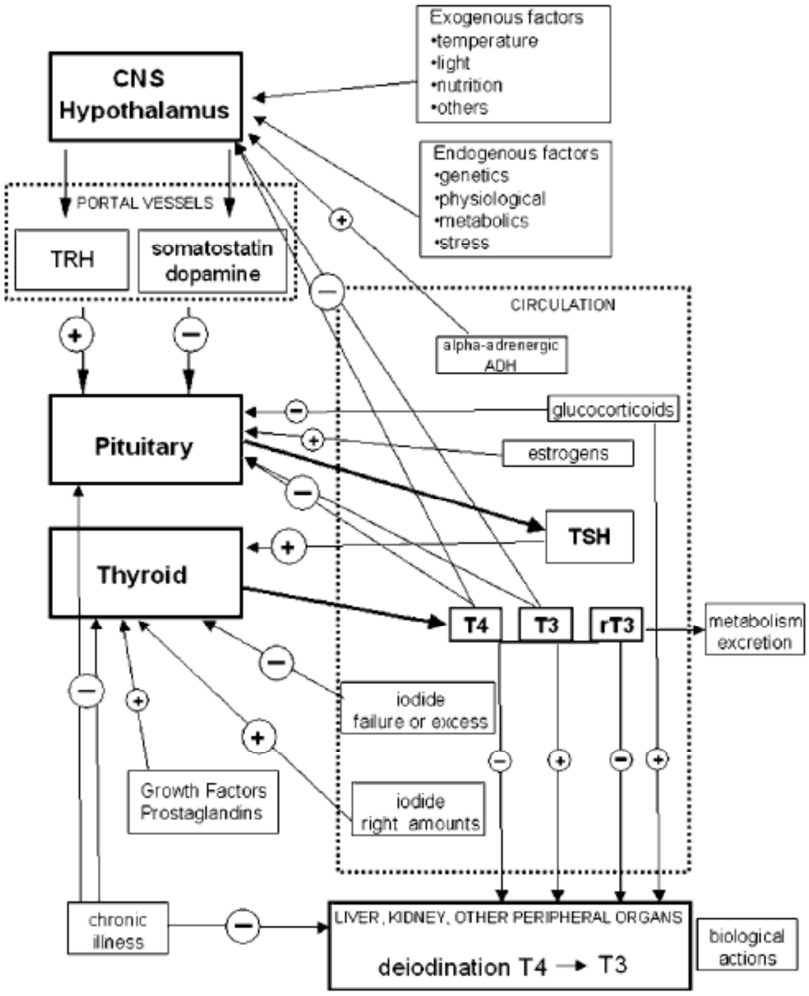


Figure 2 Schematic representation of TH regulation (Todini *et al*. 2007)

THs are lipid soluble hormones transported through the vascular system bound to plasma proteins; thyroxine-binding globulin (TG), albumin and thyroxine-binding prealbumin. The amount of free THs in plasma is very low, but in equilibrium with the protein-bound THs in plasma and in tissues. It is in the free form THs can bind to TH receptors (TR) and assert biological actions such as

inhibition of pituitary secretion of TSH. The equilibrium between free and bound THs shifts when there is a sustained increase in the concentration of thyroid binding proteins, but adjustments can occur rapidly following a decline in the rate of metabolism or with stimulation of TH production by TSH, where a new equilibrium may then eventually be reached. Consequently, species with elevated or decreased concentrations of binding proteins, particularly TG, are usually euthyroid (Ganong, 2001).

Thyroid hormone synthesis

This section aims to present the molecular aspects of TH synthesis and release from the thyroid. The thyroid gland is a capsule of connective tissue primarily containing thyroid cells which form follicles around a glycoprotein consisting mass called colloid (Figure 3). THs are stored within the colloid and secreted from the follicular cells into capillaries within the gland.

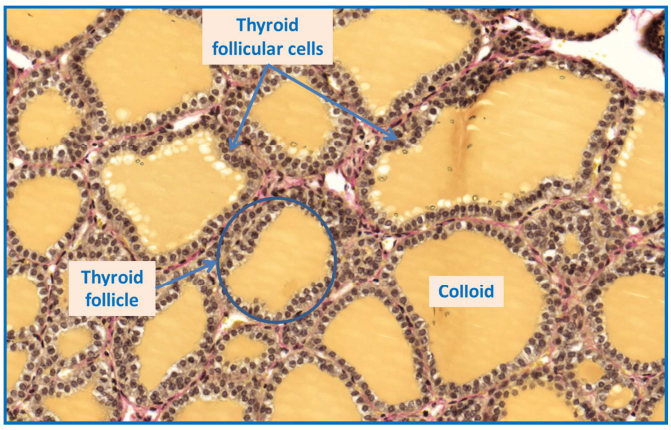


Figure 3 Microscopic view of thyroid tissue consisting of colloid distended thyroid follicles embedded in connective tissue (Representation by Author)

Dietary iodine is essential for TH production and is converted into iodide in the intestinal tract and transported to the thyroid. Within the thyroid follicular cell, iodide is oxidized back into iodine and the coupling of iodinated tyrosine molecules result in the formation of either thyroxine (T4) or triiodothyroxine (T3). When two fully iodinated tyrosine (diiodotyrosine, DIT) molecules couple, they form T4 and when a diiodotyrosine couples with a monoiodotyrosine (MIT) they form T3 (Figure 4).

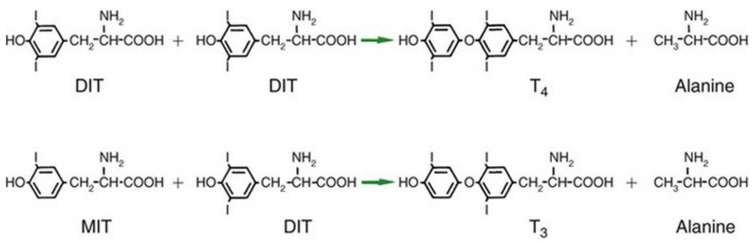


Figure 4 Production of T3 and T4 by the coupling of iodinated tyrosyl residues with thyroglobulin molecule. (Cunningham, 2002)

Figure 5 depicts the steps involved in synthesis and release of THs. Follicle cells trap the iodide through active transport, by the sodium-iodide symporter, encoded by the *SLC5A5* gene (1). When iodide passes into the follicular lumen it is oxidized back into iodine (2) and the amino acid tyrosine form a chain and make up the glycoprotein thyroglobulin precursor which is transported into colloid (3), where iodine is attached to the ring structures of the tyrosine molecules and MIT and DIT become coupled within TG (4), so the final TG contains both MIT, DIT, T3 and T4 (5). All of these processes are catalyzed by the enzyme thyroxid peroxidase (TPO). TSH stimulated secretion of T3 and T4, begins with the phagocytosis of TG (6). Lysosomal enzymes cleaves the TG molecule and T3 and T4 is actively transported to circulation by SLC16A2 and SLO1C1 (8), while iodinated tyrosines are deiodinated by a microsomal iodotyrosine deiodinase (IYD), an enzyme encoded by the *IYD* gene (9). The iodide and remaining tyrosine molecules are recycled to form new TG (10) (Yen, 2001; Cunningham, 2002).

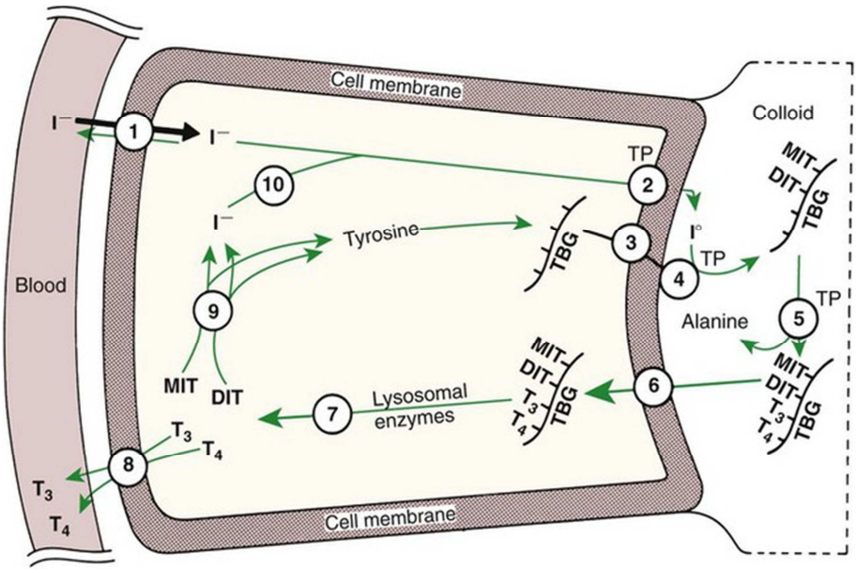


Figure 5 Depiction of follicular cell showing steps in the synthesis and release of T3 and T4. The numbers identify the major steps: (1) trapping of iodide; (2) oxidation of iodide; (3) exocytosis of TG; (4) iodination of TG (TBG); (5) coupling of iodotyrosines; (6) endocytosis of TG; (7) hydrolysis of TG; (8) release of T3 and T4; (9) deiodination of MIT and DIT (mono- and diiodotyrosine; (10) recycling of iodide, TG and TP (TPO) (Cunningham, 2002).

Peripheral thyroid hormone signaling and effects

THs from the circulation reach target cells and within the nucleus they bind to TRs and form a complex to upregulate target gene expression (Figure 6). Several transporters have been identified that mediate cellular entry of THs, many of which are not specific for THs but two TH specific transporters have been identified, SLO1C1 and SLC16A2 (Jansen *et al*., 2005). T3 is the biological active form of THs and deiodinase enzymes catabolize intracellular conversion of T4 to T3. In

humans, about 80% of plasma T3 is produced outside the thyroid gland and the remaining 20% is secreted directly by the thyroid (Hennemann *et al.*, 2001). There are three iodothyronine deiodinases: D1, D2 and D3, encoded by the *DIO1*, *DIO2* and *DIO3* genes, respectively. D1 and D2 catalyze T4 to T3 conversion and D1 is predominant in the liver and kidneys, whereas D2 is predominant in brain, pituitary, brown adipose tissue and skeletal muscle (Yen, 2001). D3 catalyzes the conversion of T3 and T4 into biologically inactive metabolites by removing an iodine atom from the tyrosyl ring to form T2 and rT3 respectively. D3 is especially credited for keeping fetal TH levels repressed but subsides to background levels in most tissues postnatally, except in skin and the central nervous system (Ueta *et al.*, 2012). Upon transport into the cell the active T3 binds to TRs (*TRa* or *TRb*) which in turn binds to TH response elements in the promoter regions of target genes. In the absence of T3, TRs recruit corepressors such as *NCoR* and the silencing mediators of retinoid and thyroid receptors (*SMRT*), which together with transducing β-like protein 1 (*TBL1*) and histone deacetylase 3 (*HDAC3*) form a complex with histone deacetylase activity on the promoters of target genes that repress basal transcription (Sinha *et al.*, 2012).

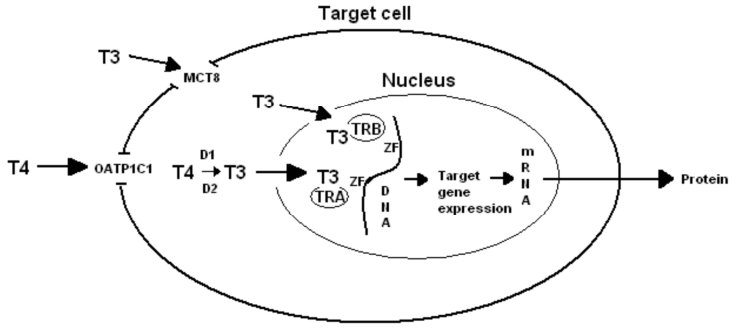


Figure 6 Simplified model of thyroid hormone transport into and action within the target cell. T3 and T4 are actively transported into the target cell by membrane transporters, T4 is deiodenized to T3 by D1 or D2, T3 binds to thyroid receptors TRA or TRB, this complex then binds to DNA via zinc fingers (ZF) and increase or decrease target expression (Representation by Author).

Gender differences in thyroid function

Circulating TH levels are influenced by breed, season, and physiological state and there may even be a minor diurnal variation. Reports on gender related difference in THs of small ruminants vary tremendously, from no differences in sheep (Eshratkhah *et al.*, 2010); to relatively more T4 in male sheep (Carlos *et al.*, 2015); to higher T4 and equal T3 of female sheep (Sharma & Kataria 2008) and higher TH levels in female goats (Todini *et al.*, 1992). The few data in literature on small ruminants and circadian rhythms in THs are discordant due to seasonal differences and difference in gender as well as physiological state (Todini 2007). Humane data do not clarify any tendencies; in fact, it has not been possible to find work that examined basal gender differences between men and

women, without considering the influence of some treatment. Thyroid illness is diagnosed using population-based wide reference ranges (Andersen *et al.,* 2002), which to our knowledge do not discern between men and women (menstruating or menopausal). Individual variations in TH concentrations in men are maintained within narrow limits but there are large variations among individuals (Andersen *et al.,* 2002). This is probably also true for menstruating women, where TH levels are stable throughout a normal cycle (Weeke & Hansen 1975). No difference in circadian variations between men and women have been found (Weeke 1973), but how levels of women are related to that of men, remains elusive. It seems commonly accepted that estrogen influence thyroid function, but the impact on circulating TH levels is unclear. Estrogen increases the binding capacity of the primary TH binding protein in the blood and affect thyroid cells directly and is attributed some causation for the increased prevalence of thyroid disease in women (Santin & Furlanetto 2011). But nowhere, it seems, are references tested to simply compare men and women. Female ruminants and women have gestation and lactation specific thyroid profiles (Riis & Madsen 1985; Leung 2012; Neville *et al*., 2002). To summarize, small ruminants and humans vary in thyroid state according to age, and in females also with gestation and lactation, and inconsistent gender differences have been reported. The significance and accuracy of gender differences remains elusive.

The HPT axis and intertwined systems

Hypo- and hyper secretion of THs, hypothyroidism and hyperthyroidism are common thyroid diseases which also underscore the interplay between the HPT axis and related metabolic systems. Both hypo- and hyperthyroidism is commonly related to renal- and cardio-vascular-and hepatic dysfunction. Hypothyroidism has been associated with increased serum creatinine and decreased glomerular filtration rate, common markers of renal dysfunction and conversely, hyperthyroidism has been associated with increased glomerular filtration rate and decreased serum creatinine (Dousdampanis *et al.*, 2014). The cardiovascular system responds to minimal, but persistent, changes in circulating thyroid hormone levels, typical of individuals with subclinical thyroid dysfunction (Fazio *et al*., 2004). Mild thyroid gland failure, evidenced solely by elevation of serum TSH concentration, have been associated with cardiovascular disease, and subtly decreased myocardial contractility and in subclinical hypothyroidism, both cardiac structures and function remain normal at rest, but impaired ventricular function as well as cardiovascular and respiratory adaptation effort may present during exercise. These changes have been reversible upon restoration of euthyroidism (Kahaly, 2000). In the opposite situation cardiovascular manifestations of TH excess, include tachycardia, a widened pulse pressure, a brisk carotid and peripheral arterial

pulsation and a hyperkinetic cardiac apex (Biondi *et al*., 2002). Both overt and subclinical hypothyroidism is linked to hypercholesterolemia and non-alcoholic fatty liver disease, whereas hyperthyroidism has been linked to hepatic oxidative stress and hyperglycemia, again restoration of euthyroidism have been shown to counteract these adversities (Sinha *et al*., 2014). All observed relations are essentially mediated or accompanied by altered TR binding/activation in target tissues, which may originate in altered circulating TH concentrations or TH availability within the target tissue, the latter possibly mediated by altered deiodinase function. Thus, it should be evident that fetal or early-life programming of the thyroid or related metabolic systems, may interchangeably act in concert to manifest in the adult phenotype of metabolic syndrome.

Materials and methods

The data presented in this thesis originate from two experiments. The experimental design for both is briefly described in the following, whereas detailed description of each experiment can be found in the respective papers. Experiment 2 was preceded by a pilot study to elaborate the protocol for a thyroxine tolerance test, and this pilot study is described below. Furthermore a protocol for thyroid histological evaluation, using advanced image analysis software was also developed as part of this thesis.

Experiment 1 – Prenatal undernutrition and early-life overnutrition

A comprehensive description of the treatments has been presented in Nielsen *et al*. (2013). 21 Twin-bearing Shropshire ewes were fed one of two diets for the last 6 weeks of gestation. The diets either fulfilled energy and protein requirements (NORM) or a diet reduced 50% in energy and protein (LOW). Twin-lambs were following divided onto each their postnatal nutrition scheme; either fed a low-fat conventional sheep diet to achieve moderate growth rates (CONV) or fed a diet high in fat and easily digestible carbohydrates (HCHF). At 6 months-of-age, all lambs were subjected to a fasting tolerance test, during which blood was collected and half the lambs were then slaughtered and autopsied. All remaining lambs were thereafter fed the same low-fat grass-based diet until 2 years-of-age, at which time they were subjected to another fasting tolerance test and then slaughtered and autopsied. Blood from 1 day, 6 months- and 2 years-of-age were analyzed for total T4 and T3, and gene expression levels were assessed by qRT-PCR for key genes relating to thyroid function within the thyroid and peripheral target tissues; liver, cardiac muscle, biceps femoris, longisimus dorsi, subcutaneous and abdominal fat.

Experiment 2 – Prenatal under or overnutrition and early-life overnutrition

A comprehensive description of the treatments has been presented in Khanal *et al*. (2014). 36 Twin-pregnant Texel ewes were assigned one of three diets, the last six weeks of gestation: NORM (fulfilling 100% of energy and protein requirements), HIGH (fulfilling 150% of energy and 110% of protein requirements) or LOW (50% of energy and protein requirements). Twin lambs were assigned either to a moderate conventional diet (CONV) or a high-carbohydrate-high-fat (HCHF) diet from day 3 to 6 months of age. The lambs were subjected to a fasting tolerance test, during which blood was collected for metabolic fasting profiles, and half the lambs were then slaughtered and autopsied. Remaining lambs were raised on a low-fat hay-based diet until 2½ years-of-age, upon which they were subjected to a T4 tolerance test and another fasting tolerance test and then

slaughtered and autopsied. Blood samples from 1 day-, 6 months- and 2½ years-of-age were analyzed for TSH, T4 and T3, and expression levels for key genes relating to thyroid function within the hypothalamus, pituitary and thyroid, as well as TH signaling in peripheral target tissues; liver, cardiac muscle, biceps femoris, longisimus dorsi, subcutaneous and abdominal fat were analyzed by qRT-PCR.

Pilot study

Aim: We wished to establish if potential programming of central HPT axis and peripheral thyroid function would translate into gross physiological alterations. It was therefore the intention to compare metabolic changes and energy expenditure among the sheep during a thyroxine challenge. We decided to measure energy expenditure, heart rates and body temperatures in adult sheep, before and during a thyroxine tolerance test.

Prerequisites: To do this we needed to establish 1) solubility and solution medium for thyroxine, 2) response time from injection to equilibrium and 3) dose concentration.

Background literature: McBride & Early (1989) and T.E.C. Weekes (1992) both induced experimental hyperthyroidism in sheep by daily injections of T4 subcutaneously for 3-5 weeks and plasma concentrations of T3 and T4 were two-/three-fold higher after this period. We wished to uncover differences in TH metabolism between the treatment groups rather than examine effects of a hyperthyroid state on metabolic phenotype and therefore this method was not desirable. Furthermore the sheep had to be normalized for further experimentation a few months after and we therefore wished to perform the tolerance test, based on a single bolus injection of T4. Peeters *et al*. (1992) found that intravenous injection of TSH increased plasma levels of T3 from 1-4 h after injection and T4 only after 4 hours in neonatal and growing lambs as well as normal, pregnant and lactating adult ewes. In comparison injection of 400 µg T4 in euthyroid pregnant ewes, comparable to non-pregnant ewes, increased plasma levels of T4 within the first hour upon injection, but failed to increase plasma T3 within the four hour sampling post-injection. The quick T3 response to TSH was attributed to stimulation of preferential intrathyroidal conversion of T4 to T3 (Peeters *et al*., 1992). It was not possible to acquire TSH, so it was determined to use T4 to experimentally increase metabolism. Single-dose thyroxine injections may not be detected as increases in plasma T3 due to intracellular conversion of T4 to T3, but may be presumed to act intracellular as the initial increase in plasma T4 normalized. Thus, we concluded that by injecting T4 we should be able to measure a response in energy expenditure and body-temperature.

The best suited concentration for a single injection of thyroxine per MBW was not directly obtained through the literature review, nor the exact maximum tissue response time, as most literature have

measured the response as plasma concentrations. Weeks (1992) reported that sheep with a 2.4 fold increase of T4 in blood plasma were moderate hyperthyroid. Control sheep in this experiment had ~200 μg T4/L plasma and we aimed to raise this concentration three times to mimic the hyperthyroid sheep of the study. We calculated the dose using the following equation:

$$T_4 = \Delta plasma\ conc\ * plasma\ vol * 2$$

Where the plasma volume was assumed equal to 5% body weight and multiplied by 2, based on the assumption that 50% of the injected thyroxine would be in other body pools than plasma (Riis & Madsen, 1985).

Among different available T4 products, we chose L-Thyroxine sodium pentahydrate (product no. T2501) from Sigma-Aldrich (Brøndby, Denmark) due to purity and availability. The manufacturer had only tested solubility in 4M ammonium hydroxide in methanol and found solubility up to 50 mg/ml. This solvent is not desirable for intravenous injection, but a solution with saline proved unsuccessful, and ethanol was chosen as solvent for preparation of a stock solution.

Trial #1

Thus, on the day of each trial, a stock solution of L-thyroxine dissolved in 70% ethanol was prepared in the lab under sterile conditions and stored in darkness. The thyroxine had a concentration of 4 mg/ml ethanol. Before injection we diluted 1 ml of the thyroxine solution with 19 ml of saline immediately prior to injection, to minimize the possible discomfort and impacts of the ethanol solute whilst ensuring preservation of bioactivity of the thyroxine. Before the injection the solutes were filtered through a sterile 0.22 PES membrane. The filtration rendered the solute transparent, compared to a milky-white color beforehand, indicating that L-Thyroxine was withheld in the filter. We planned to assess dose response by measuring core temperature by using the VitalSense® system and Jonah™ capsules (Minimitter, USA) and we considered the temperature rise significant, if the body core temperature passed 40^{o}C degrees. In trial #1, using two female sheep, we did not see any increase in temperature for the 10 hours we measured.

Trial #2

Based on Trial #1, saline was discarded as solvent and instead we performed spectroscopy analysis of L-thyroxine dissolved in ethanol or methanol, using a saline solution as reference. 0.8 mg/ml L-thyroxine was dissolved in either 70% methanol or ethanol and compared to a 0.025 mg L-thyroxine/ml saline (0.9%) solution. The absorbance of T4 is 230 nm and absorbance was measured before and after sterile filtration (see above). As suspected, the saline did not successfully dissolve L-thyroxine, which was withheld in the filter, whereas filter retention for thyroxine dissolved in methanol and ethanol solutions was negligible. Based on the spectroscopy and capacity as a solvent,

methanol was chosen. The solvent might potentially give rise to some discomfort upon injection, however the amount injected (i.e. 2 ml for an 80 kg sheep) were quite low and did not give rise to any signs of discomfort. A further adjustment was a doubling of the initial thyroxine dose and time measuring response. The test conducted in Trial #1 was then repeated. During the 17 hour period post-injection, we did not see measurable effects on core temperatures or heart rates. We concluded this was probably due to longer T4 half-life and response times than expected in combination with too small concentrations to measure within a reasonable time-frame, when using a bolus injection.

Trial #3

Knight et al (2004) administered thyroxine to dairy cattle in a concentration of 0.1 mg/MBW for several days and we decided to try this dose in a single bolus IV injection, still using methanol as a solvent. The physical setup for the study was the same as the previous two trials. The solvent was prepared the day before the pilot study and kept in the refrigerator until the following morning, where it was transported under dark conditions to the farm. The L-thyroxine solute was administered at 10.00 AM and after two hours core temperatures exceeded 40°C and increased steadily throughout the following 10 hours. We estimated, based on preliminary data that a maximal response was would occur around 12 hours after injection. Whole body metabolic responses were assessed from rectal temperatures on an hourly basis throughout the tolerance test combined with measurements of heart rates manually using a stethoscope. EE was to be estimated using the ^{13}C-bicarbonate technique. 46 animals were divided into 12 subgroups of four and the experiment would last for three weeks, with 4 groups studied per week. A schematic presentation of the planned progress per group can be seen in Figure 7. The final experimental set up is fully described in Paper II.

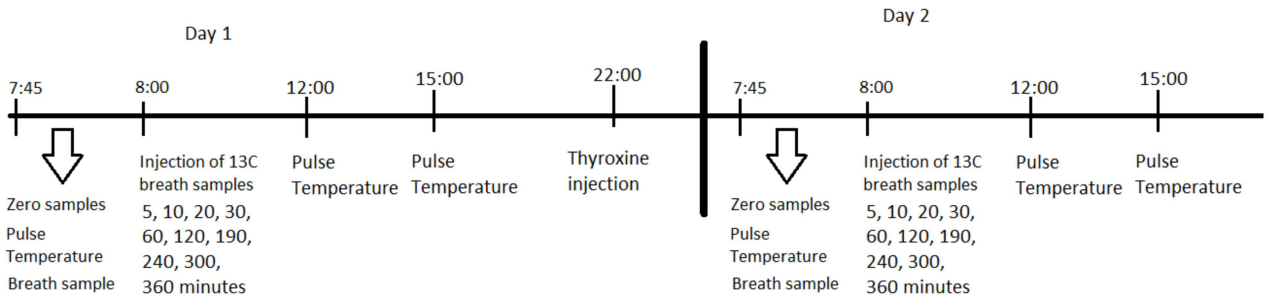


Figure 7 schematic presentation of the planned experiment, where baseline blood samples, temperature, heart rate and energy expenditure was measured on Day 1 and L-thyroxine (0.1 mg/kg LW) was injected in the evening. 12 hours later, on Day 2, all parameters were tested again, when thyroxine response was assumed at its highest.

Thyroid histological assessment

Histology

A cross section from the middle of the thyroid gland was excised and the thyroid tissue was saturated in 4% PFA for about 24h at room temperature where after tissues were transferred into 1% PFA for paraffin embedding. At 25-30 μm depth into the sample 5 μm thick sections were cut from the paraffin blocks on a Leica SM2000R Sliding Microtome (Leica Microsystems, Nussloch, Germany) and transferred to superfrost glass slides (Superfrost® WHITE; Hounsen Laboratorieudstyr, Denmark). After sectioning, the mounted slides were dehydrated in a heating oven at 50°C for 40 min. Van Gieson staining was performed following the protocol reported by Kongsted *et al.* (2014). Slides were deparaffinized by immersion in xylene for 3×10min and then rehydrated with a decreasing gradient of ethanol solutions (99%-70%). Slides were then incubated at room temperature in Lillie Weigerts iron-haematin (Th-Geyer, Roskilde, Denmark) for 5 min, followed by 10 min under running water and 4 min incubation in Pikrinacid-acidfuchsin (WVR, Herlev, Denmark). Finally, the slides were dehydrated with an increasing gradient of ethanol solutions (70%-99%), cleared with xylene and mounted with DPX Mountant (Fluka, Switzerland). The Van Gieson staining protocol results in a black staining of endothelial cells and cell nuclei and red staining of collagen/fibroblasts, whereas cell cytoplasm and other tissue components will receive a yellow stain (see Figure 3).

Quantitative image analysis

The stained slides from 6 months old lambs were scanned by a Panoramic MIDI whole slide scanner (3DHISTECH, Hungary) and the slides from the 2½ year-old sheep were whole slide scanned on the Axio Scan.Z1 (Zeiss, Germany). The slides were then analyzed using the Visiomorph™ function in Visiopharm VIS 6.4.1 (Visiopharm®, Hørsholm, Denmark). A protocol was developed that could detect and separate background, follicular cytoplasm, colloid and collagenous fibres in a manually drawn region of interest excluding cysts and otherwise compromised areas. Pre-processing steps in training the protocol involved picking the best fit of color deconvolution and classification of various areas, in this case follicular cytoplasm/follicular cells, colloid and collagenous tissue and background, Figure 8 gives an example of the labeling. Various post-processing steps were applied to remove noise from the labels and avoid overlapping counts. As the analysis runs on the whole section and as these may not be identical in size between animals, we wished to compare thyroid follicular cytoplasm:collagenous tissue ratio.

As a measure of colloid, the protocol was designed to measure the area of follicles, defined by area colloid per object and create a follicle size distribution. Unfortunately, due to hardware and software malfunction it was not possible to analyze the slides, before submission of this thesis.

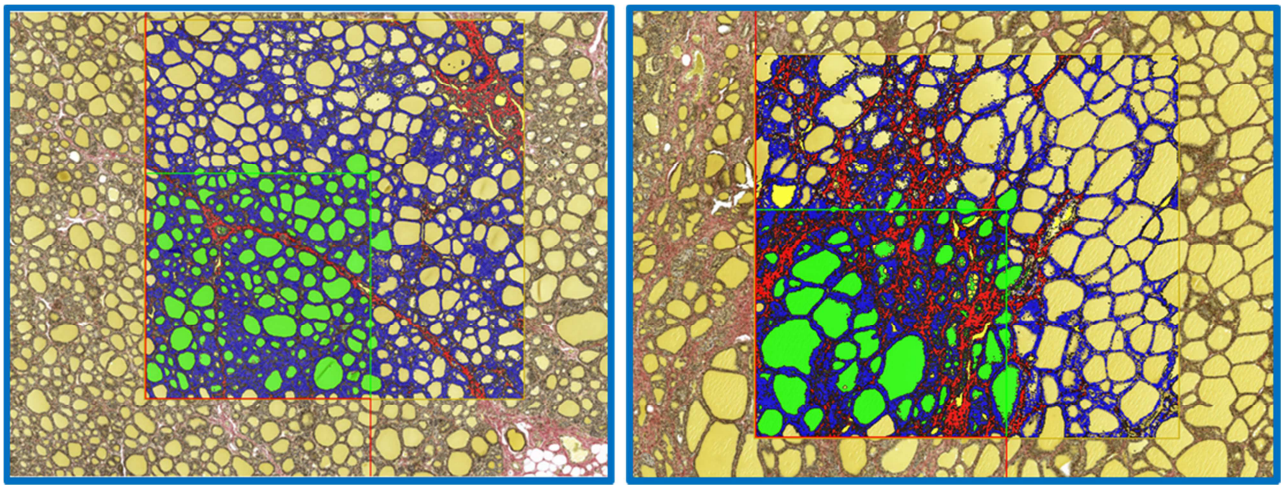


Figure 8 Example of labelling and area identification in the Visiopharm software within an applied counting frame in wholeslide scans of thyroid tissue from two different animals. Colloid is labelled green, follicular cytoplasm blue and collagenous tissue red.

Summary of presented papers

Paper I: Prenatal undernutrition and postnatal overnutrition alter thyroid hormone axis function in sheep

Fetal undernutrition is a risk factor for adult metabolic disease and there is evidence of fetal metabolic programming of thyroid function, however there are few evaluations of long-term effects in non-rodent models. Thus, we aimed to test the hypothesis that thyroid function could be programmed by late gestation undernutrition and that early-life overnutrition would exacerbate adverse effects solicited by fetal metabolic programming.

In a 2×2 factorial design, 21 twin-bearing sheep were fed one of two diets during late gestation: NORM (fulfilling energy and protein requirements) or LOW (50% of NORM). Blood was sampled for T4 and T3 evaluation on postnatal day 1. Twin lambs were assigned either a conventional (CONV) or high-carbohydrate-high-fat (HCHF) diet from day 3 to 6 months-of-age. At 6 months T4 and T3 was tested in response to a fasting tolerance test and half the lambs were slaughtered and autopsied. Remaining sheep (all female) were raised on the same moderate diet until 2-years of age, to assess 1) if there were lasting effects of the obesogenic diet after 1½ year diet correction and 2) if there were lasting effects of prenatal undernutrition, not present in 6 month-old lambs. At 2 years-of-age, the adult females were again subjected to a fasting tolerance test and adult T4 and T3 response was tested and all sheep were slaughtered and autopsied. Serum total T4 and T3 was analyzed using a human enzyme immunoassay and genes relevant for thyroid function was analyzed by qRT-PCR in tissue samples of thyroid, liver, cardiac muscle, biceps femoris, longisimus dorsi, abdominal and subcutaneous adipose tissue.

Fetal undernutrition was associated with increased adult T4 and T3 and increased TR expression in liver, cardiac and longisimus dorsi muscles, but decreased TR expression in visceral and subcutaneous adipose tissues. The postnatal obesogenic diet increased TH levels in adolescent lambs, but this was reversed after diet correction, and not evident in adult females.

Prenatal undernutrition programmed thyroid function at the secretory level and thyroid response differentially in target tissues, which was increasingly manifested with age. The differential impact on TH signaling in adipose versus other tissues may be part of a mechanism whereby fetal malnutrition can predispose for obesity and other metabolic disorders.

Paper II: Late gestation over- or undernutrition and early postnatal overnutrition affect thyroid function and metabolic phenotype in adult sheep

Prenatal under- and overnutrition and early-life overnutrition mediate metabolic programming, a risk factor of adult metabolic disease and thyroidal function is a target of programming. However, it is not known if programming of the thyroid axis manifest phenotypically and it is unknown if fetal originating metabolic disease should be functionally divided according to nutritional history.

In a 2×3 factorial design, 36 twin-pregnant sheep were either adequately nourished (NORM), undernourished (50% energy and protein requirements) (LOW) or overnourished (150% energy and 110% protein requirements) (HIGH) in the last trimester of gestation. Resulting twin lambs were subdivided onto each their diet for the interval, day 3 to 6 months-of-age, where one received a conventional diet (CONV) and the other a high-carbohydrate, high-fat diet (HCHF). At 6 months blood was drawn for thyroid hormone evaluation. From 6 months- until 2½ years-of-age, all sheep were fed the same, low-fat diet.

At 2½ years-of-age, voluntary feed intake was assessed following a 72 hour fast and baseline heart rate, rectal temperature and energy expenditure was measure before and during thyroxine tolerance test. Measurements took place 12-19 hours after a bolus injection of 0.1 mg T4/kg BW and energy expenditure was assessed by the ^{13}C-bicarbonate tracer technique, where exhaled breath was collected at defined intervals after a bolus injection of ^{13}C-bicarbonate and analyzed using an infrared isotope analyzer. TSH, T4 and T3 were measured using a double-antibody radioimmunoassay.

LOW, HIGH and HCHF treatments affected adult thyroid axis responsiveness and suppressed T4→T3 conversion during the tolerance test. When challenged, the HIGH:HCHF sheep increased serum-T3 and -T4, as well as T3:T4 and T3:TSH ratios and decreased serum TSH, while NORM:HCHF sheep responded directly opposite. HCHF decreased basal adult EE in prenatal NORM and HIGH sheep and increased adult feed intake of NORM and LOW sheep.

Fetal under- and overnutrition programmed thyroid function differently, with lasting effects on heart rate, body temperature, feed intake and energy expenditure, but without affecting basal thyroid status. NORM adults were adversely affected by the postnatal obesogenic diet. In HIGH, the postnatal CONV diet seemed to be a "mismatch" diet throughout prepubescent development. Programming in LOW sheep was primarily prenatal and did not interact with postnatal nutrition in a long-term perspective.

Paper III: Effects of late gestation under- or overnutrition and early postnatal overnutrition on the hypothalamic-pituitary-thyroidal axis in sheep

Fetal nutrition is a risk factor for adult metabolic disease including thyroid function, however there are few evaluations of long-term effects, and few, if any, have evaluated the effect of both prenatal under- and overnutrition in combination with early-life overnutrition, jointly.

36 twin-pregnant sheep were adequately nourished (NORM), undernourished (LOW; 50%) or overnourished (HIGH; 150%) according to recommendations, in the last trimester of gestation. Twin lambs were divided to a conventional diet (CONV) or a high-carbohydrate, high-fat diet (HCHF) from day 3–6 months-of-age, yielding 6 treatment groups: NORM:CONV, NORM:HCHF, HIGH:CONV, HIGH:HCHF, LOW:CONV and LOW:HCHF. At 6 months-of-age, thyroid hormone fasting response was tested and half the lambs were euthanized and autopsied. Remaining sheep were kept on the a moderate diet until 2½ years-of-age where another fasting tolerance test was performed and the sheep were euthanized and autopsied.

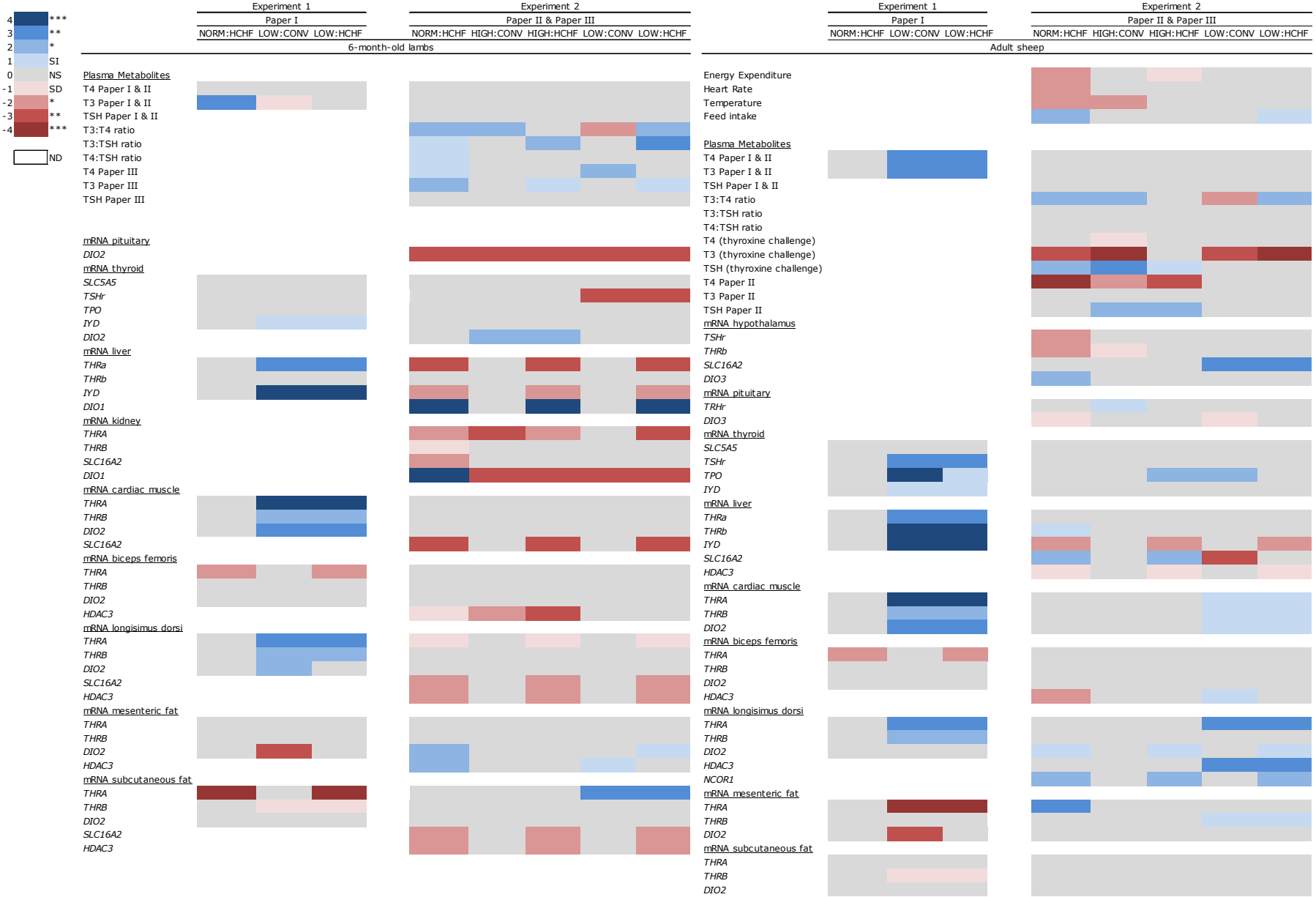
We examined thyroid stimulating hormone, thyroxine and triiodothyronine in blood from postnatal day 1, 6 months- and 2½ years-of-age using a double-antibody radioimmunoassay and examined central and peripheral gene expression of genes key to thyroid function by qRT-PCR in tissue from hypothalamus, pituitary, thyroid, liver, kidney, cardiac muscle, biceps femoris, longisimus dorsi, subcutaneous and abdominal fat. Included genes for testing were *TRHr, TSHr, TPO, TG, THRA, THRB, SLC5A5, IYD, DIO1, DIO2, DIO3, SLC16A2, NCOR1* and *HDAC3.*

Neonates: T4 increased with birth weight and TSH increased with decreasing birth weight and T3 was significantly increased in HIGH lambs compared to LOW.
Lambs: T4 and TSH response to prenatal and postnatal diets was sexually dimorphic in all treatment groups but NORM:CONV and all treatments increased T3 compared to NORM:CONV. Prenatal treatments affected thyroidal gene expression and both prenatal and postnatal diets affected peripheral gene expression.
Adult sheep: Sexual dimorphism was no longer visible. NORM:HCHF sheep had central hypothyroidism and HIGH sheep had developed overt hypothyroidism whereas LOW sheep appeared euthyroid. All adult sheep, but NORM:CONV displayed altered metabolic profiles in response to fasting. There were lasting effects of treatments on adult gene expression, now shifted central to the HPT axis and less related to peripheral thyroid function. Effects were discernible in programmed sheep according to postnatal nutrition.

General discussion

As reviewed there are still few studies on prenatal and early-life programming of the HPT axis and outcomes were not unidirectional and time of assessment varied greatly. Thus, in this study we aimed to examine programming affects through prenatal under- and overnutrition alone or in combination with an obesogenic postnatal diet, on central and peripheral HPT axis function, in a long-term perspective. The presented studies sought to test the underlying hypotheses that maternal malnutrition through either under- or overnutrition would induce HPT axis dysfunction in sheep and that a postnatal obesogenic diet would exacerbate those effects. Paper I focused on effects of late-gestation undernutrition primarily expressed in TH levels and thyroidal and peripheral gene expression of genes closely related to thyroid function. Paper II centered on adult endocrine adaptability to a thyroxine challenge, as well as basal metabolic phenotype expressed in energy expenditure, heart rate, temperature and feed intake capacity in adults sheep with a history of prenatal under- or overnutrition and/or early-life overnutrition. Paper III had a similar focus to Paper I, but included fetal overnutrition and a range of tissues and genes not examined previously. Table 2 presents an overview of the examined parameters in Paper I–III, where results differing from NORM:CONV sheep of each study is color-coded according to direction of change and level of significance. The table does not include response to fasting or thyroxine challenges and the table does not depict in-group difference between HIGH and LOW sheep according to postnatal nutrition. This discussion will focus on similarities and diverging results from the three papers presented in this thesis.

Table 2 Overview of results – Paper I–III



Effects of prenatal and early postnatal malnutrition in lambs

Overall, in Experiment 1 there were very few effects of the early postnatal overnutrition in 6-month-old lambs as compared to Experiment 2 and prenatal effects of late gestation undernutrition were predominant. The main reason for the pronounced difference in effects of the postnatal overnutrition on thyroid function, between Experiment 1 and 2, must be ascribed very different levels of overnutrition. HCHF lambs in experiment 1 were fed a maximal 0.5 L of 38% dairy cream/day, whereas HCHF lambs in experiment 2, were fed a maximal 2.5 L a day of 50% milk replacer and 50% dairy cream (38%). In Experiment 1, predominantly males were slaughtered at 6 months, whereas data from 6-month-old lambs in Experiment 2 had an equal gender distribution. Off course this is only relevant for tissue related results, as blood samples were performed on all animals and between the experiments there was a similar tendency in lambs to raise T3 and T4 in response to the obesogenic diet. Thus, it does not seem that this difference should be strictly gender related. Ewes of the Shropshire breed were used in Experiment 1 and cross-bred Texel ewes in Experiment 2. The observed difference between the two studies, are not readily explained by the difference in breeds, as both are meat sheep breeds with similar maternal heritability of growth traits and carcass traits such as muscle and fat depth (Maxa *et al*., 2007a; Maxa *et al*., 2007b). A difference between the two experiments was the difference in body condition of NORM and LOW ewes, 6 weeks prepartum, which was app. 8 kg in Experiment 1, compared to app. 2 kg in Experiment 2 (Nielsen *et al*., 2013; Khanal *et al*., 2014). This initial difference did not appear to affect birth weight of lambs of either group, as these are relatively comparable between the two experiments. However, it is possible that the initial group weight difference between NORM and LOW ewes in Experiment 1 is accountable for some of the difference we see in thyroid function between LOW animals of the two experiments. Furthermore, the digestible crude protein intake, which was approximately halved in NORM:CONV and LOW:CONV and reduced to ¼ in NORM:HCHF and LOW:HCHF, between postnatal day 3–8 weeks-of-age and approximately halved in all these groups from 8 weeks- to 6 months-of-age in Experiment 2, compared to Experiment 1 (Nielsen *et al*., 2013; Khanal *et al*., 2014). Thus, the reduced protein intake throughout postnatal development in Experiment 2, may have contributed to affect thyroid function differentially between the two experiments, specifically effects of the HCHF diet may have been more pronounced, when protein intake was also decreased. Lastly, fewer parameters were assessed in Experiment 1 compared to 2, meaning that there could theoretically be more effects of the HCHF diet in experiment 1, than reported, which seems plausible since the HCHF diet did also increase T3, as also reported in Paper III. In 6 months old lamb there were few to no effects of prenatal

nutrition on serum TH levels, meaning that THs are not a potential marker for HPT axis programming in young lambs. However, the T3:T4 ratio might reveal the programming of thyroid metabolism in the quantitatively significant tissues, such as liver and kidneys, where thyroid related genes were found to be generally downregulated in 6 month-old-lambs (Paper III) and be predictive of adult thyroid risk, as this parameter was unaltered with age.

Long-term effects of prenatal and early postnatal malnutrition in adult sheep

In Experiment 2, the postnatal HCHF diet created very distinct columnar pattern in Table 2 in 6-month-old lambs, which was less pronounced in 2½ year old sheep, but still, after 2 years on the conventional diet, there were surprisingly many lasting effects of the postnatal diet. Conversely in Experiment 1 there were hardly any effects of postnatal nutrition to HPT axis function at either age. In experiment 2 the early-life obesogenic diet programmed for a distinct thyroid phenotype of central hypothyroidism, with decreased energy expenditure, heart rates and body temperature as well as increased feed intake. This pronounced effect on adult metabolism in Experiment 2, must largely be ascribed to the before mentioned more severe postnatal overnutrition scheme and possibly to a lesser extent that the sheep were 6 months older.

There was a basal endocrine discordance between Paper I and II: Upon analysis of basal adult serum we did initially reach the same conclusions in Paper I that NORM:HCHF were programmed for adult central hypothyroidism and that HIGH sheep were programmed for overt hypothyroidism, but the decision to analyze serum samples together with the lambs obscured this effect. Upon analysis of data in Paper II, it did not make sense to analyze 6 month and 2½ year serum results in one model, since half of the animals had been slaughtered at 6 months-of-age. Perhaps it would also have been a truer representation of adult thyroid state, if we had avoided to include data from the animals when they were 6-month-old. In both Paper II and III however, HIGH sheep, regardless of postnatal diet, had the same overall thyroid phenotype expressed in serum thyroid status, but HIGH:CONV and HIGH:HCHF sheep had differentiated responses, when compared to each other, in almost every parameter examined. Even visual inspection of Table 2 reveal a differentiated response of HIGH sheep compared to NORM:CONV, revealing great interaction between prenatal and postnatal nutrition in overnourished lambs, which manifest in adulthood.

The LOW sheep do seem as though they are more capable in recovering from adverse effects of postnatal overnutrition, as the programming between LOW:CONV and LOW:HCHF seem similar in adult animals and is primarily strictly prenatal. In experiment 1, adult females presented with adult hyperthyroidism, completely different from the euthyroid–subclinical hypothyroid adult LOW sheep from Experiment 2. This could be rooted in the differences in initial ewe body conditions and

early-life protein intake, as previously discussed, but there was also a difference in body composition or growth from 6 months and upwards, although they were ad libitum fed a grass-based hay diet, adult sheep from Experiment 2 were far less lean, at time of slaughter than females of Experiment 1. This indicates that LOW sheep in Experiment 2 probably experienced a higher dietary intake throughout development, which could partly explain the difference in adult thyroid state. Another difference were that adult sheep of experiment 1 were all female, compared to results from both male and female adult sheep in Experiment 2. The sexual dimorphism observed in lambs and reported in Paper III, could indicate a gender difference and perhaps account for the different results between Paper I and III, although there were no obvious indications of gender differences in adult sheep from paper III, not statistically or by visual inspection of raw data. However, prenatal undernutrition induced greater metabolic alterations in adult females than males in Experiment 2 (Khanal *et al*., 2016), so LOW sheep may be more prone to gender specific programming.

Conclusions

Present thesis support that late gestation maternal malnutrition, either through under- or overnutrition, as well as early-life nutrition has the ability to program both central and peripheral HPT axis function, in a life-time perspective. Fetal under- as well as overnutrition did not cause similar fetal metabolic programming of the HPT axis per se; regardless of differences between adult LOW sheep in Experiment 1 and 2, LOW sheep who received either postnatal treatment were mostly identical as adults and programming effects in these sheep were primarily prenatal. Prenatal overnourished sheep (HIGH) were predisposed for adult overt hypothyroidism, an independent risk factor for development of obesity and related endocrine disorders.

Early-life overnutrition alone did not affect adult thyroid state in Paper I but predisposed for adult central hypothyroidism in Paper II, due to a more severe postnatal overnutrition scheme in Experiment 2 as compared to Experiment 1.

Early-life nutrition can be an additive risk factor for adverse effects of fetal programming, as exemplified by the reported increased feed intake and decreased energy expenditure in adult LOW:HCHF and HIGH:HCHF sheep, respectively. Overall, long-term consequences was rooted in prenatal nutrition and relied on type of postnatal nutrition and vice versa. In this way, energy expenditure, thyroxine challenge response and pituitary gene expression patterns and T3 response to fasting indicated that a conventional diet in HIGH sheep may be considered a "mismatch" diet in fetal overnourished lambs.

Paper I and II, revealed that programming through prenatal and early-life metabolic programming affects expression of genes related to thyroid function both central and peripheral to the thyroid axis and altered central and peripheral thyroid function could possibly explain a diverged energy metabolism of different tissues in programmed subjects.

Overall, distinct interactions between prenatal and postnatal treatments, do not allow us to generalize between NORM, HIGH and LOW sheep, rather we must relate each prenatal treatment to type of postnatal nutrition.

Future studies could elucidate if fetal undernutrition programs adult HPT axis function in a sexual dimorphic manner, which is relevant when considering that thyroid disease is more prevalent in women compared to men. Furthermore, this study does not test how the HIGH and LOW individuals tolerate a HCHF diet in adulthood, which is important since this is when a range of long-term effects appear.

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Appendice

Additional publications during the PhD

Johnsen L, Heerup-Larsson AC, Hellgren LI and Nielsen MO: Fetal programming of the hypothalamus and brain lipid content in response to late gestation under- or overnutrition and early life obesity – observations from sheep. **In preparation.**

Nielsen MO, Hou L, **Johnsen L**, Khanal P, Axel AMD, Leidesdorff CJ, Kongsted AH, Vaag A & Hellgren LI: Smaller adipocyte size (a risk factor for insulin resistance) in subcutaneous adipose tissues has a fetal origin in sheep. Accepted *Clin Nutr Exper*. 2016.

Khanal P, Axel AMD, **Johnsen L**, Hansen PW, Kongsted AH, Lyckegaard NB and Nielsen MO: Long-term consequences of late gestation malnutrition and an early postnatal high-fat diet on growth characteristics and fasting metabolism in adult sheep. PLoS One. 11(6). e0156700.

Khanal P, Axel AM, Kongsted AH, Husted SV, **Johnsen L**, Pandey D, Pedersen KL, Birtwistle M, Markussen B, Kadarmideen HN, Nielsen MO: Late gestation under- and overnutrition have differential impacts when combined with a post-natal obesogenic diet on glucose-lactate-insulin adaptations during metabolic challenges in adolescent sheep. *Acta Physiol*. 2015.

P. Khanal, S. V. Husted, A. M. D. Axel, **L. Johnsen**, K. L. Pedersen, M. S. Mortensen, A. H. Kongsted, M. O. Nielsen: Late gestation over- and undernutrition predispose for visceral adiposity in response to a post-natal obesogenic diet, but with differential impacts on glucose–insulin adaptations during fasting in lambs. Acta Physiologica. 2013

Nielsen MO, Kongsted AH, Tygesen MP, Strathe AB, Caddy S, Quistorff B, Jørgensen W, Christensen VG, Husted S, Chwalibog AC, Sejrsen K, Purup S, Svalastoga E, McEvoy F, **Johnsen L**: Visceral adiposity and preference for a high-fat diet are affected by late gestation undernutrition in sheep and exacerbated by a high-fat diet in early life. British J Nutr. 2012

Conference and seminar presentations

Heerup C, **Johnsen L**, Nielsen MO & Hellgren LI (2015): The Impact of Early Life Over- and Undernutrition on Fatty Acid Composition in Brain Lipids and their Association to Leptin-Related Gene. 2nd Nordic Congress on Obesity in Gynaecology and Obstetrics (NOCOGO), 27-

29 August, Middelfart, Denmark (oral presentation; available from Heerup, Christine at: http://www.nocogo2015.dk/presentations/)

Johnsen L, Axel A, Khanal P, Kongsted AH & Nielsen MO (2014): Long-term implications of late-gestation malnutrition for the hypothalamic-pituitary- thyroidal axis in sheep. Proc. Scand Physiol Soc Meeting, Karolinska Institutet Campus, Stockholm August 22th – 24th, 2014. p50 (oral presentation).

Johnsen L (2013): Konsekvenser af føtal programmering og tidlig ernæring i Får. Møde i Dansk Selskab for Reproduktion og Fosterudvikling (DSRF). Rigshospitalet. November 11th 2013.

Johnsen L, Kongsted AH, Christensen VG, Nielsen MO (2012): Late gestation under-nutition and early postnatal life over-nutrition have long-term implications for thyroid hormone axis function in sheep. Benzon Symposium No. 58 Adipose Tissue in health and disease. August 27th-30th 2012, Copenhagen, Denmark. (abstract)

Johnsen L, Kongsted AH, Nielsen, MO (2012): Late gestation under-nutition and early postnatal life over-nutrition have long-term implications for thyroid hormone axis function in sheep. 82nd Annual Meeting of the American Thyroid Association. September 19th-23rd, 2012. Québec, Canada (abstract)

Papers I–III

Paper I: Prenatal undernutrition and postnatal overnutrition alter thyroid hormone axis function in sheep

Prenatal undernutrition and postnatal overnutrition alter thyroid hormone axis function in sheep

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Abstract

Mounting evidence led us to hypothesize that i) function of the thyroid hormone (TH) axis can be programed by late gestation undernutrition (LG-UN) and ii) early-postnatal-life overnutrition (EL-ON) exacerbates the fetal impacts on TH axis function. In a 2×2 factorial experiment, 21 twin-bearing sheep were fed one of two diets during late gestation: NORM (fulfilling energy and protein requirements) or LOW (50% of NORM). From day 3 to 6 months after birth (around puberty), the twin lambs were assigned to each their diet: conventional (CONV) or high-carbohydrate, high-fat, where after half the lambs were killed. Remaining sheep (exclusively females) were fed the same moderate diet until 2 years of age (young adults). At 6 months and 2 years of age, fasting challenges were conducted and target tissues were collected at autopsy. LG-UN caused adult hyperthyroidism associated with increased thyroid expression of genes regulating TH synthesis and deiodination. In one or more of the target tissues, liver, cardiac muscle, and longissimus dorsi muscle, gene expressions were increased by LG-UN for TH receptors (*THRA* and *THRB*) and deiodinases but were decreased in visceral and subcutaneous adipose tissues. EL-ON increased TH levels in adolescent lambs, but this was reversed after diet correction and not evident in adulthood. We conclude that LG-UN programed TH axis function at the secretory level and differentially in target tissues, which was increasingly manifested with age. Differential TH signaling in adipose vs other tissues may be part of a mechanism whereby fetal malnutrition can predispose for obesity and other metabolic disorders.

Key Words
- Development
- gene expression
- sheep
- thyroid hormone metabolism
- nutrition

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Introduction

It has become increasingly clear from both epidemiological (Phillips *et al*. 1998, Laitinen *et al*. 2004, Rich-Edwards *et al*. 2005) and experimental animal studies (Woodall *et al*. 1996, Ozanne *et al*. 2003, Bol *et al*. 2009) conducted over the last 20 years that malnutrition during fetal life may predispose for adverse health outcomes later in life, including obesity, type 2 diabetes (T2D), and cardiovascular disease (CVD). The phenomenon associating adverse exposures during fetal life with altered body functions and health outcomes later in life is often referred to as fetal metabolic programing (FMP). Experience from developing countries undergoing rapid economic transition indicates that individuals who have been adversely programed during fetal life have an increased risk of developing these diseases when exposed to a mismatching overnutrition situation later in life. In certain developing

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countries, the incidence of intrauterine growth restriction may be as high as 50% and evidence suggest that as income levels rise, obesity, T2D, and CVD become more prevalent among children and young adults (Amuna & Zotor 2008). It is therefore important to get deeper insight into the (molecular) biological mechanisms underlying fetal programing in order to develop targeted and efficient intervention strategies to prevent or reverse later adverse health outcomes. The major endocrine systems in focus in research relating to fetal programing have been the hypothalamic–pituitary–adrenal axis function and the glucose–insulin axis including pancreatic endocrine function and the development of insulin resistance (Bloomfield *et al*. 2004, Ozanne *et al*. 2005, Limesand *et al*. 2006). Only a few studies have addressed the implication of fetal malnutrition for hypothalamic–pituitary–thyroid function later in life and results have been contradictory as presented in the following.

Thyroid hormones (THs) are required for normal function and development of nearly all tissues and regulate oxygen consumption and overall metabolic rate. THs increase RNA synthesis of target genes and increase mitochondrial oxidation. There are reports of altered thyroid function associated with both development of the metabolic syndrome and a history of fetal growth restriction. In patients with the metabolic syndrome, obesity, and insulin resistance have been found to stimulate thyroid cell proliferation and increase thyroid volume and stimulate the production of THs (Sari *et al*. 2003, Pergola *et al*. 2008, Rezzonico *et al*. 2008, Ayturk *et al*. 2009). In a birth cohort study, Kajantie *et al*. (2006) found that a small body size at birth and during childhood increases the risk of spontaneous hypothyroidism in adult women, whereas Brix *et al*. (2000) found, in a population-based twin case–control study, that low birth weight is not associated with thyroid autoimmunity or nonautoimmune thyroid disease. Thyroid gland metabolism has been shown to be downregulated in both nutrient-restricted suckling rats (Bonomo *et al*. 2008, Lisboa *et al*. 2010) and in fetal growth-restricted lambs (Rae *et al*. 2002); in the rat study by Lisboa *et al*. (2010), thyroid gland metabolism remained downregulated into adulthood. Others (Dutra *et al*. 2003, Lisboa *et al*. 2008) have reported that neonatal protein and energy restriction in suckling rats led to adult hyperthyroidism and increased liver deiodinase activity. However, long-term effects of fetal exposures on thyroid function in other species than the rat have not been thoroughly established and the interactive effects of fetal undernutrition followed by postnatal overnutrition has to our knowledge never been examined in any species.

In this study, we aimed to test the hypothesis that function of the TH axis is a target of fetal programing induced by late gestation undernutrition (LG-UN) that contributes to predispose for adverse health outcomes later in life. Bearing in mind that postnatal overnutrition has been reported to exacerbate the adverse negative outcomes of FMP, we further hypothesized that early-postnatal-life overnutrition (EL-ON) will exacerbate the consequences of fetal programing on the TH axis function.

We chose our recently developed Copenhagen sheep model (Nielsen *et al*. 2012) as an experimental animal model for this study since the late gestation period was in focus, where the major quantitative fetal growth takes place. As discussed by Nielsen *et al*. (2012), sheep are more comparable to humans in terms of physiological maturity at birth than rodent offspring. In humans and sheep, thyroid formation occurs during the first trimester and the final maturation (primarily increase in thyroid epithelia) during the last trimester before birth (Bocian-Sobkowska *et al*. 1997, Hájovska 2002, Kratzsch & Pulzer 2008). In rodents, the hypothalamic–pituitary–thyroidal axis continues to develop simultaneously into the *post partum* period and is not concluded until 3–4 weeks after birth independent of intrauterine–placental influences (Dussault & Labrie 1975).

In the Copenhagen sheep model, twin-pregnant sheep were subjected to 50% energy and protein restriction during the last trimester and twin lambs were raised on a special high-carbohydrate, high-fat (HCHF) diet from 3 days to 6 months of age. We have previously shown in this model that exposure to LG-UN can predispose for increased appetite for a high-fat diet early in postnatal life and predispose for visceral obesity by altering fat deposition patterns (Nielsen *et al*. 2012), and in this study, we aimed to relate these phenotypic changes to serum TH levels and TH signaling in major target tissues. Serum levels of total tri-iodothyronine (TT_3) and total thyroxine (TT_4) were therefore measured at different time points in postnatal life in growing lambs and young adult sheep with different nutritional histories in late prenatal and early postnatal life. Tissue samples were obtained from subgroups of animals killed at 6 months and 2 years of age. Gene expression in the thyroid gland was determined for key targets implicated in the regulation of TH synthesis and release (TSH receptor (*TSHR*), thyroglobulin (*TG*), thyroid peroxidase (*TPO*), solute carrier family 5 (sodium iodide symporter), member 5 (*SLC5A5*), type II iodothyronine deiodinase (*DIO2*), and iodotyrosine deiodinase

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(*IYD*)). In addition, expression of genes encoding for targets involved in TH signaling (TH receptor α (*THRA*), *THRB*, and *DIO2*) was determined in the major target tissues liver, heart, two skeletal muscles, and two adipose. *IYD* expression was also determined in liver.

Materials and methods

Experimental animals and experimental design

The Copenhagen sheep model used in this project has been described in detail by Nielsen *et al*. (2012). All experimental animal handling and procedures were approved by The Danish National Committee on Animal Experimentation. In summary, the experiment was a 2×2 factorial design with two prenatal and two postnatal nutritional treatments (Fig. 1). Twin-pregnant ewes (Shropshire breed; $n=21$) were during the final 6 weeks of gestation (term=147 days) fed diets meeting either 100% (NORM, $n=10$) or 50% (LOW, $n=11$) of the daily requirements for energy and protein. The twin lambs were assigned to each their postnatal treatment and individually fed a conventional (CONV) or an obesogenic HCHF diet from day 3 to 6 months *post partum*, resulting in four treatment groups: NORM:CONV ($n=10$), NORM:HCHF ($n=10$), LOW:CONV ($n=11$), and LOW:HCHF ($n=11$). The CONV treatment consisted of good-quality artificially dried hay, which was supplemented with a commercial milk replacer until weaning at 56 days of age and daily allowance was adjusted weekly to achieve moderate weight gains of ∼225 g/day. The HCHF diet consisted of dairy cream (38% fat; with a maximal daily allowance of 0.5 l/day), high-starch popped maize (maximal daily allowance of 1.0 kg/day), and commercial milk replacer (maximal daily allowance was 2.0 l/day until 56 days of age and 0.5 l/day thereafter). All animals had free access to water and a vitamin–mineral mix. At 6 months of age (around puberty), half of the animals from each treatment group were killed, which included all males and three females. Only female offspring (a total of 18) continued in the experiment after 6 months of age and they were fed the same moderate grass-based diet until the age of 2 years (young adults), where they were subjected to a fasting challenge and then killed. After killing, thyroids and target organs were quickly excised and weighed and tissue samples were snap-frozen for later RNA extraction. Target tissues included liver (left lobe), two different skeletal muscles (the longissimus dorsi dominated by glycolytic type II fibers and biceps femoris dominated by oxidative type I fibers; Jørgensen *et al*. (2009), cardiac muscle

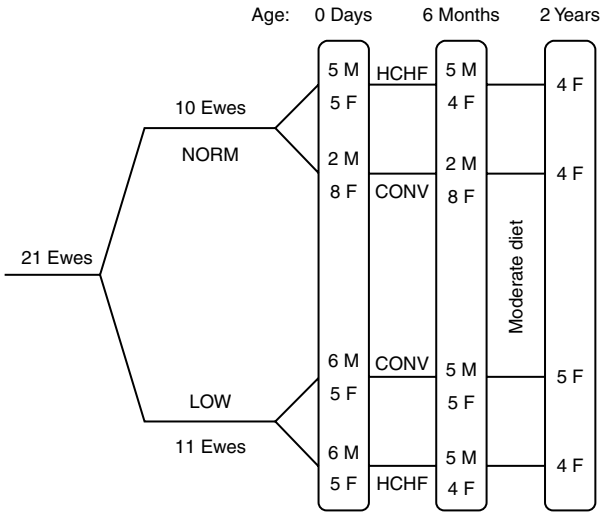

Figure 1
Study design as previously reported in detail by Nielsen *et al*. (2012). Twin-pregnant ewes were exposed to diets fulfilling 100% (NORM) or 50% (LOW) of energy and protein requirements during the last 6 weeks of gestation (term=147 days). Twin offspring were assigned to each their experimental diet from 3 days to 6 months *post partum*; a conventional moderate hay-based diet (CONV) adjusted to achieve moderate growth rates of ∼225 g/day or an obesogenic high-carbohydrate, high-fat diet (HCHF) based on maize and a milk-dairy cream mix. All males were killed at 6 months of age. Only female sheep continued in the experiment after that, and from 6 months to 2 years of age, they were fed the same moderate (and for HCHF sheep: body fat correcting) grass/hay-based diet. Animal numbers and sex (M, males; F, females) included at different time points are shown.

(central part of ventriculus sinister cordis), and two adipose tissues (visceral and subcutaneous sampled above the central part of longissimus dorsi).

Blood sampling and serum TT_4 and TT_3 analysis

Baseline blood samples were drawn from lambs at 1 day, 56 days, and 6 months of age and from female sheep at 1 and 2 years of age. At 2 years of age, catheters were inserted into both jugular veins, as described previously (Husted *et al*. 2008), and sheep were subsequently subjected to a 3-day period of fasting. Blood was sampled 0, 24, and 48 after the feed was withheld and 1 h after re-feeding at 72 h after initiation of the fasting. The sheep had free access to water during the fasting period. Blood was collected in serum tubes and allowed to coagulate at room temperature for ∼30 min. Serum was separated by centrifugation at 1800×G_{av} (at 4 °C for 15 min) and subsequently stored at −20 °C until analyzed. Serum TT_4 and TT_3 concentrations were assessed using a human enzyme immunoassay (DRG Diagnostics, Marburg, Germany). Six out of 195 and 9/195

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samples had an intra-assay variation >10% but below <15% for TT_3 and TT_4 respectively. The interassay variation was 20% for TT_3 (the kit supplied control values with a variation of up to 20–30% for concentrations in the range of 1.30–2.50 ng/ml) and 7.5% for TT_4. Samples from a given animal were analyzed within the same assay kit. Excluding the few samples that yielded large variations from statistics did not affect results.

Relative quantification of gene expression

mRNAs for *SLC5A5*, *IYD*, *TPO*, *DIO2*, *TG*, *TSHR*, *THRA*, and *THRB* were quantified using quantitative reverse transcriptase PCR and procedures were according to manufacturer's guidelines. Total RNA was extracted with TRIzol Reagent (Invitrogen) and 1-bromo-3chloropropane (Sigma–Aldrich) and cleaned up using SV Total RNA Isolation (Promega) from tissue samples from thyroid, liver, heart muscle, longissimus dorsi, biceps femoris, subcutaneous fat, and visceral fat. Total RNA concentrations and sample purity were established with NanoDrop ND-1000 u.v.-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and RNA integrity was confirmed by bioanalysis (Agilent 2100 Bioanalyzer; Agilent Technologies, Santa Clara, CA, USA). RT was performed using 0.5–2 μg (depending on tissue) total RNA with oligo-dT and random hexamer as primers and with MMLV reverse transcriptase (Promega). The cDNA was pooled to make standard curves and calibrator for each plate. Calibrator, samples, and negative controls were performed in triplicate. Standard curves were made using serial dilutions of cDNA (1:2, 1:4, 1:8, 1:16, 1:32, and 1:64) to determine the efficiency of each primer set within the resulting linear regression. Efficiencies of primers were between 1.8 and 2.1 (this equals to an increase between 80 and 110% of target nucleic acid in each amplification cycle; Table 1) and all coefficients of determination ≥0.99. Primer sequences were derived from ovine or bovine cDNA sequences obtained from the National Center for Biotechnology Information (NCBI) and primer sequences, NCBI accession numbers, and annealing temperatures are listed in Table 1. SYBR Green (SYBR Green master mix; Roche) was used as the fluorophore, and qPCR was performed using the LightCycler 480 System (Roche). Melt curve analysis was conducted on each sample after the final cycle to ensure that a single product was obtained. Peptidylprolyl isomerase B (cyclophilin B; *PPIB*) was used as reference mRNA for all tissues except for the two adipose tissues where β-actin (*ACTB*) was a better match; the identical first-strand cDNA was used for quantification of specific mRNAs of interest to circumvent any between-run variation. Data were analyzed using the advanced relative quantification method provided by LightCycler 480 instrument version 2.0 Software (Roche). The qPCR products were cleaned up by Wizard SV Gel and PCR clean up system (Promega) and sequenced on ABI3130XL (Applied Biosystems) with BigDye terminator v3.1 cycle sequencing

Table 1 Primer sequences and accession numbers for applied genes

Gene	Primer sequence	NCBI accession no.	Annealing temperature (°C)	Efficiency
Cyc B	F: GATCCAGGGTGGAGATTTCAC R: GGCCCATAGTGTTTAAGCTTG	AJ865374 (Oa)	60	1.88
ACTB	F: ACCCAGATCATGTTCGAGACCTT R: TCACCGGAGTCCATCACGAT	AY141970 (Bt)	60	1.83
SLC5A5	L: CGGAATCATCTGCACCTTCT R: GGACAACCCAGAAACCACTC	XM_581578.5 (Bt)	60	1.87
IYD	L: TTCTCCCACAGTCGATACCC R: ATCTGGGTCCTTCACAACCA	NM_001102165.1 (Bt)	60	2.19
DIO2	L: GTGGCTGACTTCCTGTTGGT R: GCATCGGTCTTCCTGGTTC	NM_001010992 (Bt)	60	1.97
TPO	L: ATCACGGATTCCAACTCCAA R: GGGTCCACTTCATCCTCACA	XM_603356.5 (Bt)	60	1.91
TG	L: GAGCAGGTTTCCAGAGGTGT R: AGAGTGGTCTCAGCGAAGGT	NM_173883.2 (Bt)	60	2.00
TSHr	L: GGGAGTGAGGAGATGGTGTG R: GAGGATGACCAGGACGAAGA	NM_001009410.1 (Oa)	60	1.97
THRA	L: CCTCTTCTCTCCTCCCTCTC R: TTGTCCGCTCTTAGTTCTCC	NM_001100919.1 (Oa)	60	1.92
THRB	L: GAAGCTCGTGGGAATGTCT R: GCCTTTGCACTTCTTCTCCT	NM_001190391.1 (Oa)	57	2.18

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kit and Hi-Di formamide (Applied Biosystems). Sequences were confirmed using the NCBI blast logical alignment search tool.

Statistical analysis

It should be noted that in the data based on tissue samples obtained at killing, sex differences cannot be distinguished at the two different ages, as no males were killed at 2 years of age and only three females were killed in the 6-month old group (they were all from the NORM-CONV group). Inclusion of gene expression data for these three, 6-month old female NORM-CONV lambs did not impact outcomes of the statistical tests and their data are therefore included in the results presented here. The variable sex is therefore eliminated from the model for data derived from tissue samples in 2-year-old sheep.

For all other data, gender-specific responses to the late gestation and early postnatal life nutrition exposures could be evaluated for growing lambs up to 6 months of

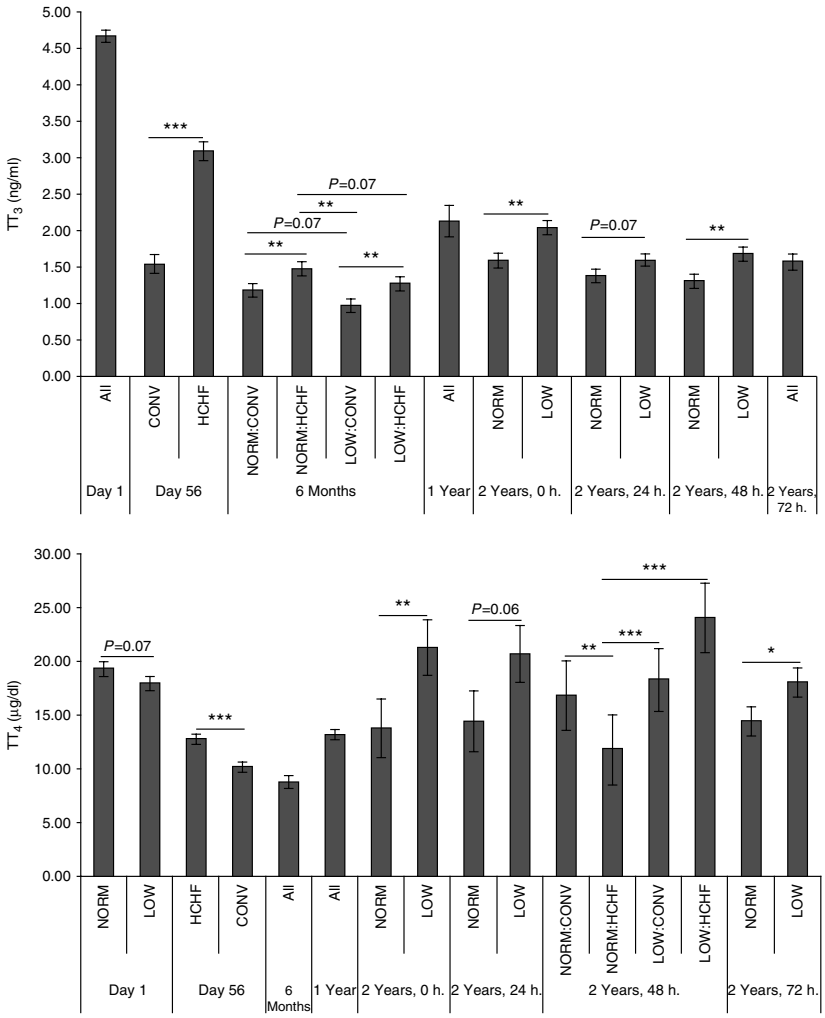

Figure 2

Basal serum concentrations of total T_3 (TT_3, ng/ml) and total T_4 (TT_4, µg/dl) obtained from 1-day, 56-day, 6-month, 1-year, and 2-year-old sheep, as affected by nutrition received during late fetal life and the first 6 months of postnatal life. Samples from 1-day to 6-month-old sheep were obtained from both males and females. All males were killed at 6 months of age, and only female sheep were included in the experiment thereafter. NORM and LOW refer to the plane of nutrition offered to twin-pregnant ewes during late gestation, and CONV and HCHF refer to the postnatal diet fed to either of the twin lambs from day 3 to 6 months of age (see legends to Fig. 1 for further details), thus giving rise to four treatment groups: NORM-CONV, NORM-HCHF, LOW-CONV, and LOW-HCHF. At 2 years of age, time point 0 h indicates samples taken from the female adult sheep in the fed state, 24- and 48-h samples were obtained after 24 and 48 h of fasting respectively and 72-h sample was taken 1 h after re-feeding. All other samples were taken from non-fasted animals. Significant differences are $*P \leq 0.05$, $**P \leq 0.01$, or $***P \leq 0.001$.

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age; and differences in the manifested responses with age could be evaluated for the females that were studied until adulthood at 2 years of age.

All statistical models were derived from the same multifactorial model:

$$Y_{ijlo} = \mu + \alpha_i + \alpha\beta_{ij} + \gamma_k + \alpha\beta\gamma_{ijk} + \kappa_l + \varepsilon_{ijkl}$$

where Y_{ijlo} is the specific factor, described by all the qualitative explanatory variables, μ is the overall mean, α_i is the effect of the prenatal NORM or LOW treatment, β_j is the effect of the postnatal CONV or HCHF treatment, $\alpha\beta_{ij}$ is the interaction between the two treatments, γ_k is the effect of age (or sex; male or female), $\alpha\beta\gamma_{ijk}$ is the three-way interaction between the two treatments and age (or sex), κ_l is the random effect of offspring, and ε_{ijkl} is the residual variation $\sim n(0,\sigma 2)$. The universal sample space of the qualitative explanatory variables are $i=\{1,2\}$, $j=\{1,2\}$, $k=\{1,2\}$ (TH's: $k=\{1,\ldots,5\}$), and $l=\{1,2\}$.

All models were tested in R 2.10.1 (R Development Core Team 2010 GNU Project, http://www.r-project.org) using 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 box-cox. Following this, the model was reduced by testing significance of any interactions by two-way ANOVA. All variables that showed no significance were eliminated from the model in this way. Estimates and significance of the remaining factors were calculated by the function lsmeans in R 2.10.1.

Results

No effects of prenatal nutrition, postnatal nutrition, or sex were observed unless specifically stated. The basic phenotypic characteristics and performance of the Copenhagen sheep model used in this experiment have been described in detail by Nielsen *et al.* (2012), but some of the most relevant findings for interpretation of results from this study will be summarized here. When compared with NORM controls, LOW animals exposed to LG-UN had reduced birth weights, had a preference in the very early postnatal period for high-fat dairy cream rather than starch-rich maize, and an increased susceptibility to develop visceral obesity. After conversion to the moderate diet from 6 months to 2 years of age, total body fat content was normalized in the adult HCHF females and became similar to that of CONV sheep, which had been raised on a moderate plane of nutrition throughout the postnatal period.

TT_3 and TT_4 in serum

Prenatal nutrition had no impact on TT_3 serum concentrations in the new born lambs (Fig. 2a), whereas TT_4 levels tended to be reduced in LOW lambs compared with NORM lambs at day 1 after birth (Fig. 2b). At 56 days of age, the HCHF diet led to significantly higher serum TT_3 and TT_4 in lambs compared with the CONV lambs at 56 days of age ($P=0.0002$ and $P=0.0004$ respectively) and at 6 months of age lambs receiving the postnatal HCHF diet continued to have higher TT_3 concentrations compared with their twin on the CONV diet ($P=0.01$), whereas TT_4 concentrations was no longer affected by the postnatal diet at this or older ages. At 1 year of age (where all animals had been fed the same moderate diet for 6 months), postnatal diet effects on TT_3 had also disappeared and there were no impacts of prenatal nutrition either. However, in adult females at 2 years of age, an effect of LG-UN became evident, as prenatal LOW sheep had significantly higher levels of both TT_3 and TT_4 ($P=0.007$ for both) compared with NORM females that had been adequately nourished during late gestation. Furthermore, prenatal LOW sheep continued to have higher concentrations of circulating TT_3 and TT_4 during a 48-h fasting period. One hour after re-feeding (after

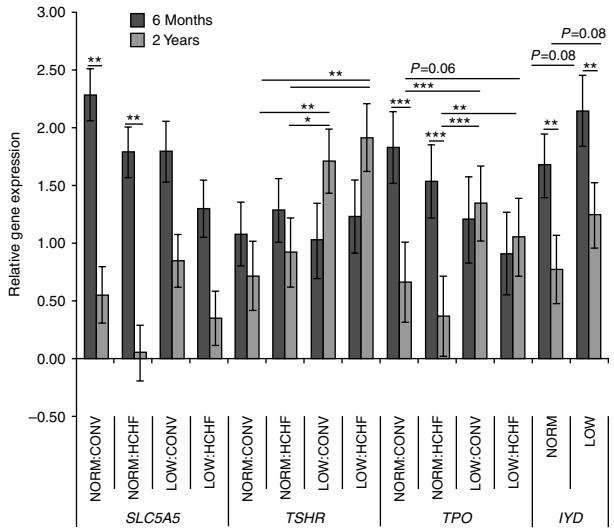

Figure 3
Group means of relative gene expression in the thyroid, for the genes *SLC5A5*, *TSHR*, *TPO*, and *IYD* from lambs at 6 months (predominantly males) and 2 years (exclusively females) of age, as affected by nutrition received during late fetal life (NORM or LOW) and the first 6 months of postnatal life (CONV or HCHF). The experimental design and dietary treatments have been fully described in legends of Figs 1 and 2. Significant differences are $*P\leq 0.05$, $**P\leq 0.01$, or $***P\leq 0.001$.

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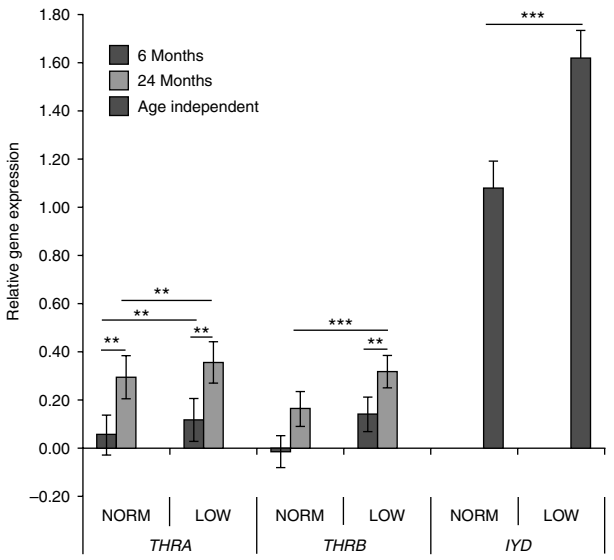

Figure 4
Group means of relative gene expression in the liver, for the genes *THRA*, *THRB*, and *IYD* from lambs at 6 months (predominantly males) and 2 years (exclusively females) of age, as affected by nutrition received during late fetal life (NORM or LOW) and the first 6 months of postnatal life (CONV or HCHF). The experimental design and dietary treatments have been fully described in legends of Figs 1 and 2. Significant differences are $^{**}P \leq 0.01$, or $^{***}P \leq 0.001$.

3 days of fasting), all animals had equal circulating levels of TT_3, but TT_4 remained increased in LOW sheep (Fig. 2a and b).

Gene expression in the thyroid gland

There were no effects of late gestation nutrition on *TSHR* and *TPO* expression in 6-month-old predominantly male lambs, but an effect of LG-UN became evident in adulthood, as 2-year-old LOW females had higher *TSHR* and *TPO* expression compared with NORM females ($P=0.008$–0.05 and $P=0.0005$ respectively; Fig. 3). *IYD* expression tended to be significantly upregulated in LOW compared with CONV animals, which was observed in both 6-month-old lambs and 2-year-old sheep.

There were no effects of postnatal diet on expression of any of the abovementioned genes and no effect of neither pre- nor postnatal treatment on *DIO2*, *TG*, and *SLC5A5* expression in thyroid tissues from 6-month-old lambs or 2-year-old adult sheep. The 2-year-old female sheep generally had lower expression levels of *IYD* compared with the (mostly male) 6-month-old lambs ($P=0.008$). In NORM animals, *TPO* ($P=0.0004$) and *SLC5A5* ($P=0.003$) was also lower in adult females compared with the predominantly male lambs, but in LOW animals, expression levels of these two genes were similar in lambs and adult sheep.

Gene expression in TH target tissues

Liver Expression of *DIO2* could not be detected in the liver; therefore, no results are presented for this gene. LOW animals with a history of prenatal undernutrition had higher expression levels compared with NORM animals for *THRA* ($P=0.008$) and for *IYD* ($P=0.0002$) at both 6 months and 2 years of age and such an effect of prenatal undernutrition also became evident for *THRB* in adult female sheep at 2 years of age ($P=0.009$) (Fig. 4). Expression of *THRA*, *THRB*, and *IYD* was generally higher in 2-year-old females compared with 6-month-old (mostly male) lambs. The postnatal diet had no influence on expression of any of these genes in the liver.

Muscle **Cardiac** Animals subjected to the LOW prenatal treatment had significantly upregulated *DIO2*, *THRA*, and *THRB* expression in cardiac muscle at both 6 months and 2 years of age compared with NORM animals ($P=0.01$, $P=0.0001$, and $P=0.04$ respectively; Fig. 5).

Longissimus dorsi *DIO2* was significantly upregulated in longissimus dorsi in the LOW:CONV-fed lambs at 6 months of age compared with all other treatment groups ($P=0.02$–0.01), but in 2-year-old females, there were no

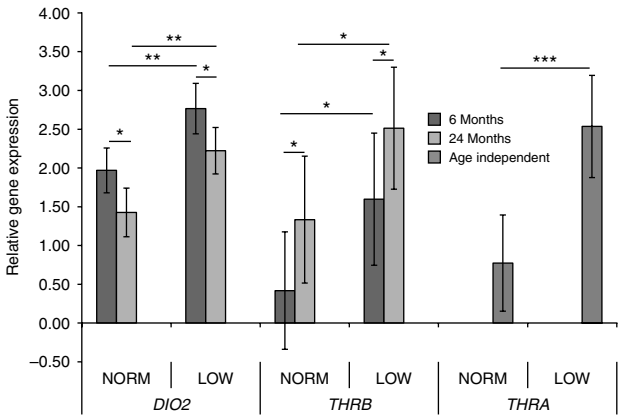

Figure 5
Group means of relative gene expression in cardiac muscle, for the genes *THRA*, *THRB*, and *DIO2* from lambs at 6 months (predominantly males) and 2 years (exclusively females) of age, as affected by nutrition received during late fetal life (NORM or LOW) and the first 6 months of postnatal life (CONV or HCHF). The experimental design and dietary treatments have been fully described in legends of Figs 1 and 2. Significant differences are $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, or $^{***}P \leq 0.001$.

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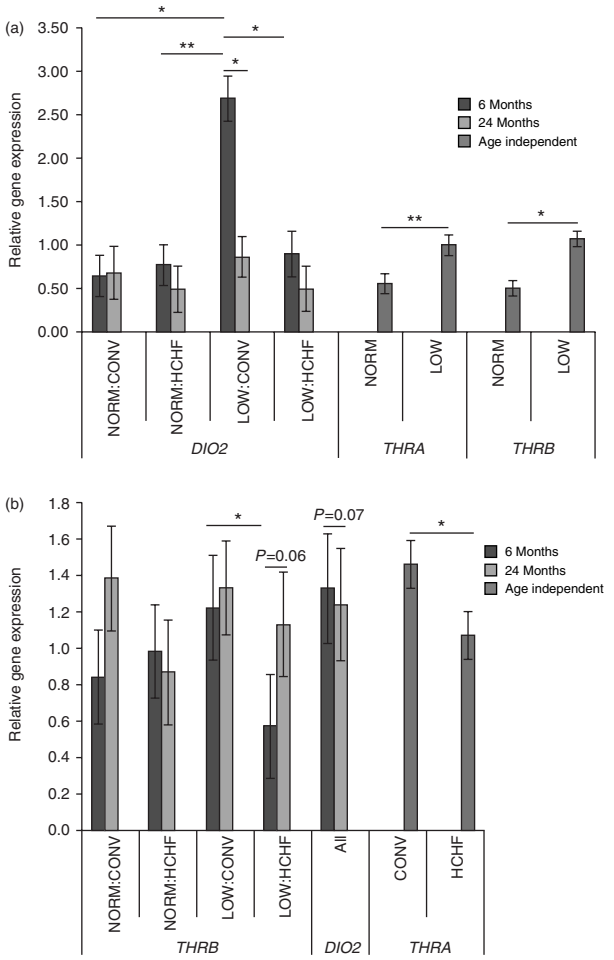

Figure 6
Group means of relative gene expression in longisimus dorsi (a) and biceps femoris (b) for the genes *THRA*, *THRB*, and *DIO2* from lambs at 6 months (predominantly males) and 2 years (exclusively females) of age, as affected by nutrition received during late fetal life (NORM or LOW) and the first 6 months of postnatal life (CONV or HCHF). The experimental design and dietary treatments have been fully described in legends of Figs 1 and 2. Significant differences are $*P \leq 0.05$, $**P \leq 0.01$.

differences in *DIO2* expression between treatment groups. As for cardiac muscle, both *THRA* and *THRB* expression were upregulated in longissimus dorsi in LOW animals compared with NORM animals ($P=0.009$ and $P=0.05$ respectively; Fig. 6a) and in both 6-month-old (mostly male) lambs and in 2-year-old adult female sheep. The postnatal nutrition had no impact on expression patterns of any of these genes in either cardiac or longissimus dorsi muscle.

Biceps femoris This muscle had a different response to early life nutrition exposure compared to cardiac muscle and longissimus dorsi. *THRA* expression was not affected by prenatal diet in this muscle, but it was significantly downregulated in animals fed the HCHF diet compared with those fed the CONV diet and this was observed not only in 6-month-old lambs, when they were exposed to the different diets, but also in 2-year-old female sheep after they had been fed for 1½ years on the same moderate diet ($P=0.04$). The HCHF diet had a similar depressive effect on *THRB* expression in 6-month-old LOW lambs ($P=0.04$), but it was not observed in the NORM lambs or in the adult females after diet correction. *DIO2* expression was not affected by neither pre- nor postnatal nutrition but was lower in the 2-year-old females compared with the 6-month-old (mostly male) lambs ($P=0.07$; Fig. 6b).

Adipose tissue **Subcutaneous** *THRA* expression in subcutaneous adipose tissue was influenced by the diet received in postnatal life but not by prenatal nutritional history. At 6 months of age, *THRA* expression was reduced in lambs fed the HCHF diet compared with lambs fed the CONV diet ($P=0.001$–0.01). In the adult female sheep, which had been fed 1½ years on the same moderate diet, there was also an effect of nutrition exposure in early postnatal life on *THRA* expression, but the effect was opposite to that observed in lambs, as expression levels were highest in the adult sheep that were previously fed the HCHF diet ($P=0.02$; Fig. 7a). *THRB* in contrast to *THRA* was not affected by postnatal nutrition but tended to be affected by prenatal nutrition with LOW having reduced expression levels compared with NORM animals both at 6 months and 2 years of age (Fig. 7a). *DIO2* expression was not affected by pre- or postnatal nutritional treatments in animals at any of the two ages.

Visceral *THRA* expression in visceral adipose tissue was not affected by pre- or postnatal dietary treatments in 6-month-old lambs. But in 2-year-old sheep, expression levels were negatively affected by a history of both LG-UN and EL-ON in an apparently additive way ($P=0.03$–0.001). In animals that had been adequately nourished as fetuses (NORM), *THRA* expression levels were higher in the 2-year-old female sheep compared with the 6-month-old (mostly male) lambs ($P=0.005$). However, in adult sheep with a history of LOW nutrition prenatally, expression levels were substantially depressed relative to 6-month-old lambs (Fig. 7b). *THRB* expression was not influenced by pre- or postnatal nutritional treatments or age (results not shown). *DIO2* expression was significantly reduced in LOW compared with NORM animals that had been fed the CONV diet in postnatal life and this was across both age groups ($P=0.009$; Fig. 7b). This influence of prenatal

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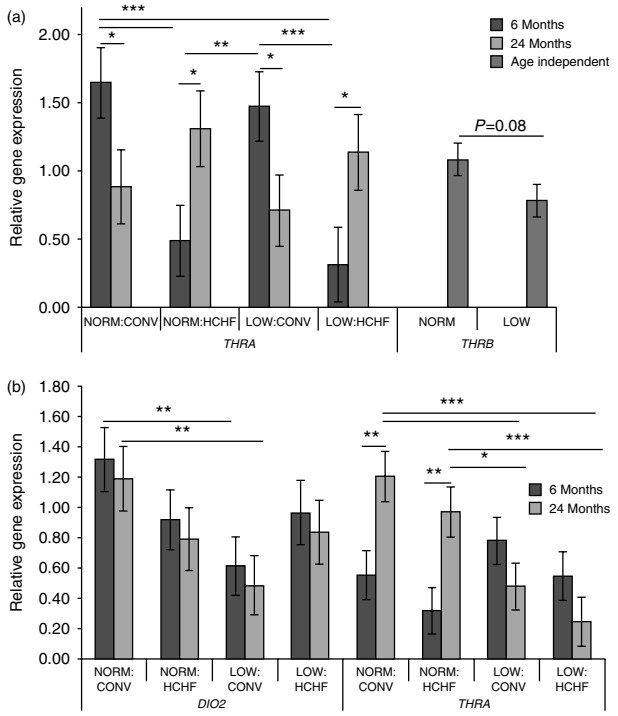

Figure 7
Group means of relative gene expression in subcutaneous adipose tissue (a) and visceral adipose tissue (b), for the genes *THRA*, *THRB*, and *DIO2* from lambs at 6 months (predominantly males) and 2 years (exclusively females) of age, as affected by nutrition received during late fetal life (NORM or LOW) and the first 6 months of postnatal life (CONV or HCHF). The experimental design and dietary treatments have been fully described in legends of Figs 1 and 2. Significant differences are $*P \leq 0.05$, $**P \leq 0.01$, or $***P \leq 0.001$.

nutritional history on *DIO2* expression was not observed in lambs or sheep that were exposed to the HCHF diet in early postnatal life. Expression levels of *DIO2* were of similar magnitude in the two age groups.

Discussion

Fetal origins of health and disease have been quite extensively studied during the last couple of decades (see e.g. review by Correia *et al.* (2012)), but there is a scarcity of studies focusing on the TH axis. Using our new Copenhagen sheep model (Nielsen *et al.* 2012), we aimed to study the impacts on TH axis function and signaling in target tissues of global energy and protein malnutrition during late gestation, combined with different nutrition exposures in the early postnatal period, which included an obesogenic HCHF diet.

Although the experimental design does not allow us to make direct comparisons between sexes with respect to target tissue responses, it was noteworthy that gene expression patterns in 6-month-old male lambs with a history of LG-UN were also quite persistently observed in the 2-year-old adult females subjected to LG-UN. The consistent findings between the LOW male lambs and LOW adult females suggest that the long-term implications of fetal nutrition are hardly restricted to any specific sex.

Effects of LG-UN in growing lambs

We did not find evidence to suggest that LG-UN significantly affected serum TH levels in female or male lambs, neither at birth nor during the growth period up until puberty. In another sheep study, De Blasio *et al.* (2006) reported that placental restriction reduced plasma TT_4 and increased plasma TT_3 in growing lambs. We did see a close-to-significant effect toward TT_4 being lowered in 1-day-old LG-UN lambs, but placental restriction (removal of the majority of endometrial caruncles) and global feed restriction may not be directly comparable interventions to induce undernourishment and growth restriction of the fetus. To the best of our knowledge, we are the first to report impacts of nutrition in prenatal life on TH signaling-related genes in multiple target tissues and we have convincingly demonstrated that the TH axis was indeed a target of programing in response to LG-UN exposure, as distinct changes in gene expression encoding factors involved in TH signaling in target tissues were observed in the 6-month-old LOW compared with NORM lambs. These effects were manifested irrespectively of which diet the lambs received in early postnatal life, and we found no indications to suggest that the postnatal diet can exacerbate the impacts of a fetal programing of the TH axis neither in lambs nor in adult sheep. Interestingly, the change in gene expression in response to a history of undernutrition in late fetal life was target tissue specific. Thus, LG-UN-induced upregulation of both deiodinase activity and THRs in metabolically important lean tissues, i.e. liver, cardiac muscle, and longissimus dorsi. This would suggest that the sensitivity toward TH in these tissues was increased in LOW lambs. However, in adipose tissues, there was a tendency for the opposite response with downregulation of deiodinase and THRs in LOW lambs (see Fig. 8). Based on these results, it is tempting to raise the question whether reduction of the sensitivity in adipose tissue toward catabolic actions of THs is one mechanism whereby LG-UN can increase the predisposition for development of (visceral) obesity later in life?

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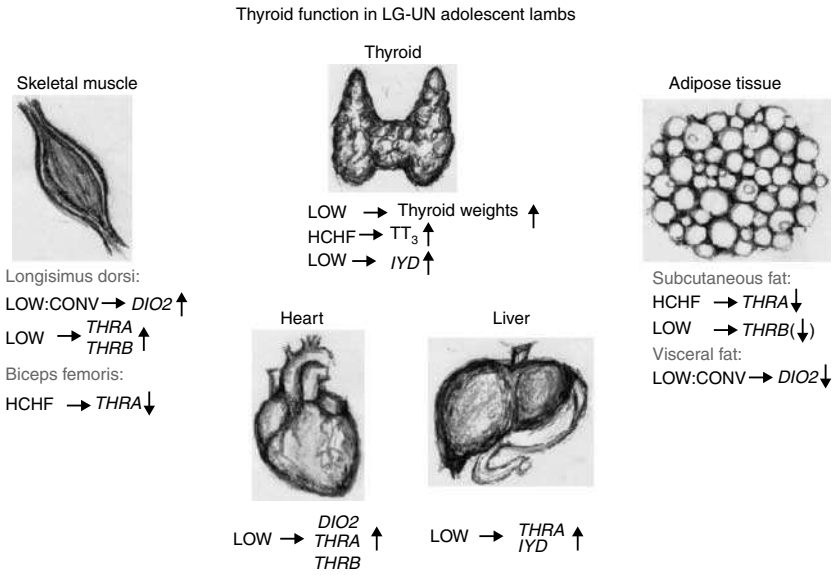

Figure 8

Overview of the significant alterations mediated by the late gestation undernutrition (LOW) diet or postnatal high-carbohydrate, high-fat (HCHF) diet on thyroid function in adolescent lambs. The experimental design and dietary treatments have been fully described in legends of Figs 1 and 2. We previously reported that exposure to the prenatal LOW compared with NORM diet close to significantly increased weight (as a proportion of total body weight) of thyroids in both growing and adult animals ($P=0.055$; Nielsen *et al*. 2012). This dietary consequence has therefore been included in the figure. Illustrations by Ms Rikke Lenitha Larsen.

THs have also been shown to stimulate GH/insulin-like growth factor (IGF1) axis signaling and expression of target genes in bone (Yen 2001). Exposure to undernutrition during fetal life results in earlier cessation of growth and a smaller adult body size in sheep (Schinckel & Short 1961) and humans (Jones 2004), and this was also the tendency in the present experiment (Nielsen *et al*. 2012). We did not investigate TH signaling in bone tissue in the present experiment, but in a recent sheep study by Lanham *et al*. (2011), thyroidectomy at 105–110 days of gestation caused fetal growth retardation and the hypothyroid state was associated with significant changes in metatarsal bone structure and strength when analyzed later in gestation (130 and 144 days; term = 147 days). Future studies are therefore needed to clarify the implications of early programing of TH function also on skeletal development and health later in life.

Effects of LG-UN in adult sheep

Effects of LG-UN on gene expression patterns in the thyroid and TH target tissues observed in growing male lambs were also quite consistently observed in adult female sheep (Figs 8 and 9), but in the adult sheep, impacts of LG-UN were also evident for a range of new parameters at both thyroid and target tissue levels (see Fig. 9). Adult females with a history of LG-UN showed signs of hyperthyroidism with increased TH serum level both in the fed and fasted state. Dutra *et al*. (2003) and Lisboa *et al*. (2008) have reported similar findings in rats, where nutrient restriction of neonatal rat pups result in hyperthyroid adults. Our adult LOW sheep had upregulated gene expression in the thyroid for the key TH biosynthesis genes *TSHR*, *IYD*, and *TPO* and upregulated THR expression, *THRA* and *THRB*, in heart muscle, liver, and LD, concomitant with increased baseline and fasting serum levels of THs and these findings, along with gross physiological evidence provided in the following, strongly support that these animals were hyperthyroid. The upregulation of THRs *THRA* and *THRB* in these major target tissues concomitant with upregulated (cardiac muscle) or unaltered (Biceps Femoris (BF) and Longissimus Dorsi (LD)) expression of *DIO2* indicates that the hyperthyroid state is not induced by peripheral TH resistance. Rather, we speculate that the regulation of thyroid secretion has been programed at a higher level of the Hypothalamic-Pituitary-Thyroidal (HPT) axis, with increased sensitivity toward TSH and TH biosynthesis in the pituitary.

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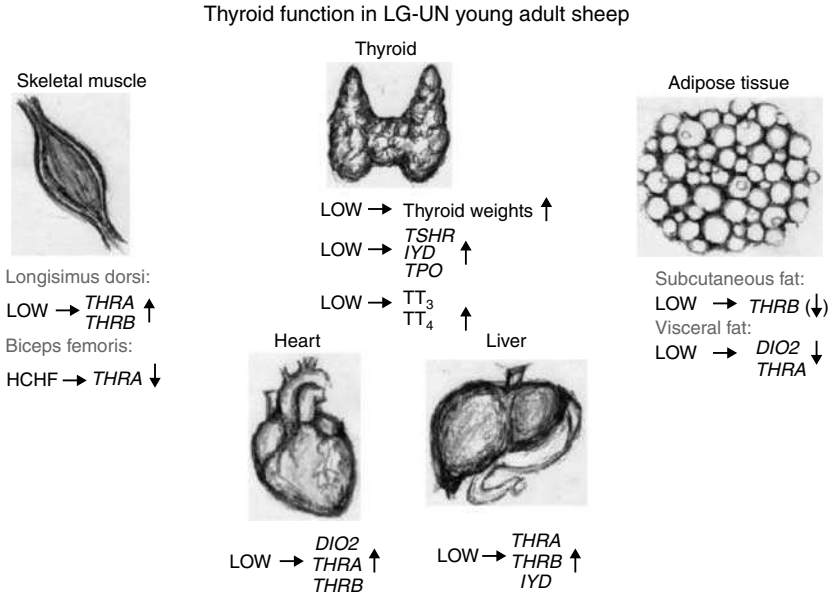

Figure 9

Systemic overview of the significant alterations mediated by the late gestation undernutrition (LOW) diet or postnatal high-carbohydrate, high-fat (HCHF) diet on thyroid function in adult sheep. The experimental design and dietary treatments have been fully described in legends to Figs 1 and 2. We previously reported that exposure to the prenatal LOW compared with NORM diet close to significantly increased weight (as a proportion of total body weight) of thyroids in both growing and adult animals ($P=0.055$; Nielsen *et al*. 2012). This dietary consequence has therefore been included in the figure. Illustrations by Ms Rikke Lenitha Larsen.

On a gross physiological scale, we know that the transfer of HCHF females to a moderate and body fat correcting diet from 6 months of age resulted in a loss of ~10% body weight in LOW:HCHF females from 6 to 12 months of age, whereas the NORM-HCHF female sheep maintained their bodyweights during this period (Nielsen *et al*. 2012). This could reflect an overall higher metabolic rate of the hyperthyroid LG-UN sheep, and indeed Kiani *et al*. (2008) reported that LG-UN sheep had larger energy expenditures per kilogram Metabolic Body Weight (MBW) compared with sheep that were adequately nourished during fetal life.

That LG-UN programs for hyperthyroidism could offer additional mechanistic explanations to some of the known hallmark symptoms of FMP and associated metabolic disorders later in life, such as fasting hyperglycemia, impaired fasting glucose, impaired glucose tolerance or insulin resistance, systemic inflammation, hypertension, and hyperlipidemia (Fernandez-Twinn & Ozanne 2010). Hyperthyroidism increases endogenous glucose production, induces hepatic insulin resistance, causes hypertension, and has been associated with low HDL-cholesterol and hyperlipidemia (Cachefo *et al*. 2001, Biondi *et al*. 2002, Tancevski *et al*. 2008, Klieverik *et al*. 2009). Indeed, we found that LG-UN female sheep at 2 years of age cleared insulin significantly slower during insulin challenge than NORM females (Kongsted 2011). Another hallmark symptom of FMP is obesity. Hyperthyroidism would normally be associated with loss of body weight, but interestingly in this study, we found that *THRA*, *THRB*, and/or *DIO2* were downregulated in adipose tissue, opposite to the upregulation observed in other target tissues, and we hypothesize that this can impair the potential catabolic actions of THs in adipose tissue in LG-UN individuals and thereby increase their susceptibility for development of obesity when nutrition is abundant.

Effect of early life overnutrition in growing lambs

Male and female lambs fed the obesogenic HCHF diet had, as expected, significantly higher serum TT_3 levels compared with their twin lambs fed the moderate CONV diet during the time they were exposed to this diet (Fig. 8). It is well documented that accumulation of body fat is positively correlated with adipose leptin synthesis and leptin concentration in plasma and leptin can, in turn

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upon binding to specific receptors in the arcuate nucleus of the hypothalamus, induce neuroendocrine changes resulting in increased release of TRH from the hypothalamus, TSH from the pituitary gland, and thereby TH from the thyroid glands (Costa da Veiga *et al*. 2004).

There were remarkably few effects of the extreme (for a ruminant animal) HCHF diet on expression of genes encoding for factors involved in TH signaling in target tissues. In fact, only two significant results were found and both regarded *THRA*, which was downregulated in both BF muscle and subcutaneous adipose tissue. The different responses in muscles to pre- and postnatal dietary exposure could not be related to whether the muscles contained predominantly glycolytic (LD) or oxidative (cardiac and BF) muscle fibers (Jørgensen *et al*. 2009).

Effect of early life overnutrition in adult sheep

The increased TH concentrations observed in the 6-month-old male and female HCHF fed lambs were clearly related directly to the dietary intake at that time, as there were no indications of any postnatal dietary impacts on serum TH levels in the same females after they had been fed a moderate (and body fat correcting) diet for 6 months or more. This is in line with the results from Sari *et al*. (2003), who found that weight loss in women could restore TSH levels to normal.

LG-UN resulted in upregulated gene expression in LD and cardiac muscle regardless of age, whereas only one long-term implication of EL-ON could be detected, and it was downregulation of *THRA* expression in BF, as also observed in the male lambs at 6 months of age. It could be speculated that the HCHF diet-reduced expression of *THRA* in BF facilitates an increased capacity for triglyceride uptake and/or accumulation in this predominantly oxidative muscle. Thus, the observed differentiated effects of LG-UN and EL-ON on TH signaling in the different muscles suggest that quite specific long-term metabolic adaptation can be induced in different tissues depending on the nutritional environment in particularly pre- but also postnatal life.

In conclusion, LG-UN unmistakably programed the TH axis resulting in adult hyperthyroidism associated with increased thyroid expression of genes regulating TH synthesis and deiodination and increased THR and deiodinase expression in one or more of the target tissues liver, cardiac muscle, and longissimus dorsi muscle but decreased THR and deiodinase expression in adipose tissues. LG-UN thus appears to program for differentiated TH response or sensitivity in major target tissues. The programing effects mediated by LG-UN on TH signaling in target tissues were evident early in life, before LG-UN effects could be detected in circulating levels of TT_3 and TT_4 in serum. LG-UN effects were permanent and became even more strongly expressed in adulthood. Further studies are required to establish whether the apparently differential TH signaling in adipose vs other tissues can be part of a mechanism linking LG-UN to altered growth trajectories and increased predisposition for visceral obesity and associated disorders. Early postnatal dietary treatment effects on TH axis parameters were in general remarkably few and reversible and had no impact on the expression of fetal programing of the TH axis function. This suggests that the time-of-birth is a critical set point for when long-term programing of TH axis function can occur and this should be considered in the choice of experimental animal models when late gestation impacts are in focus.

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 has been in charge of all the laboratory procedures, statistical evaluation of data, and for writing the manuscript. Post doc A H K has contributed with valuable inputs in the interpretation of results and manuscript revision. Associate Prof. M O N, vice-director of the Danish Centre for Fetal Programming, was the overall responsible person for designing the experiment and developing the experimental sheep model, for evaluating the results, and finalizing the manuscript. All three authors were actively involved in the underlying experimental animal work.

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Paper II: Late gestation over or- overnutrition and early postnatal overnutrition affect thyroid function and metabolic phenotype in adult sheep.

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Late gestation over- or undernutrition and early postnatal overnutrition affect thyroid function and metabolic phenotype in adult sheep

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Background: In adult sheep, the hypothalamic-pituitary-thyroidal (HPT) axis is a target for programming by prenatal and early postnatal nutrition. Present experiments were designed to test; 1) If fetal under- or overnutrition programs the HPT axis, with lasting effects on energy metabolism and thyroid status, and 2) If an early-life obesogenic diet promotes effects of the over- or under nutritional diets applied. **Methods:** Three groups of 12 (36) twin-pregnant sheep were either i) adequately nourished (NORM), ii) undernourished (50% energy and protein requirements) (LOW) or iii) overnourished (150% energy and 110% protein requirements) (HIGH) in the last trimester of gestation. Resulting twin lambs were subdivided onto each their diet for the interval, day 3 to 6 months-of-age, where one received a conventional diet (CONV) and the other a high-carbohydrate, high-fat diet (HCHF). At 6 months blood was drawn for thyroid hormone evaluation. From 6 months- until 2½ years-of-age, all sheep were fed the same, low-fat diet. At 2½ years-of-age, feed intake was assessed and a thyroxine tolerance test was performed and heart rate, rectal temperature and energy expenditure was measured, before and during the test. **Results:** LOW, HIGH and HCHF treatments affected adult HPT axis responsiveness and suppressed T4→T3 conversion during the tolerance test. When challenged, the HIGH:HCHF sheep increased serum-T3 and -T4, as well as T3:T4 and T3:TSH ratios and decreased serum TSH, while NORM:HCHF sheep responded directly opposite. HCHF decreased basal adult EE in prenatal NORM and HIGH sheep and increased adult feed intake of NORM and LOW sheep. **Conclusions:** Fetal under- and overnutrition programmed HPT axis function differently, with long lasting effects on heart rate, body temperature, feed intake and energy expenditure (EE), but without effecting basal thyroid status. NORM were adversely affected by the postnatal obesogenic diet. In HIGH, the postnatal CONV diet seemed to be a "mismatch" diet throughout prepubescent development. Programming in LOW sheep was primarily prenatal and did not interact with postnatal nutrition in a long-term perspective.

Keywords: Fetal Development, Thyroid Function Tests, Thyroid Hormone Metabolism, Animal Physiology

Introduction

Prenatal under- and over-nutrition and early postnatal nutrition can be mediators of metabolic programming with life lasting effects and presents a risk factor for developing metabolic disease like obesity and type 2 diabetes (1, 2, 3). In light of the increasing global prevalence of metabolic disease (4) it is of great interest to learn if metabolic disease originating in fetal life should be divided into functional subcategories, which potentially call for different types of treatments or interventions when diagnosed later in life. Research has mainly focused on programming of the hypothalamic-pituitary-adrenal axis function (5), glucose-insulin axis function (6) and more recently programming of the hypothalamic-pituitary-adipose axis (7, 8, 9),

but the HPT axis is another endocrine axis subject to programming. The HPT axis regulate metabolism through stimulation of mitochondrial oxygen consumption, affect carbohydrate and lipid metabolism, are essential for normal growth and development, affect the nervous system and the cardiovascular system (10, 11). However, few publications have been concerned with fetal programming of the HPT axis and/or phenotypical metabolic traits and none have evaluated long-term programming effects comparing fetal under- and overnutrition.

The limited amount of reports on programming of thyroid state as well as overall metabolic state, reach adverse results, or do not include long term evaluation; Ayala-Moreno *et al.* (12) found that fetal undernutrition in rats resulted in lower resting EE and subclinical hypothyroidism in adulthood, possibly making these rats more susceptible to develop obesity. Oppositely, fetal and early overnutrition in rats, stimulated the HPT axis at weaning, but long-term effects were not assessed (13). Fetal undernutrition upregulated thyroid hormone (TH) axis function in adult female sheep (14) and Kiani *et al*. (15) reported that late gestation undernourished sheep had elevated EE compared to sheep that had been adequately nourished during fetal life. Jørgensen *et al.* (16) reported that small for gestational age adult men lowered their EE in response to fasting, suggesting increased adaptability to periods of sparse nutrition. In a study where effects of pre- and postnatal mismatching diets were assessed, in ovo programming induced by maternal protein restriction followed by posthatch catch up growth lead to elevated adult metabolic rates, in zebra finches (17). Sun *et al.* (18) found that large for gestational age children, had significantly higher EE than children born appropriate for gestational age. These observations that adverse nutritional programming alters EE support the possibility that fetal overnutrition can program the HPT axis to a degree resulting in phenotypical manifestations later in life. Thus, different adverse nutritional conditions, such as fetal under- and overnutrition, can apparently either upregulate or downregulate the HPT axis and/or EE, but these two phenotypic outcomes have only been evaluated jointly in the study by Ayala-Moreno *et al*. (12), in the rat model with fetal undernutrition. No studies have so far included evaluation of the long-term consequences. The aim of this study was to compare third trimester under- and overnutrition effects on HPT axis function in sheep, and assess if early postnatal obesity development impacts the phenotypic manifestation of fetal programing.

We hypothesized that: i) fetal under- and overnutrition programs the HPT axis differently, with respect to long lasting effects on energy metabolism phenotype and thyroid status, ii) late gestation under- and overnutrition may alter postnatal sensitivity to early postnatal life obesity development and alter the phenotypical manifestation of the fetal programming of HPT axis function and energy metabolism. The *Copenhagen sheep model* (3) was used here, since sheep display fetal growth trajectories and offspring maturity at birth, 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 a high fat, high carbohydrate diet for the first six months (around puberty) of life. In adulthood, EE, heart rate and body temperature were tested in these offspring with different pre- and early postnatal nutrition histories, before- and during-, a thyroxine (T4) tolerance test.

Materials and methods

Experimental animals and experimental design

A total of 38 sheep were included in this study. An extensive description of the Copenhagen sheep model and the experimental design was published by Khanal *et al*. (9). All experimental animal procedures were approved by The Danish National Committee on Animal Experimentation. Overall, the experimental

sheep had been exposed to different levels of nutrition during the last 6 weeks prior to parturition by feeding their twin pregnant mothers 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 NRC (2007) for sheep in the last trimester of gestation. The twin lambs were assigned to each their postnatal diet from 3 days till 6 months (after puberty) of age: a conventional moderate hay based diet (CONV) or a high carbohydrate, high fat diet (HCHF). Blood was drawn for later analysis and thereafter they were raised as gender divided flocks and all fed the same low-fat diet consisting of artificially dried green hay fed ad libitum supplemented with rolled barley (see ref. 19) until 2½ years-of-age (adulthood). The experimental groups were: 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). A measure of voluntary feed intake capacity was assessed in the adult sheep by determining weight of ingested hay offered ad libitum over a 4 hour period following a 72 hour period of fasting.

Thyroxine tolerance test and metabolic measurements

The tolerance test lasted two consecutive days where collection of baseline values for EE, heart rate and body temperature in un-manipulated sheep was carried out the first day (Day 1) and the T4 tolerance test on the following day (Day 2). Adult sheep were placed in individual pens that allowed for limited physical contact with the neighboring sheep through the bars of the pen and they had a day to adjust to the pens before testing. They were fasted overnight and during the experiment. On Day 1, each sheep received a single bolus injection into the jugular vein of a 4 mg/kg MBW dose of a 50 mg/ml ^{13}C-bicarbonate (Sigma-Aldrich, product no. 372382) solution in sterile 0.9% saline. Exhaled breath was collected in 1L TECOBAGs (Tesseraux, Burstad, Germany) using an anaesthetic mask (large 271435 or X-large 271436 from KRUUSE A/S, Demark) with a mounted two-way non-rebreathing valve system (Hans Rudolph Inc., Kansas City, MO, USA).

This was done to determine whole body EE by the ^{13}C-bicarbonate tracer technique, which has been validated in several species and can be used for free ranging animals (20, 21, 22, 23). The breath samples were analyzed using IRIS (infrared isotope analyzer, ^{13}C Wagner Analysen Technik). Breath samples were collected immediately before and 5, 10, 20, 30, 60, 120, 190, 240, 300, 360 minutes after the ^{13}C-bicarbonate injection. Heart rate and rectal temperature was measured before injection of ^{13}C-bicarbonate, 8.00AM and again at 12.00PM and 3.00PM. At approximately 8.00PM, Day 1, a basal blood sample was collected into a serum tube from the jugular vein by venipuncture and they received an intravenous bolus injection of 0.1 mg T4/kg BW. The solution was prepared by dissolving L-Thyroxine sodium pentahydrate (T2501, Sigma-Aldrich) in methanol (4mg/ml) under sterile conditions and filtering it through a 0.22 µm polyethersulfone membrane filter, immediately prior to use. Blood samples were collected 12, 16 and 19 hours post T4 injection, i.e. at 8.00AM, 12.00PM and 3.00PM on Day 2. A new round of measurements of EE, heart rate and rectal temperature was carried out at these same time points in exactly the same way as Day 1.

The serum tubes samples coagulated at room temperature for ~20 minutes before the tubes were centrifuged (1800×G, 15 minutes at 4°C) and serum separated and stored frozen in cryotubes at -20°C until analysis. Concentrations of T3, T4 and TSH in serum were measured using a double-antibody radioimmunoassay (24, 25). The sensitivity was 0.12 nM for T4 and 0.02 ng/ml for T3 and TSH. The intra-assay variation for T4, T3 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 ^{13}C-bicarbonate tracer technique data handling has been thoroughly presented in (21). In short, the infrared ^{13}C isotope analyzer, IRIS (Wagner Analysentechnik, Bremen, Germany), was used to measure the ^{13}C:^{12}C ratio. This delta value $\delta^{13}C, ‰,$ was used to compute the atom percentage of ^{13}C, which was then used to calculate the atom percentage excess in expired air at basal condition and the given time points, following the isotope injection. The atom percentage excess forms an exponential degrading curve over time. The area under the curve was then used to calculate the CO_2 production, RCO_2 (mol/min) as in (eq. 1).

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

D is the dose of bicarbonate and RF is the fractional ^{13}C recovery in expired air. Under basal conditions it varies between approximately 0.6–0.8 across species and a value of 0.7 was assumed for the first day (23, 26, 27). For experimental day 2, RF was set to be 1, based on the assumption that ^{13}C recovery increases to almost 100% after the increase in metabolism post T4 injection, as it has been seen during exercise (28). Using RCO_2, EE was found by a modified Brouwer equation (29) (eq. 2).

$$EE\left(\frac{kJ}{day}\right) = 5{,}16 * RCO_2\left(\frac{L}{day}\right) + 16{,}18 * \frac{RCO_2}{RQ} - 5{,}9 * N_u \quad (2)$$

A RQ of 0.91 was assumed, as found for equal size sheep fed a similar diet of high quality dried green hay (30). N_u is the nitrogen excreted with the urine and methane CH_4 was excluded, since it was not possible to assess and have been found insignificant in previous studies (21, 31).

Statistics

All statistical models were derived from the same multi-factorial model:

$$Y_{ijlo} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \gamma_k + \alpha\beta\gamma_{ijk} + \alpha\beta\gamma\delta_{ijk} + \kappa_l + \varepsilon ijkl$$

Where Yijlo is the specific factor, described by all the qualitative explanatory variables, μ is the overall mean, αi is the effect of the prenatal NORM, LOW or HIGH nutrition treatments, βj is the effect of the postnatal CONV or HCHF dietary treatments, αβij is the interaction between pre- and postnatal nutrition treatments, γk is the effect co-variates, i.e. age, time, day, sex, birthweight, metabolic bodyweight and/or basal blood concentration, αβγijk is the three-way interaction between pre- and postnatal treatments and age, day or time, αβγδijk is the four-way interaction between pre- and postnatal treatments, day and time and κl is the random effect of twins and εijkl is the residual variation ~ N(0,σ2). The universal sample space of the qualitative explanatory variables are: i = {1,..,3}, j = {1,2}, k = {1,2} and l = {1,2}. 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 LS-means and presented as Least Square Means ± SEM and considered significant when $P<0.05$ and a tendency was declared when $P<0.10$.

Results

Overall, there were no effects of pre- or postnatal nutrition treatments or their interactions or of gender unless specifically stated in the following. When interpreting the gender results or lack of same, it must be borne in mind that there was an uneven number of males and females across treatment groups and few of either of them in certain groups.

Serum T4, T3, 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 T3 (<8%). Males had lower concentrations of T4 and T3 than females (P=0.01 and P=0.009, respectively) at both 6 months and 2½ years of age, but equal TSH concentrations (Table 1). The T3:T4 concentration ratios were significantly lower than in LOW:HCHF, NORM:HCHF and HIGH:CONV sheep (P=0.02, 0.03 and 0.08, respectively and P=0.05 for the pre- and postnatal interaction) at both 6 months and 2½ years of age, which could be ascribed to the numerically lowest levels of T3 in LOW:CONV sheep at both ages. The T3:TSH ratios were generally increased in HCHF sheep at 6 months of age (P=0.001 for the postnatal diet and age interaction), where NORM:CONV had lower ratios than NORM:HCHF, HIGH:HCHF, and LOW:HCHF (P=0.0009, 0.003 and 0.01, respectively), HIGH:CONV had significantly lower ratios than HIGH:HCHF (P=0.0009) and LOW:CONV had lower ratios than HIGH:HCHF and LOW:HCHF (P=0.006 and P=0.0009), these difference did not pertain into adulthood. The ratio between serum T4:TSH was also increased by the postnatal HCHF diet at 6 months of age (P=0.01 for the postnatal diet and age interaction). NORM:CONV, HIGH:CONV and LOW:CONV had significantly lower T4:TSH concentration ratios than their respective paired prenatal groups, NORM:HCHF, HIGH:HCHF and LOW:HCHF (P=0.03 for all). Males had lower ratios than females (P=0.03), reflecting the naturally higher T4 concentrations observed in females.

Hormonal responses during the thyroxine tolerance test

The mean serum concentrations determined 12–19 hours after intravenous L-thyroxine injections of T4, T3, TSH and the ratios between them, are shown in Figure 1, Figure 2, Figure 3 and Figure 4, respectively. The L-thyroxine injection induced an approximately tenfold increase in serum T4 in all treatment groups, but lower levels were reached in females compared to males (~3800 and ~4200 ng/ml, respectively, P=0.001) although the L-thyroxine dose was given relative to metabolic body weight. The dramatic increase in T4 resulted in a doubling of T3 (P<0.001) 16 hours post-injection and a decrease in TSH from a basal level of 0.16 ng/ml to 0.13 ng/ml (P<0.001).

Through the sampling period from 12 to 19 hours post-injection, serum T4 concentration decreased slowly (P<0.001), but HIGH sheep (with a fetal overnutrition history) had longer serum thyroxine half-life (70.23 hours) compared to NORM (57.75 hours; P=0.06) and LOW (51.0 hours; P=0.02) sheep. Increased T3 and decreased TSH levels remained stable over the sampling period from 12-19 hours post-injection in all groups. Although T4 levels decreased over the sampling period, the serum levels must have remained sufficiently high to elicit a constant (maximal) stimulation of T3 formation and suppression of TSH secretion.

The serum concentrations of T4 (Figure 1; P=0.009), T3 (Figure 2; P<0.0001), TSH (Figure 3; P=0.003) and the concentration ratios between them (Figure 4; P<0.04) after L-thyroxine injection all depended on the specific combination of nutrition in pre- and early postnatal life.

<u>NORM sheep:</u> NORM:CONV sheep reached the highest serum levels of T4 and T4:TSH (together with NORM:HCHF and LOW:CONV sheep), T3 and T3:T4 (with HIGH:HCHF sheep), and T3:TSH (with LOW:CONV sheep), and consistent with the high T3 levels, TSH levels were reduced to the lowest levels in this group (with LOW:CONV and LOW:HCHF sheep) after L-thyroxine administration. NORM sheep exposed to the obesogenic HCHF diet in early life, had significantly lower levels of T3 (P=0.0002), T3:T4 (P=0.001) and T3:TSH (P<0.0001) ratios, and hence higher levels of TSH

(*P*=0.02) after L-thyroxine administration compared to NORM:CONV sheep.

HIGH sheep: in general, maintained the highest TSH levels compared to NORM:CONV and both groups of LOW sheep after L-thyroxine administration (Figure 3). HIGH:CONV sheep had lower levels of T3 post-injection (*P*=0.001) and lower ratios of T3:T4 (*P*=0.001), T3:TSH (*P*=0.0003) as well as T4:TSH (*P*=0.03) compared to NORM:CONV sheep, resembling the levels observed in NORM:HCHF sheep. In HIGH sheep, the HCHF diet in early postnatal life had the opposite effect compared to what was observed in NORM sheep. Thus, responses after L-thyroxine administration in T3 serum levels and ratios of T3:T4 and T3:TSH were increased in HIGH that had been fed the HCHF compared to CONV diet in early postnatal life, and they reached the same high levels as observed in NORM:CONV sheep. Opposite responses were also observed for TSH, where levels were reduced in HIGH sheep previously fed the HCHF compared to CONV diet, whereas higher TSH levels were observed in NORM sheep fed the HCHF diet compared to CONV diet. The basal serum hormone levels did not reveal these pre- and postnatal dietary interactions.

LOW sheep: were among the groups with the lowest levels of TSH but this was also associated with low levels of T3 compared to the other groups after L-thyroxine administration, and the HCHF diet had no effect on T3 and only a quantitatively small positive long-term effect on TSH. This picture deviated from what was observed in NORM and HIGH sheep, where the postnatal diet induced more pronounced changes and in opposite directions of T3 and TSH. LOW:CONV sheep had lower levels after L-thyroxine administration of T3 and T3:T4 (*P*=0.004 and 0.001 respectively) compared to NORM:CONV sheep, which was also evident from the basal hormone concentrations. The major differences relating to HIGH:CONV sheep were lower levels of TSH (P=0.0002), resulting also in increased T4:TSH and T3:TSH ratios (*P*=0.0003 and 0.0006, respectively).

Birth weight correlated with the response of T4, T3 and TSH (*P*=0.0002, *P*<0.0001, *P*=0.004, respectively) and basal hormonal level correlated with T3 and TSH response (*P*<0.0001 and *P*=0.01, respectively).

Rectal temperature

Rectal body temperatures ranged from 38.3-39.5 before the L-thyroxine tolerance test (Day 1). After L-thyroxine administration body temperature was raised (Day 2) on average by 0.2°C in all treatment groups (*P*=0.001) (Figure 6). Temperatures dropped over the day on Day 1 and increased on Day 2 (*P*<0.001 for the Time*Day interaction), where serum T4 and T3 levels had increased in response to the L-thyroxine tolerance test. The response in rectal temperatures Day 2, after L-thyroxine administration in the different treatment groups, as well as rectal temperatures prior to this administration, resembled the response pattern after L-thyroxine treatment for T3 (Figure 2) and T3:T4 concentration ratios (Figure 4), with the highest temperatures and increments in temperature (relative to Day 1) observed in the NORM:CONV and HIGH:HCHF groups. Thus, rectal temperatures prior to L-thyroxine administration as well as temperature increments after administration were reduced in NORM sheep exposed to the HCHF diet in early postnatal life (*P*=0.02), in HIGH sheep that had been exposed to the CONV diet (*P*=0.03) and numerically in all LOW sheep irrespective of the diet received in early postnatal life (*P*=0.001 for the pre- and postnatal nutrition interaction).

Heart rate

Heart rates were within the range of 60-140 beats per minute (BPM), higher in the morning than in the afternoon (*P*<0.0001), and males had lower heart rates, around 85.0 BPMs, compared to a mean of 93.9 BPM for females (*P*=0.005). On Day 1, there were no major group differences in basal heart rates except for faster heart rates in NORM:CONV and HIGH:HCHF as compared to NORM:HCHF

(P=0.04). The L-thyroxine treatment increased heart rates overall from a mean of 90 BPMs before administration to 95 BPMs after administration (P<0.0001) (Figure 7). After L-thyroxine administration, the same response pattern was observed on Day 2 for increments in heart rates as for T3, T3:T4 and rectal temperature, where the highest heart rates were observed in the NORM:CONV and HIGH:HCHF sheep (P<0.0001 for the pre- and postnatal treatment interaction). Only HIGH:CONV sheep did not have a numerical increase in their heart rate after L-thyroxine administration.

Energy expenditure

EE results are shown in Figure 8. The daily EE was on average 496.5 kJ/MBW/d prior to L-thyroxine administration (Day 1), and affected by the early postnatal but not prenatal diet, reflecting lower EE in NORM:HCHF and HIGH:HCHF compared to their paired prenatal groups, NORM:CONV and HIGH:CONV, respectively (P<0.05). L-thyroxine administration increased EE by 23.8% to 652.1 kJ/MBW/d (Day 2) (P≤0.003), and this obliterated any group differences except for a more pronounced increment in EE in LOW:HCHF compared to all other groups (although only significantly compared to LOW:CONV (P<0.05) and close to significant compared to NORM:HCHF (P=0.06)). Such a response pattern to L-thyroxine administration across the treatment groups was not observed for any of the other measured parameters.

Voluntary feed intake

As a measure of feed intake capacity, voluntary feed intake was determined over a 4-hour period immediately after a 72 hour period of fasting. This feed intake was increased in NORM and LOW (but not HIGH) sheep that had been fed the HCHF diet in early postnatal life (P=0.02 and 0.03, respectively). Males tended to have a higher feed intake than females (~200g average, P=0.08) and MBW and birthweight were significant co-variates for adult feed intake (Figure 5).

Discussion

The aim of this experiment was to examine long-term consequences of different prenatal and early-postnatal nutrition exposure combinations on HPT axis function and overall metabolic traits in sheep. In general, basal levels of TH and TSH did not reveal functional changes to the HPT axis but an L-Thyroxine tolerance test revealed lasting functional changes to HPT axis function. 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) (32). Long term effects of prenatal and postnatal nutrition on HPT axis function and overall metabolism relied specifically on type of postnatal nutrition in both HIGH and CONV sheep, but not in LOW sheep, as will be discussed in the following.

The effect of an early postnatal obesogenic diet in NORM sheep

Although, there was no significant difference in basal serum concentrations between NORM:CONV and NORM:HCHF, NORM:HCHF sheep had lower basal metabolism, expressed in EE, heart rate and temperature, and less increment of these parameters in response to L-Thyroxine injection. Only a minor part of circulating T3 stems directly from thyroidal production, most is produced peripherally by deiodination of T4 (39). NORM:HCHF sheep had significantly lower T3:T4 ratios during the challenge, which may reflect a reduced proficiency for T4→T3 conversion, central to the thyroid or peripheral or both. A reduced ability to convert T4 into its active form T3, could explain the observed lower basal metabolism of NORM:HCHF sheep. It is a well-known phenomenon in humans that weight loss following obesity decrease EE (40), which is instinctively counter-productive to maintaining lipobody-homeostasis. Even though NORM:HCHF sheep spent 2 years on the same moderate diet as NORM:CONV sheep, they had a reduced adult EE. It is not completely understood what triggers this lasting decrease in EE, probably

because of the many diverse complex inputs to the hypothalamic-systemic energy balance circuit, but remodeling (gliosis) of the hypothalamus has been identified in obese mice and humans (41, 42). Hypothalamic remodeling has been reported as reversible in obese mice (43), but it seems that overnutrition in the growing lamb may remodel hypothalamic function irreversibly. Together with a significantly higher spontaneous post-fasting feed intake capacity in NORM:HCHF as compared to NORM:CONV this may result in a phenotype predisposing for obesity. Under normal conditions, leptin maintains a satiety response in the hypothalamus and decrease food intake as well as it acts to increase EE in animals and humans (44). However, overnutrition has the potential to induce lasting leptin resistance (7, 42) and this could further explain the increased feed intake capacity in of NORM:HCHF sheep as well as their decreased EE, compared to NORM:CONV sheep.

Responses to postnatal nutrition in sheep exposed to prenatal overnutrition (HIGH)

In general, long-term consequences of exposure to the early postnatal HCHF as compared to CONV diets in HIGH sheep were opposite to those observed in NORM sheep. HIGH:CONV sheep had the most increased TSH levels and lowest T3:T4 ratios during the tolerance test compared to both HIGH:HCHF and NORM:CONV sheep. HIGH-CONV just like NORM:HCHF, thus failed to suppress TSH to the extent of other groups during the challenge, which as mentioned above may reflect less T4->T3 activation (peripheral, thyroidal or both) and thus less negative feedback on TSH by the active T3. In humans it is a common trait of obesity, to present with isolated elevated TSH. The mechanism behind this hyperthyrotropinemia is not quite known, but in humans it is apparently not explained by changes in peripheral activity of TH (45). Instead it is thought that isolated elevated TSH may represent a possible hypothalamic-pituitary hormone resistance and disturbed negative feedback signaling (45, 46). Maternal overnutrition cause leptin resistance in rats through decreased STAT3 and SOC3 gene signaling in the arcuate nuclueus (13) and increased leptin concentrations have the capability to stimulate pro-TRH formation and thus trigger pituitary TSH stimulation (47). The same mechanisms may be at play in both NORM:HCHF sheep and HIGH:CONV sheep, although only revealed during the T4 challenge, as these sheep at time of challenge were not obese. It was surprising that HIGH:HCHF responded very alike in the examined parameters to NORM:CONV sheep, and likewise NORM:HCHF and HIGH:CONV responded alike in spite of contrasting postnatal dietary exposures. This lead us to speculate whether the CONV diet was a "mismatch" diet throughout early postnatal development for the HIGH sheep, leading to a permanent downregulation of T4->T3 activation mechanisms as an adaptive response? Moreover, there were also indications from this study that HIGH sheep had a superior ability compared to others to recover normo-adipocity and normal metabolic function after development of obesity in early postnatal life by correcting their diet later in life (9). In support of this, findings in mice have shown 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 changed to a normal chow diet during lactation (48). The fetal high fat/high sucrose treatment also offered better protection against hyperleptinemia in adult offspring upon exposure to a high fat diet.

In addition to a possible primary or secondary central hypothalamic-pituitary programming of the TH axis, the decreased T3:T4 ratios in HIGH:CONV sheep also point to a decreased T4->T3 conversion compared to HIGH:HCHF and NORM:CONV sheep. This was actually reflected even in the basal hormone concentrations, is evidence to a programming down-stream of the HPT-axis. This sort of peripheral programming has also been seen in Japanese macaques and female sheep, where

maternal high fat diets disrupted fetal TH receptors and their downstream regulators (49, 14). This interference with peripheral TH function in tissues quantitatively important in energy metabolism is in line with the observation that HIGH:CONV sheep were incapable of increasing their heart rate in response to a L-thyroxine surge.

Although the postnatal HCHF diet was accompanied by opposite alterations in most traits studied in the HPT axis in HIGH and NORM sheep, they did respond similarly to the HCHF diet with reduction in EE. Early postnatal obesity development, although transient, thus appears to be a particular a risk factor for obesity development later in life, which does not appear to be directly linked to alterations in the HPT axis.

Late gestation undernutrition in relation to postnatal normal and overnutrition

In general, the two LOW groups has similar responses in the tested parameters, which was unexpected since fasting tolerance tests in these sheep revealed pronounced interactions between pre- and postnatal treatments, where LOW:HCHF sheep were predisposed to adult hypercholesterolaemia, hypercreatinaemia and fasting-induced hyperureamia (9). LOW sheep who received a CONV diet after birth had an equal reduced T4->T3 activation to NORM:CONV and HIGH:CONV sheep, but contrary to NORM and HIGH, T3 levels of LOW sheep were constantly decreased, no matter the type of postnatal nutrition and these presented alongside the numerically lowest TSH concentrations. This picture was different from that observed in NORM and HIGH sheep, where the impact of postnatal nutrition during the T4 tolerance test exposed opposite responses in T3 and TSH. This may confirm what was indicated by the basal serum levels; that activation of T4->T3 may not be as effective in LOW:CONV as NORM:CONV, but the low T3 concentrations do not result in increased serum TSH, as in both NORM and HIGH. This could point to a reduced sensitivity and/or transcriptional failure of the hypothalamus/pituitary to increase TSH production. It was solely in the LOW:CONV sheep that the functional changes to the HPT axis was indicated by the basal serum values alone. During the T4 tolerance test, LOW:HCHF sheep increased EE more than LOW:CONV sheep, possibly relating to the greater capacity for T4→T3 conversion and lesser TSH depression during the tolerance test and the HCHF diets overall tendency to permanently down-regulate EE was not evident in LOW sheep. Although prenatal undernutrition did not alter basal metabolic phenotype, early postnatal exposure to the HCHF diet resulted in an increased voluntary feed intake of LOW sheep after fasting which in LOW sheep may be an additive risk factor for development of adult obesity. Noticeably, during fasting-, glucose- and insulin- tolerance tests LOW-HCHF sheep also had increased plasma levels of BUN, cholesterol, creatinine, lactate and the lipid parameters TG and NEFA, in one or more of the tolerance tests (19), so although functional programming of the HPT-axis seem to be primarily prenatal, other axis' were indeed affected by early postnatal overnutrition. In LOW sheep there seems to be less evidence of long-term consequences of the early postnatal overnutrition, after switching to a conventional diet. Overall, hormonal data suggest a down-regulated adult HPT axis in late-gestation undernourished sheep, but this did not translate into metabolic phenotype. From a different study, we have previously reported increased basal TH levels of late gestation undernourished adult female sheep (14), but this was not confirmed in the present study. Albeit differentially directed consequences, one similarity was that the long-lasting programming in the axis was of prenatal origin and there were very few effects of postnatal nutrition in the adult females of the previous study. The two studies were different in numerous aspects; the sheep in the present study had a different HCHF diet and were ad libitum fed after 6 months of age and reached higher body condition scores (9) at the time of slaughter, instead of more moderate body

conditions reached in the previous study (3), furthermore they were different breeds and included both females and males. We cannot rule out that the end-point difference in body condition scores and race differences impacted on the differential TH levels found in the two studies.

Our study had limitations in small group sizes and the earlier mentioned gender confounding, which could explain that patterns observed in basal THs, were mostly insignificant (Table 1). Gender and age differences in TH and TSH levels, as well as HPT axis response in functional tests, have been reported in studies of different species including humans. These data are not univocal for humans or animals and there have been reports of unaltered, increased or decreased TH or TSH-levels or -sensitivity of one gender compared to the other (33, 34, 35, 36, 37, 38). The reasons behind the different outcomes of the diverse studies are not readily identifiable, but in present study females had higher basal T4 and T3 both at 6 months- and 2½ years-of-age and concentrations increased with age and we did not, in spite of gender confounding, see indications of differentiated effects in males compared to females. In general, group differences and tolerance test induced changes in temperature and heart rate follow patterns of TH serum concentrations well, but EE response during the tolerance test did not clearly comply with the other measurements, which could be because we measured EE prematurely, before we had reached a new steady state, as implied by the constant rise in temperatures during the tolerance test.

In conclusion, late-gestation nutrition as well as prepubescent postnatal nutrition, can program HPT axis function permanently, with lasting effects on energy metabolism phenotype but not basal thyroid status. Early-life nutrition can be an additive risk factor for adverse effects of fetal programming but long-term consequences was rooted in prenatal nutrition and relied on type of postnatal nutrition and vice versa. In this way NORM sheep were adversely affected by a postnatal HCHF diet, whereas the postnatal CONV diet actually seemed to be a "mismatch" in HIGH sheep and HIGH sheep who received a "matching" postnatal high energy diet throughout prepubescent development, resembled NORM:CONV sheep in adulthood. Early life overnutrition permanently decreased basal adult EE in prenatal NORM and HIGH sheep and increased the voluntary feed intake capacity in NORM and LOW sheep, both possible risk factors for developing obesity. LOW sheep who received either postnatal treatment were mostly identical as adults and programming in these sheep were therefore primarily prenatal and did not interact with postnatal nutrition in a long-term perspective. Overall, distinct interactions between prenatal and postnatal treatments, do not allow us to generalize between NORM, HIGH and LOW sheep, rather we must relate each prenatal treatment to type of postnatal nutrition.

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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																	
T4	276.4±34.4	249.6±36.9	266.3±30.0	306.2±31.5	263.7±30.9	266.1±26.4	233.77±22.08^{a}	308.98±28.34^{b}	0.44	0.76	0.39	0.30	0.54	0.005	-	0.10	0.93
T3	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^{a}	1.69±0.19^{b}	0.85	0.23	0.41	0.14	0.14	0.01	-	0.40	0.03
TSH	0.110±0.019	0.124±0.021	0.127±0.017	0.123±0.018	0.123±0.017	0.111±0.015	0.120±0.012	0.119±0.016	0.79	0.74	0.65	0.17	0.42	0.98	-	0.68	0.63
T3:T4	0.0051±0.0007	0.0062±0.0008^{b}	0.0057±0.0006^{b}	0.0050±0.0006	0.0045±0.0006^{a}	0.0059±0.0005^{b}	0.0055±0.0005	0.0053±0.0006	0.86	0.14	0.05	0.36	0.55	0.60	-	0.46	0.02
T3:TSH	7.64±2.58^{a}	13.84±2.58^{b,d}	11.16±2.42^{a,d,e}	17.36±2.30^{b}	9.13±2.13^{d,a,c}	15.33±2.04^{b,e}	10.96±1.38	13.52±1.21	0.89	0.10	0.69	0.001	0.07	0.07	-	0.65	0.29
T4:TSH	1686.4±461.4^{a,c}	2460.3±457.7^{b,d}	1825.4±424.8^{a,d,e}	2599.3±405.9^{b,c,f}	1869.7±399.9^{a,d,f}	2643.7±387.7^{b,c,e}	1868.1±315.2^{a}	2493.5±393.6^{b}	0.96	0.82	0.32	0.01	0.17	0.03	-	0.79	0.97
2½ years-of-age																	
T4	365.4±33.9	338.5±35.5	355.3±34.2	395.2±29.7	352.6±28.4	355.1±33.1	322.74±30.66^{a}	397.96±21.12^{b}	0.44	0.76	0.39	0.30	0.54	0.005	<0.0001	0.10	0.93
T3	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^{a}	1.78±0.14^{b}	0.85	0.23	0.41	0.14	0.14	0.01	0.01	0.40	0.03
TSH	0.147±0.019	0.160±0.020	0.164±0.019	0.160±0.017	0.160±0.016	0.148±0.019	0.157±0.017	0.156±0.012	0.79	0.74	0.65	0.17	0.42	0.98	0.002	0.68	0.63
T3:T4	0.0046±0.0007	0.0057±0.0007^{b}	0.0052±0.0007^{b}	0.0045±0.0006	0.0040±0.0006^{a}	0.0054±0.0007^{b}	0.0050±0.0006	0.0049±0.0004	0.86	0.14	0.05	0.36	0.55	0.60	0.01	0.46	0.02
T3:TSH	14.13±2.47	12.69±2.56	11.43±2.40	9.99±2.41	13.47±2.22	12.03±2.25	11.03±1.37	13.59±1.22	0.89	0.10	0.69	0.001	0.07	0.07	0.87	0.65	0.29
T4:TSH	2942.2±439.3	2400.9±453.3	3081.2±427.7	2539.9±428.8	3125.6±420.2	2584.2±426.8	2466.3±424.8^{a}	3091.7±298.8^{b}	0.96	0.82	0.32	0.01	0.17	0.03	0.01	0.79	0.97

Table 1 Mean basal serum levels of T4 (nM), T3 (ng/ml) and TSH (ng/ml) and T3:T4, T3:TSH and T4:TSH ratios, for female (n=22) and male (n=16) sheep at 6 months and 2½ years of age. Data are presented as least square means±SEM. 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 6 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).

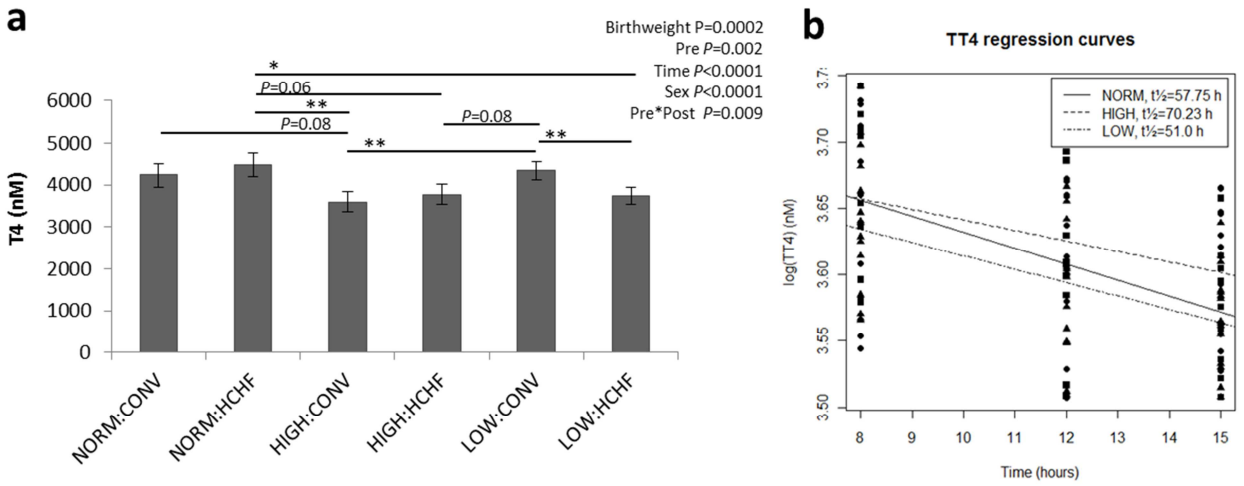

Figure 1 A) Group LSmeans of serum total T4 (nM) measured 12, 16 and 19 hours after injection of 0.1 mg thyroxine/kg LW, in 2.5 year old sheep, male and female (m: male, f: female), with a history of adequate nutrition (NORM), overnutrition (HIGH) or undernutrition (LOW) in late gestation, combined with a conventional (CONV) or a High-Carbohydrate-High-Fat (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 T4 (log(conc)) (not presented as LSmeans), in 2.5 year old sheep, male and female, where prenatal treatments NORM (■), HIGH (●) and LOW (▲) significantly impacted T4 half-life upon injection (P=0.01). Experimental design and dietary treatments have been fully described in the legend to Table 1. Significant differences are *P≤0.05, **P≤0.01, or P≤0.001.

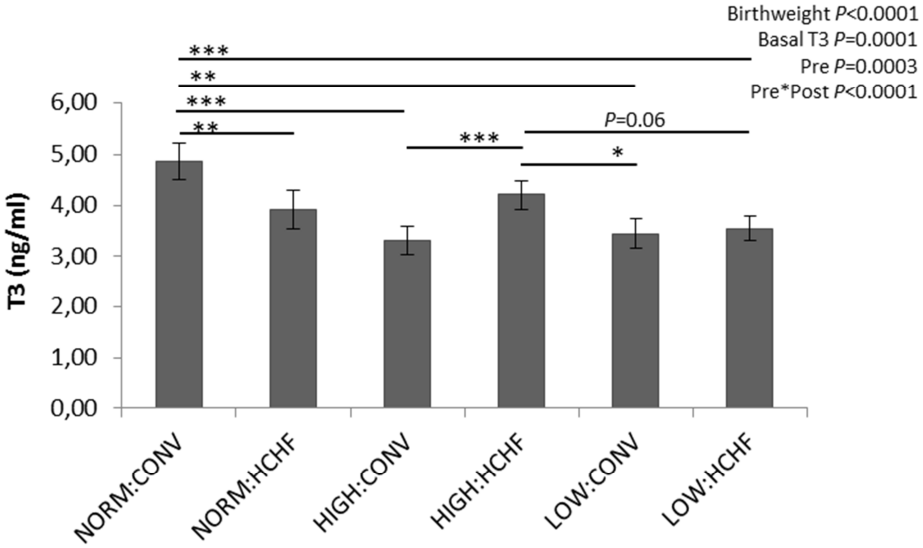

Figure 2 Group LSmeans of serum total T3 (ng/ml), measured 12, 16 and 19 hours post injection of 0.1 mg thyroxine/kg LW, in 2.5 year old sheep, male and female, with a history of adequate nutrition (NORM), overnutrition (HIGH) or undernutrition (LOW) in late gestation, combined with a conventional (CONV) or a High-Carbohydrate-High-Fat (HCHF) diet for the first 6 months postnatal months, yielding 6 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). Experimental design and dietary treatments have been fully described in the legend to Table 1. Significant differences are *P≤0.05, **P≤0.01, or P≤0.001.

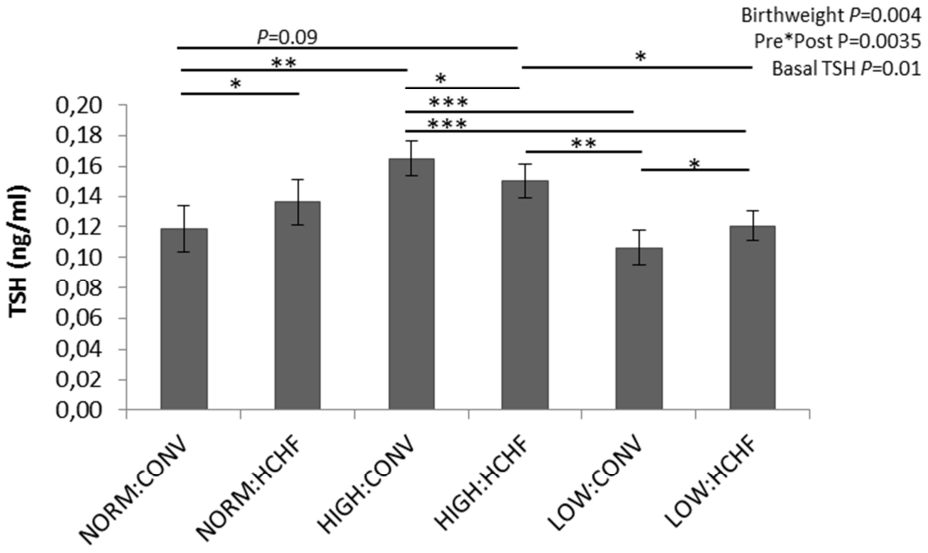

Figure 3 Group LSmeans of serum TSH (ng/ml), measured 12, 16 and 19 hours post injection of 0.1 mg thyroxine/kg LW, in 2.5 year old sheep, male and female, with a history of adequate nutrition (NORM), overnutrition (HIGH) or undernutrition (LOW) in late gestation, combined with a conventional (CONV) or a High-Carbohydrate-High-Fat (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 1. Significant differences are *$P \leq 0.05$, **$P \leq 0.01$, or $P \leq 0.001$.

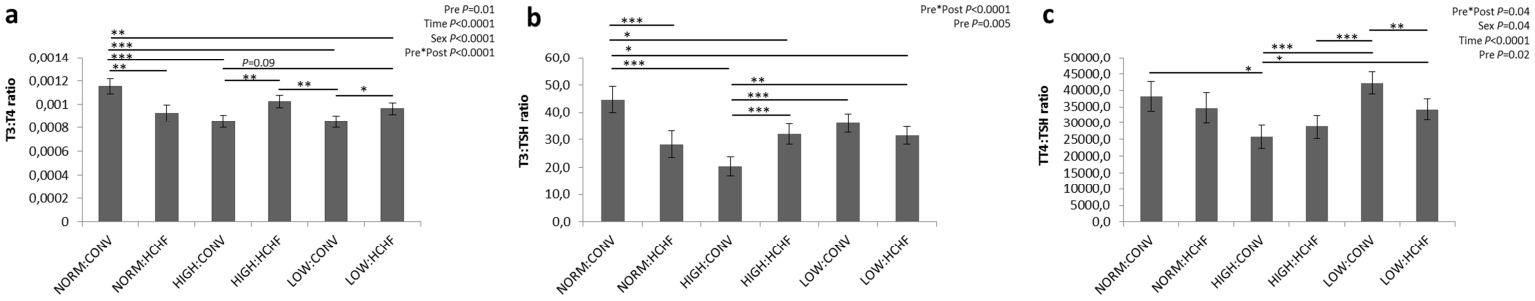

Figure 4 LSmeans of serum total T3:T4 (a), T3:TSH (b) and T4:TSH (c) ratios, 12, 16 and 19 hours post injection of 0.1 mg thyroxine/kg LW, in 2.5 year old sheep, male and female, with a history of adequate nutrition (NORM), overnutrition (HIGH) or undernutrition (LOW) in late gestation, combined with a conventional (CONV) or a High-Carbohydrate-High-Fat (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 1. Significant differences are *$P \leq 0.05$, **$P \leq 0.01$, or $P \leq 0.001$.

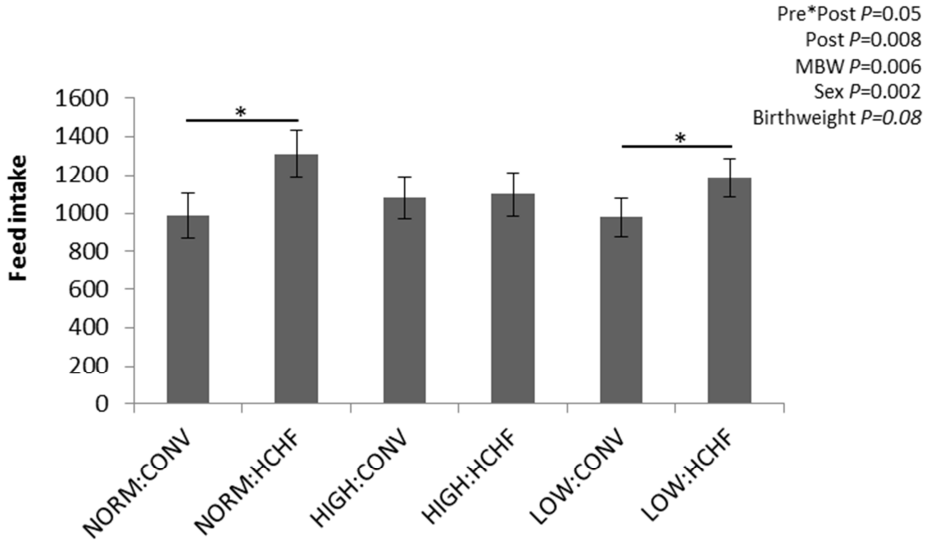

Figure 5 Voluntary feed intake based on residues of 2 kg green hay weighed 4 hours after feeding, following a 72 hour fasting challenge in 2.5 year old sheep, male and female, with a history of adequate nutrition (NORM), overnutrition (HIGH) or undernutrition (LOW) in late gestation, combined with a conventional (CONV) or a High-Carbohydrate-High-Fat (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 1. Significant differences are *$P \leq 0.05$, **$P \leq 0.01$, or $P \leq 0.001$.

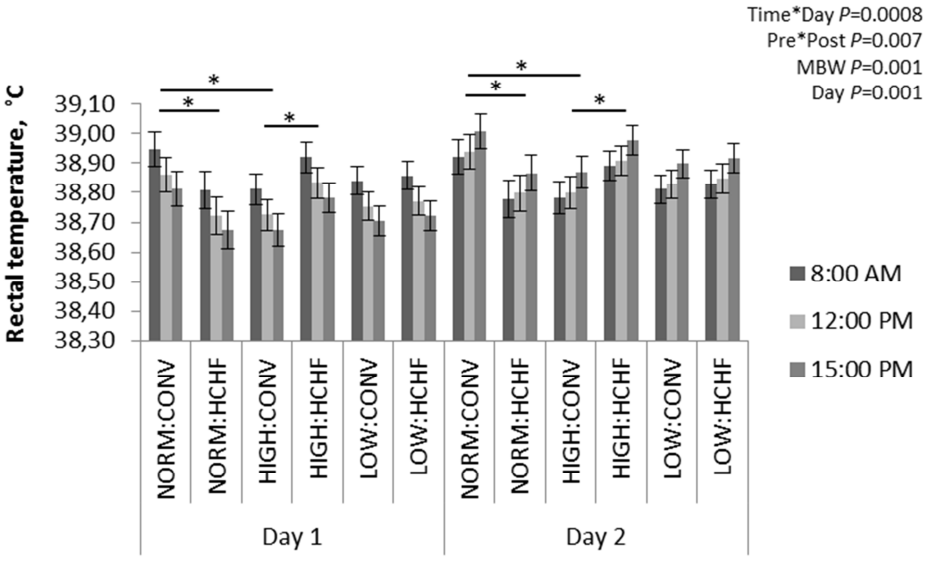

Figure 6 LSmeans of rectal temperatures measured at basal state (day 1) at 8.00 AM, 12.00 PM and 15.00 PM and after injection of 0.1 mg thyroxine/kg LW, at 12 (8.00 AM), 16 (12.00 PM) and 19 (15.00 PM) hours post injection, in 2.5 year old sheep, male and female, with a history of adequate nutrition (NORM), overnutrition (HIGH) or undernutrition (LOW) in late gestation, combined with a conventional (CONV) or a High-Carbohydrate-High-Fat (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 1. Significant differences are *$P \leq 0.05$, **$P \leq 0.01$, or $P \leq 0.001$.

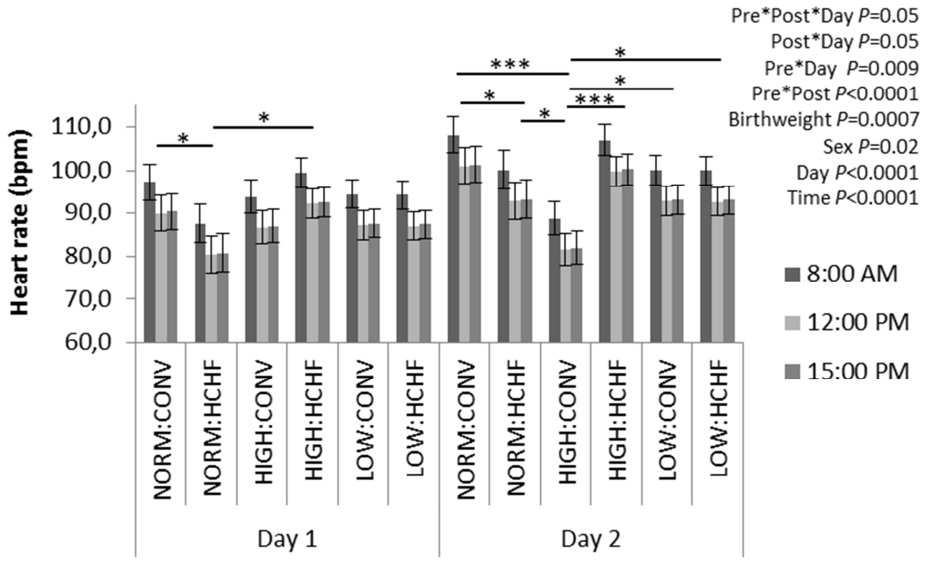

Figure 7 LSmeans of heart rates measured at basal state (day 1) at 8.00 AM, 12.00 PM and 15.00 PM and after injection of 0.1 mg thyroxine/kg LW (day 2), at 12 (8.00 AM), 16 (12.00 PM) and 19 (15.00 PM) hours post injection, in 2.5 year old sheep, male and female, with a history of adequate nutrition (NORM), overnutrition (HIGH) or undernutrition (LOW) in late gestation, combined with a conventional (CONV) or a High-Carbohydrate-High-Fat (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 1. Significant differences are **P*≤0.05, ***P*≤0.01, or *P*≤0.001.

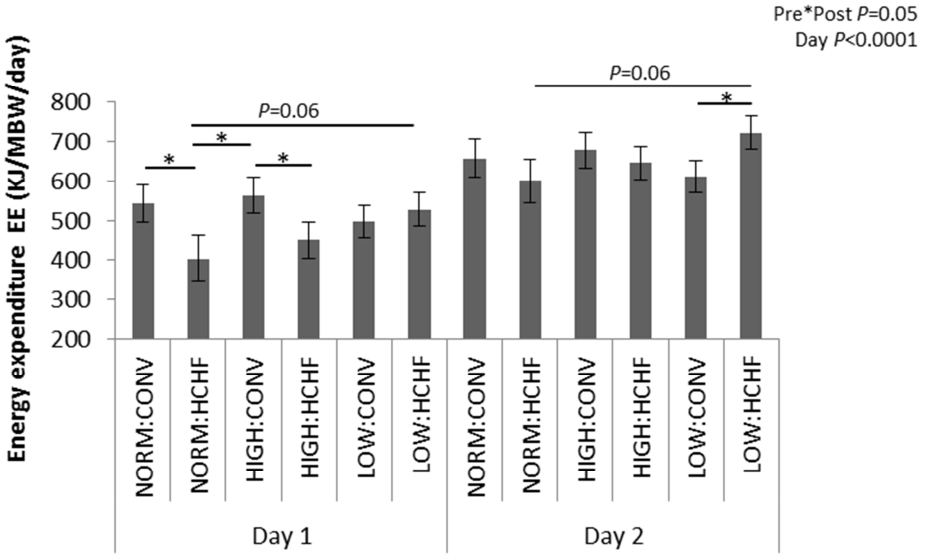

Figure 8 Group LSmeans for energy expenditure in 2.5 year old fasted sheep, male and female, with a history of adequate nutrition (NORM), overnutrition (HIGH) or undernutrition (LOW) in late gestation, combined with a conventional (CONV) or a High-Carbohydrate-High-Fat (HCHF) diet for the first 6 months 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 hours previously. Experimental design and dietary treatments have been fully described in the legend to Table 1. Significant differences are **P*≤0.05, ***P*≤0.01, or *P*≤0.001.

Paper III: Effects of late gestation under- or overnutrition and early postnatal overnutrition on the hypothalamic-pituitary-thyroidal axis in sheep.

In preparation.

Effects of late gestation under- or overnutrition and early postnatal overnutrition on the hypothalamic-pituitary-thyroidal axis in sheep

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Abstract

The hypothalamic-pituitary-thyroidal (HPT) axis is a target for fetal or early-life programming and this study aimed to test; 1) if the HPT axis was long-term affected by prenatal programming and/or early-life overnutrition in adulthood and 2) if fetal under- and overnutrition led to differentiated response in the HPT axis, when subjected to early life overnutrition. Thirty-six twin-pregnant sheep were adequately nourished (NORM), undernourished (LOW; 50/50%) or overnourished (HIGH; 150/110%) according to energy/protein recommendations, in the last trimester of gestation. Twin lambs were divided to either a moderate, low fat diet (CONV) or an obesogenic high-fat diet (HCHF) from day 3-6 months of age, yielding 6 treatment groups: NORM:CONV, NORM:HCHF, HIGH:CONV, HIGH:HCHF, LOW:CONV and LOW:HCHF. At 6 months-of-age, thyroid hormone response to fasting was tested and half the lambs were euthanized and autopsied. Remaining sheep were kept on a moderate diet until 2½ years-of-age, where another fasting tolerance test was performed and they were euthanized and autopsied. Thyroid stimulating hormone was examined, thyroxine and triiodothyronine in blood from postnatal day 1, 6 months- and 2½ years-of-age and examined central and peripheral gene expression of genes key to thyroid function. In neonates, T4 increased with birth weight, TSH increased with decreasing birth weight and T3 was increased in HIGH compared to LOW lambs. In 6 month-old-lambs, T4 and TSH response to prenatal and postnatal diets was sexually dimorphic in all treatment groups (except NORM:CONV) with males having decreased T4 and increased TSH relative to females. All treatments increased T3 compared to NORM:CONV. The HCHF diet primarily decreased expression of genes related to thyroid function in peripheral target tissues, main effects were observed in liver, kidney and subcutaneous fat. In adult sheep, sexual dimorphism was no longer visible in serum concentrations. NORM:HCHF sheep had serum profiles consistent with central hypothyroidism and HIGH sheep had developed overt hypothyroidism whereas LOW sheep appeared euthyroid. All adult sheep, but NORM:CONV displayed altered metabolic profiles in response to fasting, possibly a secondary effect of altered leptin metabolism. Effects of dietary interactions shifted to mainly affect gene expression of the hypothalamus and pituitary. In this experiment late-gestation programming through over- and undernutrition as well as early-life overnutrition alone had long-lasting effects on central and peripheral thyroid function and effects were discernible in programmed sheep according to postnatal nutrition.

Introduction

Fetal and early-life nutrition can program for adult non-communicable diseases such as cardiovascular disease, diabetes mellitus and obesity (de Gusmão Correia *et al.*, 2012). Evidence is accumulating that the HPT axis also undergo programming, and thyroid diseases are among the most prevalent endocrine disorders globally. It has been estimated that nearly 11% of Europeans and 5.9% U.S. citizens have thyroid dysfunction, with a clear prevalence of clinical and overt hypothyroidism, and half of these cases are

undiagnosed (Madariaga *et al*., 2014; Hollowell *et al*., 2002). It has long been known that thyroid hormones affect energy metabolism, but it has been regarded primarily as a maintenance signal for peripheral energy homeostasis. This view is shifting towards a central level energy balance regulation by thyroid hormones, acknowledging the THs intricate relation to energy sensors in the central nervous system (Lopez *et al.*, 2013; Vaitkus *et al*., 2015; Kim, 2008).

Early life programming of thyroid function has been recognized in several species as a consequence of diverse insults, but results are not unidirectional; undernourished neonates have been found to have high levels of T4, concurrent with low rT3 and T3. The hypothyroid state has been proposed to represent a peripheral metabolic adaptation to achieve a metabolic sparing of T4, i.e. a more energy conserving phenotype of the fetal energy restricted baby (Mahajan *et al*., 2005). De Blasio *et al*. (2006) found normal plasma T4, increased T3 and increased T4 to T3 conversion in placental restricted ~1 month old lambs. In support of this, prenatal protein restriction in heifers programmed calves to have higher plasma FT3 relative to T3 believed to attribute to the concurrent catch-up growth (Micke *et al.*, 2015). Maternal excess micronutrient supply and either under- or overnutrition in sheep, caused increases in plasma T4 without affecting T3 levels, and a possible increased conversion of T4 to T3 could be the drive for the observed compensatory growth (Vonnahme *et al.*, 2013). The long term effects of malnutrition through protein restriction versus energy-restriction were examined in rats and reported that protein restriction led to adult hyperthyroidism, whereas energy restriction led to increased serum T3, due to increased deiodination of T4 (Passos *et al*. 2002). In another rat model, mimicking maternal lactation failure in midlactation or late lactation, offspring were programmed for adult central hypothyroidism or secondary hypothyroidism, respectively (Bonomo *et al*., 2008; Lisboa *et al.*, 2010). Maternal isocaloric and moderate high-fat feeding caused hyperactivity of the thyroid axis at weaning in rat offspring, despite marked obesity in these offspring, possibly explained by leptin stimulation of TRH, a potential of T3 to increase food intake and at the same time lowered pituitary sensitivity to negative feedback (Franco *et al.*, 2012). The studies reviewed above clearly indicate that the HPT axis function can be programmed by adverse nutrition exposures in fetal or early-postnatal life. However, much remains to be known about the impact different adverse pre- and early postnatal nutrition exposures can have on HPT axis function and peripheral signaling, and the extent to which such impacts may be overcome by dietary interventions later in life. In a previous experiment, our group found evidence to suggest that prenatal undernutrition and early postnatal overnutrition increased TH levels in adult female sheep and altered gene expression of thyroid receptors and genes key to thyroid function in selected target tissues, in a tissue specific matter (Johnsen *et al*., 2013).

The aim of this experiment was to expand the examinations to possible programming of the HPT axis in response also to prenatal overnutrition and to assess whether there is long-term gender specific manifestations of early life programing of HPT axis function. The specific hypotheses we aimed to test were: 1) HPT axis function and peripheral tissue signaling is differentially programmed by late gestation under- as compared to overnutrition, 2) this has differential long-term implications for adaptation of HPT axis function upon obesity development in early postnatal life and for recovery of normal HPT axis function upon diet intervention later in life.

These hypotheses were addressed in a study using sheep, which has similar fetal development trajectories to humans, which were exposed to late gestation under- or overnutrition followed by exposure in early postnatal life to moderate or obesogenic diets. HPT axis function was assessed by evaluation of serum total T3, total T4 and TSH concentrations from birth to adulthood, and

expression-analysis of key genes in the hypothalamus, pituitary and thyroid, as well as in the target tissues; cardiac muscle, liver, kidney, subcutaneous and abdominalfat and cardiac and skeletal muscle tissues in post-pubertal adolescent lambs and in adulthood. The genes examined were *SLC5A5, IYD, DIO1, DIO2, DIO3, TPO, TG, TSHr, THRA, THRB, TRHr, SLC16A2, NCOR1, HDAC3* (see Table 1 for tissue and gene function).

Materials and methods

Experimental animals and experimental design

The Copenhagen sheep model were used to study nutrition impacts in late gestation and early postnatal life on HPT axis function, and an extensive description of the animal model and the overall design of the animal experiment was published by Khanal *et al*. (2014). All experimental animal handling and procedures were approved by The Danish National Committee on Animal Experimentation. Sheep were exposed to different levels of nutrition during the last 6 weeks prior to parturition (last trimester, gestation length=147 days) by feeding their twin pregnant mothers diets fulfilling 50/50% (LOW, N=14), 100/100% (NORM, N=9) or 150/110% (HIGH, N=13) of the NRC recommendations (2007) for energy/protein for twin pregnant sheep in the last trimester of gestation. Twin lambs were assigned to each their conventional diet; moderate hay based (CONV, N=35; 16 males, 19 females) or an obesogenic high carbohydrate, high fat diet (HCHF N=35; 18 males, 17 females). The lambs were raised on these diets from postnatal day 3 until 6 months-of-age (after puberty), where they were subjected to a 44-hour fasting tolerance test. Subgroups of lambs from each treatment group were then euthanized and autopsied (NORM:CONV: 3 (3M, 0F), NORM:HCHF: 3 (3M, 0F), HIGH:CONV: 5 (2M, 3F), HIGH:HCHF: 5 (2M, 3F), LOW:CONV: 5 (2M, 3F), LOW:HCHF: 5 (3M, 2F)). Remaining sheep were fed identically with the same low-fat, conventional diet until 2½ years-of-age (adulthood), at which time they were subjected to a 68-hour fasting tolerance test and then all sheep were euthanized and autopsied (NORM:CONV: 6 (2M, 4F), NORM:HCHF: 4 (2M, 2F), HIGH:CONV: 6 (2M, 4F), HIGH:HCHF: 6 (3M, 3F), LOW:CONV: 8 (4M, 4F), LOW:HCHF: 7 (4M, 3F)).

Blood sampling and serum TSH, total T4 &total T3 analysis

Baseline blood samples were drawn from lambs at postnatal day 1 and during fasting tolerance tests at 6 months- and 2½ years-of-age. Prior to the fasting tolerance tests, catheters were inserted into the jugular vein,as previously described (Khanal *et al.*, 2014), and lambs and sheep were subsequently subjected to 44 hours and 68 hours of fasting, respectively. Blood was sampled 0, 24, 44 (and in adults: 68) hours after the feed was withheld. The sheep had free access to water during the fasting period. The serum tubeswere placed at room temperature for ~20 minutes to coagulate and then centrifuged (1800×g_{av}, 15 minutes at 4°C) beforethe serum was separated and frozen in cryotubes at -20°C until analysis. Concentrations of T3, T4 and TSH in serum were measured using a double-antibody radioimmunoassay (as described in Zhang *et al*., 2004 and Wrutniak *et al*., 1987). The sensitivity was 0.12 nM for T4 and 0.02 ng/ml for T3 and TSH. The intra-assay variation for T4, T3 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.

Relative quantification of gene expression

Gene expression of messenger RNAs (mRNAs) for *TRHr, TSHr, TPO, TG, THRA,THRB, SLC5A5, IYD, DIO1, DIO2, DIO3, SLC16A2, NCOR1* and *HDAC3* were quantifiedin the relevant tissues, using quantitative reverse transcriptase PCR (qRT-PCR), and procedures were according to manufacturer's guidelines.Primer sequences were derived from ovine or bovine cDNA

sequences from the National Center for Biotechnology Information (NCBI) and primer sequences, tissue of relevance, function, efficiency, NCBI accession numbers, and annealing temperatures are listed inSupplementary Table 1. Total RNA was extracted, quality checked and transcribed into cDNA and qPCR was run, PCR products sequenced and qPCR analyzed using the same procedure and reagents as previously described in Johnsen *et al*. 2013.The only exception was that RNA extraction from the pituitary and hypothalamus was carried out using RNeasy®Lipid Tissue Mini Kit (QIAGEN).

Statistics

All statistical models were derived from the same multi-factorial model:

$$Y_{ijlo} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \gamma_k + \alpha\beta\gamma_{ijk} + \kappa_l + \varepsilon ijkl$$

Where Yijlo is the specific factor, described by all the qualitative explanatory variables, μ is the overall mean, αi is the effect of the prenatal NORM, LOW or HIGH treatments, βj is the effect of the postnatal CONV or HCHF treatments, αβij is the interaction between pre- and postnatal treatments, γk is the effect of co-variates, i.e. time, metabolic body weight (MBW), birth weight and/or sex, αβγijk is the three-way interaction between pre- and postnatal treatments and time or sex, κl is the random effect of twins and εijkl is the residual variation ~ N(0,σ2). The universal sample space of the qualitative explanatory variables are: i = {1,..,3}, j = {1,2}, k = {1,2} and l = {1,2}. 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. Variables that showed no significance were eliminated from the model and estimates and significance of the remaining factors were calculated by the function LS-means.Sex was confounded, due to unequal distribution of gender between adult treatment groups and for that reason only included as a co-variate, in the analysis of adult data.

Results

There were no effects of pre- or postnatal nutrition, sex birth weight, or weight at the time of blood or tissue sampling for any of the determined parameters unless specifically stated.

Serum TSH, total T3 &total T4

Postnatal day 1

Results are shown in Figure 1. T4 ranged from 282–811nM, increased with birth weight (2.050–5.830 kg) (*P*=0.02), but was not affected by prenatal nutrition. T3 ranged from 1.76–6.28 ng/ml and was lowest in LOW lambs (*P*=0.01compared to HIGH). TSH ranged from 0.09–0.35 ng/ml and LOW lambs increased TSH levels, compared to HIGH lambs (*P*=0.04) and correlated negatively to birth weight (*P*=0.04).

6-months-old lambs

Overall, basal T4, T3 and TSH ranged from ~201–310 nM, ~1.1–1.4 ng/ml and 0.10–0.14ng/ml, respectively. In all treatment groups, except NORM:CONV, males had significantly lower T4 levels compared to females within their treatment group (Figure 2a). LOW:CONV female lambs had significantly increased T4 compared to LOW:HCHF and NORM:CONV females (*P*=0.03 and *P*=0.05, respectively). The observed sexual dimorphism was less pronounced in TSH levels, with increased concentration in NORM:HCHF and HIGH:CONV male lambs compared to females within their treatment group (*P*=0.05 and *P*=0.04, respectively)(Figure 2c), which was in line with serum T4. Inversely, LOW:CONV females tended to have increased serum TSH over LOW:CONV males (*P*=0.09). Total T4

transiently increased after 24 hours of fasting (P=0.02), but dropped below baseline levels after 44 hours of fasting (P=0.07) (Supplementary figure 1a and c).
T3 significantly increased in HCHF lambs, correlated negatively to birth weight (P=0.02) and decreased throughout the 44 hour fast in all groups to approximately half of basal level (P<0.0001) (Supplementary figure 1b).

2½-year old sheep
As mentioned due to small groups and unequal gender distribution it was not possible to analyse interactions between gender, pre- and postnatal nutrition and time, in adult sheep, but sex was included as a covariate. Visual inspection of results did not seem to reveal sexual dimorphism or interactions (results not shown). T4 was increased in females compared to males in all treatment groups and not significant for T3 and TSH. NORM:HCHF sheep were hypothyroid, with decreased basal total T4 (P=0.001) and numerically decreased T3 concentrations and normal basal TSH. Prenatal overnutrition alone programmed for adult overt hypothyroidism, and HIGH:CONV and HIGH:HCHF had decreased T4 (P=0.04 and P=0.01, respectively) and increased TSH levels (P=0.06 and P=0.05), compared to NORM:CONV (Figure 3). LOW sheep appeared euthyroid.
The fasting tolerance test revealed that all treatment groups but NORM:CONV adapted strangely to the long-term fast. NORM:CONV adult sheep maintained equal T4 plasma levels, throughout the 68 hour fast, whereas all other treatments increased plasma T4 in the first 44 hours, where after plasma concentrations plateaued (P<0.0001). Every group tended to have a unique response to fasting when examining TSH and T3 levels, and very unlike the observed pattern between treatments in 6-month-old lambs. NORM:CONV sheep maintained stable serum T3 and TSH, throughout the fast. Likewise, serum T3 was not significantly affected by fasting in NORM:HCHF, HIGH:HCHF or LOW:CONV, but they did show a pattern of numerical increase in T3, which was completely opposite to the marked decrease in T3 during the fast in 6-month-old lambs. T3 levels in HIGH:CONV and LOW:HCHF increased transiently, with peak concentrations at 20 and 44 hours of fasting, respectively, where after the declined towards basal concentrations. A transient increase in TSH, followed by a decline to less than basal levels, was also observed in NORM:HCHF and HIGH:HCHF sheep, with a peak around 20 hours of fasting, similar to the observed at 6 months-of-age. However, there was no clear pattern for TSH response in HIGH:CONV sheep, a consistent decrease of TSH in LOW:CONV sheep and oppositely a consistent increase in TSH of LOW:HCHF sheep, during the 68 hour fast.

Gene expression in the hypothalamic-pituitary-thyroidal pathway

Data are only presented for genes where expression was affected by prenatal nutrition or postnatal nutrition alone or where these two interact.

Hypothalamus
In lambs, the only gene affected by any of the parameters studied was *DIO2*, where expression correlated positively to birthweight (P=0.04) and expression levels were almost twice as high in females compared to male lambs (P=0.06). The TH membrane transporter *SLC16A2* expression also tended to correlate positively to birth weight (P=0.06) (Figure 4a-c).
In adult sheep, *SLC16A2* was app. 2.5 fold higher in LOW as compared to NORM and HIGH sheep (P=0.008 and P=0.003, respectively) and correlated negatively to birth weight (P=0.004). For *TSHr* and *THRb*, expressions depended on the combination of pre- and early postnatal nutrition exposures. Thus, the HCHF diet depressed expressions in NORM sheep (P=0.02 and P=0.04 for *TSHr* and *THRb*, respectively) and for *TSHr* also in LOW sheep (P=0.03), whereas expressions were increased in HIGH sheep previously exposed to the early postnatal HCHF diet

(*P*=0.002 and *P*=0.0005 for *TSHr* and *THRb*, respectively). *DIO3* expression was upregulated by the HCHF diet in all prenatal (*P*=0.04) and males had higher expression of *DIO3* than females (*P*=0.04).

Pituitary

In 6 months old lambs, the *DIO2* and *THRa* were the only genes for which expression was affected by any of the parameters tested. Adverse nutrition exposure either prenatally (LOW or HIGH) or postnatally (HCHF) reduced *DIO2* compared to NORM:CONV lambs (*P*=0.04). *THRa* expression was affected by gender in lambs with females having almost twice as high expression as males (*P*=0.05) (Figure 5).

These early nutrition impacts did not persist into adulthood, but others emerged in adult sheep. For *TRHr* and *THRb*, prenatal overnutrition increased expression relative to NORM and LOW, and for *TRHr* the mismatching LOW:HCHF group reached similar high expression levels (*P*=003 and *P*=0.008, respectively for the Pre*Post nutrition interaction). There were similar patterns observable in *SLC16A2*, *NCOR1* and *DIO3* (although not in every case significant) that expressions were depressed by the HCHF diet in NORM but increased in the HIGH sheep, and in LOW the HCHF diet also tended to increase expressions of *SLC1A2* and *DIO3* (*P*=0.06-0.07 for Pre*Post nutrition interaction). *THRa* expression was too low in adults to be analyzed.

Thyroid

In 6 months old lambs, only prenatal nutrition affected gene expression. LOW lambs had significantly reduced *TSHr* expression compared to NORM lambs (*P*=0.007) with HIGH lambs in between. *DIO2* expression was increased in HIGH lambs compared to NORM and LOW lambs, which had similar expression levels (*P*=0.03 and *P*=0.01, respectively) and expression of TG was also significantly higher in HIGH than LOW with NORM in between (*P*=0.01).

In spite of several attempts, relative expression of *DIO1* and *DIO3* in the thyroid of adult sheep, with extreme individual variation, did not yield functional results. There were no significant effects on the examined genes in adult sheep (Figure 6f–g), except for *TPO*, which was increased by HCHF in NORM and HIGH sheep but decreased by HCHF in LOW (P=0.01), resulting in similar high expression levels in NORM:HCHF, HIGH:HCHF and LOW:CONV and lower levels in NORM:CONV, HIGH:CONV and lowest in LOW:HCHF.

Gene expression in thyroid hormone target tissues

Liver

In 6 months-old lambs, the most profound effect on gene expression was solicited by the postnatal HCHF diet. *THRb* expression tended to be down regulated in LOW:HCHF compared to LOW:CONV (*P*=0.06) and *THRa* was significantly downregulated by the postnatal obesogenic diet in all treatment groups (*P*=0.009) (Figure 7a and c). The HCHF diet also downregulated expression of *IYD* compared to the postnatal CONV diet (*P*=0.02) (Figure 7d), but significantly upregulated expression of *DIO1* (*P*=0.0002) (Figure 7e).

In 2½-year-old adult sheep, pre- and postnatal diets interacted on expression of *THRb*, where the early postnatal overnutrition tended to upregulate *THRb* expression in NORM animals (*P*=0.09) and significantly upregulated *THRb* expression in LOW:HCHF compared to LOW:CONV sheep (*P*=0.03), whereas it led to a significant upregulation in HIGH:CONV compared to HIGH:HCHF (*P*=0.01). Overall, males had higher *THRb* expression than females (*P*=0.02) (Figure 8a). The early postnatal HCHF diet increased adult *SLC16A2* expression (*P*=0.03) and the prenatal LOW diet caused adult downregulation of *SLC16A2* compared to both NORM and HIGH sheep (*P*=0.008 and *P*=0.02, respectively) (Figure 8b). Adult *IYD* expression was significantly downregulated (*P*=0.05) and *HDAC3* and

DIO2 tended to be downregulated (*P*=0.09 and *P*=0.06, respectively) by early-life overnutrition (Figure 8c, d and e).

Kidney

There were many significant effects of both prenatal and postnatal nutrition in the 6-month-old lambs. Both *THRa* and *THRb* were affected by interactions between prenatal and postnatal nutrition. *THRa* expression was downregulated in response to the HCHF diet in both NORM and LOW lambs (*P*=0.01 and *P*=0.008), but not in HIGH:HCHF and *THRa* expression in both HIGH:CONV and HIGH:HCHF lambs were significantly decreased compared to NORM:CONV (*P*=0.05 for both) (Figure 9a). Early overnutrition also affected *THRb* expression differently, according to prenatal nutrition and *THRb* was significantly or close to significantly less expressed in NORM:HCHF as compared to HIGH:HCHF, LOW:HCHF and NORM:CONV (*P*=0.03, *P*=0.05 and *P*=0.07) (Figure 9b). Expression of *SLC16A2* followed the same numerical pattern as *THRa*, but only NORM:CONV and NORM:HCHF differed significantly with the HCHF diet decreasing expression in NORM lambs (*P*=0.02) (Figure 9c). Prenatal HIGH and LOW decreased *DIO1* expression compared to NORM lambs (*P*=0.005 for both) and early postnatal overnutrition generally increased expression of *DIO1* compared to the postnatal CONV diet (*P*<0.0001) (Figure 9d). *DIO2* expression was also significantly upregulated by the early overnutrition, but only in HIGH:HCHF compared to HIGH:CONV and LOW:HCHF (*P*=0.006 and *P*=0.01) and tended to be upregulated compared to NORM:HCHF (*P*=0.09) (Figure 9e).

There were no lasting or new effects of prenatal and postnatal nutrition in adult kidney tissue.

Cardiac

Only *SLC16A2* expression was significantly affected by the postnatal overnutrition in the 6-month-old lambs, where it was downregulated in HCHF compared to CONV lambs (*P*=0.003, Figure 10a). *THRa* expression correlated positively to MBW at 3 months-of-age (Figure 10c).

Only *DIO2* expression was affected by prenatal and postnatal treatments in adult sheep, where *DIO2* was significantly upregulated in LOW:HCHF compared to LOW:CONV sheep (*P*=0.05) and furthermore *DIO2* correlated positively to MBW (*P*=0.04) and tended to correlate negatively to birth weight (*P*=0.07, Figure 10d). *THRa* expression was higher in females compared to males (*P*=0.04) and *SLC16A2* expression correlated positively to MBW (*P*=0.04, Figure 10e). All genes, appeared slightly increased in the three HCHF groups, and analyzed together the thyroid function related gene expression was increased by early-life overnutrition (*P*=0.02, results not shown).

Biceps Femoris

TR corepressor *HDAC3* expression tended to be downregulated by the obesogenic diet in HCHF lambs compared to their prenatal pairing group (*P*=0.08) and expression in HIGH:CONV was significantly lower compared to NORM:CONV (*P*=0.05) and expression in HIGH:HCHF was significantly downregulated compared to both NORM:HCHF and NORM:CONV (*P*=0.05 and *P*=0.01, respectively). *HDAC3* expression correlated positively to birth weight (*P*=0.03) (Figure 11a).

In adult sheep, both TR corepressors *HDAC3* and *NCOR1* were affected by interactions between pre- and postnatal nutrition (Figure 11c, d). Early postnatal overnutrition caused downregulation of *NCOR1* in HIGH:HCHF and LOW:HCHF compared to HIGH:CONV and LOW:CONV, respectively (*P*=0.06 and *P*=0.006), but equal expression in NORM:CONV and NORM:HCHF. Early-life overnutrition also caused downregulation of *HDAC3*, but in all HCHF groups, compared to their prenatal paired group (NORM: *P*=0.02; HIGH: *P*=0.01; LOW: *P*=0.002). DIO2 expression tended to correlate negatively to birth weight (*P*=0.08).

Longisimus Dorsi

Postnatal overnutrition tended to downregulate or significantly downregulated expression of *THRa*, *SLC16A2* and *HDAC3* (*P*=0.06, *P*=0.03 and *P*=0.04, respectively) in 6-month-old lambs (Figure 12a, b and c). *THRb* expression correlated positively to MBW at 3 months (*P*=0.01) and was 2 times higher in females compared to males (*P*=0.006, Figure 12d).

In the adult sheep *THRa* and *HDAC3* was significantly affected by the prenatal undernourishment and LOW sheep had significantly upregulated expression of these genes compared to both NORM and HIGH sheep (*THRa*: *P*=0.008 and *P*=0.01; *HDAC3*: *P*=0.007 for both) and expression was positively correlated to birth weight (*P*=0.07 and *P*=0.05, respectively) (Figure 12e and f). There were lasting effects of early-life overnutrition as both *NCOR1* and *DIO2* was upregulated in adult HCHF sheep compared to CONV sheep (*P*=0.05 and *P*=0.07) (Figure 12g and h).

Subcutaneous fat

THRa expression in subcutaneous fat was numerically upregulated by HIGH and LOW, compared to NORM, although only significant for LOW (*P*=0.02) and correlated positively to birth weight (Figure 13a). A similar expression pattern was observed in *THRb*, *TSHr*, *DIO2* and *NCOR1* expression and although it was not accompanied by significant differences between treatment groups, all genes correlated positively to birth weight (*P*=0.03, *P*=0.02, *P*=0.02 and *P*=0.01, respectively, Figure 13d and e). Both *SLC16A2* and *HDAC3* expression was downregulated in response to postnatal overnutrition (*P*=0.05 and *P*=0.02, respectively; Figure 13b and c) and numerically *NCOR1* and *DIO2* expression was oppositely affected (Figure 13d).

There were no significant direct effects solicited by either treatment in the adult sheep. *TSHr* expression correlated positively to MBW (*P*=0.02) and *NCOR1* and *HDAC3* correlated positively to birth weight (*P*=0.05 and *P*=0.07, Figure 13g).

Abdominal fat

Significant differences in gene expression in abdominal fat were few and results for both lambs and adult sheep can be found in Figure 14. In lambs, the HCHF diet significantly upregulated *DIO2* expression in abdominal fat (*P*=0.02) and to a greater extend in NORM:HCHF than HIGH:HCHF (*P*=0.005) and *DIO2* expression correlated positively to birth weight. *HDAC3* expression was also significantly upregulated in NORM:HCHF compared to NORM:CONV (*P*=0.01), which was not seen in LOW and HIGH lambs.

In adult sheep, *THRb* expression tended to be upregulated in LOW compared to NORM lambs (*P*=0.08) and increased in males compared to females. *TSHr* expression was increased in NORM and LOW sheep with a history of early-life overnutrition (*P*=0.01 and *P*=0.04, respectively), but tended to be upregulated in HIGH:CONV compared to HIGH:HCHF sheep (*P*=0.08).

Discussion

The overall, lasting effects of prenatal and postnatal treatments to the HPT axis have been compiled in Figure 15 and effects to peripheral thyroid function in Figure 16, as a visual aid to conceptualize the observed alterations in adult sheep. There were many effects of prenatal nutrition in both lambs and adult sheep, but every effect of prenatal origin, observed in 6-month-old lambs, was not present in adult animals. Instead, new and more prenatal effects appeared in adult sheep, however the effects of these seemed to pull in the same direction, with regards to expected HPT axis function and peripheral signaling. In the lambs, thyroid gene expression was a target of prenatal programming and peripheral gene expression was primarily effected by the postnatal HCHF diet. In adult sheep, effects of programming on gene expression, shifted towards altered hypothalamic and pituitary signaling and these effects, as well as effects on peripheral gene expression, were largely defined by interactions between pre- and postnatal nutrition. The difference between the observed

effects in lambs and adult sheep and the many dietary interactions observed in adults, points at extensive adaptations with aging and differentiated programming defined by the combination of prenatal and postnatal nutritional level. For this reason, the following is structured into sections discussing; i) the effect of prenatal nutrition and early-life overnutrition in lambs ii) the long-term effects of prenatal nutrition and early-life nutrition (adult sheep) and iii) sexual dimorphism in programmed lambs.

Effects of prenatal and postnatal nutrition in lambs

Prenatal undernutrition: As mentioned, there were few effects of prenatal nutrition in 6-month-old lambs. LOW lambs had decreased thyroidal *TSHr* expression and slightly decreased *TG* expression, probably related to less TSH stimulation of thyroglobulin formation and secretion. It is difficult to see this reflected in serum levels, as TSH levels in LOW male and female lambs seem relatively alike and comparable to NORM:CONV and we have not previously found that LOW affected *TSHr* and *TG* expression of male lambs (Johnsen *et al.*, 2013).

In subcutaneous fat, *THRa* was upregulated by LOW, *TSHr* was slightly increased and *NCOR1*, *THRB* and *DIO2* were all correlated to negative birth weight. T3 stimulates *THRa* expression in adipocytes to increase lipogenic enzymes and triglyceride accumulation and simultaneously lower lipolysis (Jiang *et al.*, 2004; Gambo *et al.*, 2016) and perhaps LOW lambs have upregulated TH activity in subcutaneous fat as a thermogenic adaptation. Interestingly, we have previously reported lasting morphological changesto subcutaneous fat, comprised of very small adipocytes with increased collagen infiltration and reduced lipid accumulation ability, in adult LOW sheep (Nielsen *et al*., 2016) and although gene expression related to thyroid metabolism seemed normal in adult sheep, the morphological changes may be rooted in early life adaptations in TH signaling.

Prenatal overnutrition: HIGH lambs had decreased hypothalamic and pituitary *DIO2* expression and increased thyroidal *DIO2* expression, which could reflect less hypothalamic and pituitary negative feedback of T3. Again, this change is not readily reflected in serum levels and *DIO2* expression is not affected in adults. HIGH adult sheep did however fail to decrease TSH in response to a thyroxine tolerance test (Johnsen *et al.*, 2016), which could reflect permanently altered negative feedback sensing, rooted in altered early-life *DIO2* activity.

Postnatal overnutrition: 6 months of postnatal overnourishment induced a sexual dimorphic response in T4 and TSH, which will be discussed separately elsewhere, but the HCHF diet increased T3 of males and females, and increased T3 has previously been related to overnutrition (Stichel *et al.*, 2000). There were hardly any effects of the obesogenic diet on gene expression central to the HPT axis, but many effects were solicited to peripheral gene expression, and thus the altered serum THs are most lightly reflecting altered peripheral thyroid function in the overnourished state. The majority of circulating T3 stems from *DIO1* deiodination of T4 in the liver and kidneys (Yen, 2001) and increased in T3 could relate to upregulated *DIO1* expression in these two tissues. Except for this liver and kidney specific upregulation of *DIO1*, the tendency was that the HCHF diet downregulated peripheral gene expression, especially *SLC16A2*, *HDAC3* and *THRa*. The effects solicited to the kidney, pointing to an overall decreased renal TH stimulation may be a risk factor for renal dysfunction as it could decrease renal protein turnover and cause renal growth retardation (Canavan *et al.*, 1994) and have been shown to increase plasma creatinine, reflecting poor glomerular filtration (Dousdampanis *et al.*, 2014). Indeed, HCHF lambs had increased plasma creatinine and reduced kidney growth by 20%, which at the time was best explained by the low protein content of the HCHF diet and possibly physical constraints related to concurrent extreme renal

adipose encapsulation, but could possibly also be ascribed to decreased renal TH delivery and metabolism. Decreased TH stimulation specific to the glycolytic longisimus dorsi muscle fibers may also be a risk factor for reduced peripheral insulin sensitivity, since THs regulates mitochondrial gene expression and function in skeletal muscle and reductions in T3-mediated transcription may contribute to diabetes-related impairments in oxidative metabolism (Crunkhorn & Patti, 2008). We have previously reported downregulated *THRa* expression in both biceps femoris and longisimus dorsi in 6-month-old HCHF fed lambs (predominantly male) (Johnsen *et al*., 2013), as well as altered mitochondrial function (Jørgensen *et al*., 2009).

Long-term effects of prenatal and postnatal nutrition

Adult LOW sheep appeared euthyroid with unaltered serum THs and TSH levels compared to NORM:CONV sheep, concurrent with very few effects on both central and peripheral gene expression. Prenatal overnourished (HIGH) sheep developed overt hypothyroidism with reduced T4 and increased TSH and early-life overnutrition in NORM sheep caused adult central hypothyroidism, as evidenced by decreased T4 and numerically halved serum T3. It is widely accepted that fetal programming is a risk factor for metabolic syndrome (Rinaudo & Wang, 2012; Symmonds *et al.*, 2009; Brenseke *et al.*, 2013) and the frequency of metabolic syndrome has been found to be increased in humans with overt hypothyroidism (Erdogan *et al.*, 2011). Here, both prenatal overnutrition and late-gestation overnutrition predisposed for adult overt or central hypothyroidism, which may further contribute to the adverse effects of adult metabolic syndrome of prenatal origin.

There were hardly any effects of prenatal and early postnatal overnourishment to gene expression central to the HPT axis in the lambs, but with increasing age evidence of programming manifested in both hypothalamic and pituitary gene expression, but less in the thyroid. The discrepancy between thyroid state between HIGH and NORM:HCHF sheep, seems to be rooted in pituitary specific programming where there were more effects specific to the prenatal HIGH diet and specifically increased *TRHr* expression could reflect the increased TSH levels, not present in NORM:HCHF sheep. Oddly, *TSHr* expression in the thyroid appeared downregulated in HIGH animals, which could reflect a level of thyroidal TSH resistance and perhaps relate to the decreased serum TH levels. In the thyroid, *TPO* expression was increased in HIGH:HCHF and LOW:CONV sheep, which could indicate some extent of thyroid autoimmunity, as *TPO* is also a thyroid autoantigen (Ruf & Carayon, 2006).

Although HIGH sheep present with the same overall thyroid state, they do differentiate on multiple parameters. Gene expression within the HPT axis was adversely affected in HIGH:CONV and HIGH:HCHF sheep, in spite of similar basal serum THs and TSH. With regards to hypothalamic expression, genes were affected by early-life overnutrition in the same fashion in HIGH:HCHF and NORM:HCHF, but within the pituitary the response was completely opposite, with *NCOR1*, *THRb* and *DIO3*, being upregulated in HIGH:HCHF compared to HIGH:CONV. Furthermore, T3 response to fasting in HIGH:CONV sheep varied distinctly from both HIGH:HCHF and NORM:CONV. HIGH:CONV sheep were unable to decrease TSH in response to a thyroxine tolerance test to the level of NORM:CONV and HIGH:HCHF sheep and at the same time they had decreased T3:T4 ratios, reflecting less peripheral T3 (Johnsen *et al.*, 2016). These observations indicate that the postnatal CONV diet may be a "mismatch" diet in HIGH lambs.

Hypothalamic *TSHr* has been shown to be present in hypothalamic nuclei relevant for feeding control and TSH injections to the nucleus of the solitary tract reduced feed intake of adult rats (Burgos *et al.*, 2016). We have previously reported an increased voluntary feed intake immediately post-fasting in both adult NORM:HCHF and LOW:HCHF sheep

(Johnsen *et al.*, 2016), which we ascribed to possibly perturbed central leptin action but additionally the increased feed intake may be linked to the reduced hypothalamic *TSHr* expression reported.

Regarding peripheral TH metabolism of adult animals, there were fewer effects than observed in lambs, these were different from what was observed in 6-month-old lambs and present in fewer tissues, where gene expression levels pointed to thyroid function being most affected in liver, skeletal muscles and abdominal fat. The numerous effects on gene expression in kidneys were no longer visible and adults did not have differentiated kidney weights, LOW:HCHF sheep did however show signs of lasting kidney dysfunction (Khanal *et al.*, 2016). It is not possible to say if this observation can be linked to programmed thyroid function during development.

During normal conditions prolonged fasting should instinctively manifest in decreased serum T4 and T3 and low-normal TSH levels, but NORM:CONV sheep maintained stable levels of T4, T3 and TSH throughout the 68 hour fast. This confirms that a three day fast is not a major metabolic challenge for adult ruminants due to large nutrient resources in the forestomachs, and explains the lack of decrease in TH (Khanal *et al.*, 2016; Bertoni *et al.*, 1993).

A peculiar observation was the fasting profile of HIGH, LOW and NORM:HCHF sheep, as they increased serum T4 throughout 68 hours of fasting. Decreasing leptin levels during fasting is a signal to suppress proTRH gene expression within neurons of the PVN and thus reduce hypothalamic TRH release, and systemic administration of leptin has been shown to prevent this fasting induced suppression (Légrádi *et al.*, 1997). Early-life overnutrition has been shown to have the potential to induce lasting leptin resistance and hyperleptinemia (Glavas *et al.*, 2010; Kjaergaard *et al.*, 2016) and late gestation overnourished sheep have been shown to present with increased leptin in adulthood (Long *et al.*, 2010), and prenatal undernutrition have been shown to increase leptin response throughout a 72 hour fast in adult sheep (Kongsted *et al.*, 2013). Thus, altered leptin response of adult HIGH, LOW and NORM:HCHF sheep, may account for the abnormal TH response in fasting. The altered response to fasting may be considered a secondary effect of programming to the HPT axis, rooted in altered leptin metabolism.

Sexual dimorphism in lambs

6 months of postnatal overnutrition induced sexual dimorphism in thyroid state between NORM:HCHF and HIGH males and females, where males reduced serum T4 compared to females and most increased serum TSH compared to females. Sexual dimorphism have also been observed in 100 day-old calves of heifers fed either low or high protein diets in the first or second trimester (Micke *et al*, 2015), so although we cannot offer a mechanistic explanation, thyroid programming have been shown to be affected in a sexual dimorphic manner and apparently early postnatal programming also solicited such an effect in lambs. Due to few sheep combined with unequal gender distribution between treatment groups in adult animals, it is not possible to say with absolute certainty that sexual dimorphism did not apply to adult sheep. However, sex was only a co-factor on adult T4 concentrations, where it meant that females had increased T4 compared to males, in all groups, including NORM:CONV and visual inspection of results did not imply any apparent dimorphic pattern between males and females.

Conclusions

Overall, there were pronounced effects of both late gestation and early postnatal malnutrition on central and peripheral HPT axis function. Although development of adult metabolic disease was proposed to differentiallyprogramme HPT axis function, adult thyroid state seemed to be unidirectional depressed, varying in severity. Early-life overnutrition independently predisposed for adult central hypothyroidism. Adult LOW

sheep appeared euthyroid, but altered fasting response and gene expression levels, imply that there are permanent effects of early life nutrition.Prenatal overnutrition predisposed for adult overt hypothyroidism. There were discernible effects of programming to the HPT axis depending on type of fetal and early-life nutrition, implying that there were differential long-term implications for adaptation of HPT axis function to early-life overnutrition and recovery of normal adult axis function. Namely, prenatal overnourished sheep presented pituitary gene expression patterns and T3 response to fasting indicated that a conventional diet may be considered a "mismatch" diet in fetal overnourished lambs.

Declaration of interests

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

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Author contributions

PhD Fellow LJ was in charge of the animal experimentation, laboratory procedures, statistical evaluation of data, and for writing the manuscript. Dr. PK contributed with valuable inputs in the interpretation of results and performed manuscript revision. Prof. MON, vice-director of the Danish Centre for Fetal Programming, Denmark, developed the Copenhagen sheep model and was the responsible person for designing the overall sheep experiment and has performed manuscript revision.

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Table 1 Primer sequences and accession numbers for applied genes

Gene	Full name	Overall function	Tissue	Primer sequence	NCBI Accession No.	Annealing temp. (°C)	Efficiency
CYCB	Cyclophiline B	Reference gene	All (-adipose tissues)	FW: GATCCAGGGTGGAGATTTCAC; RV: GGCCCATAGTGTTTAAGCTTG	AJ865374 (Oa)	60	1.9
ACTB	Beta actin	Reference gene	Adipose tissues	FW: ACCCAGATCATGTTCGAGACCTT; RV: TCACCGGAGTCCATCACGAT	AY141970 (Bt)	60	1.8
SLC5A5	Sodium iodide symporter	Mediates iodide uptake in thyroid follicles	Thyroid	(L)CGGAATCATCTGCACCTTCT; (R)GGACAACCCAGAAACCACTC	XM_581578.5 (Bt)	60	1.9
IYD	Iodotyrosine deiodinase	Deiodinates tyrosines	Thyroid, liver	(L)TTCTCCCACAGTCGATACCC; (R)ATCTGGGTCCTTCACAACCA	NM_001102165.1 (Bt)	60	2.2
DIO1	Deiodinase 1	Deiodinates T3/4 in target tissues	Liver, kidney, thyroid	(L)AGGCTCTGGGTCCTCTTTCA; (R)CCCATGGCCAGGATGTTCTT	XM_004001999.1 (Oa)	60	1.9
DIO2	Deiodinase 2	Deiodinates T3/4 in target tissues	Peripheral tissues	(L)GTGGCTGACTTCCTGTTGGT; (R)GCATCGGTCTTCCTGGTTC	NM_001010992 (Bt)	60	2.0
DIO3	Deiodinase 3	Deiodinates T3/4 in target tissues	Hypothalamus, pituitary, thyroid	(L)TCTACATCGAGGAAGCGCAC; (R)AGCTGCTGGAGTTGGTCATC	NM_001122650.1 (Oa)	60	1.9
TPO	Thyroid peroxidase	Iodine oxidation and binding	Thyroid	(L)ATCACGGATTCCAACTCCAA; (R)GGGTCCACTTCATCCTCACA	XM_603356.5 (Bt)	60	1.9
TG	Thyroglobulin	TH storage molecule	Thyroid	(L)GAGCAGGTTTCCAGAGGTGT; (R)AGAGTGGTCTCAGCGAAGGT	NM_173883.2 (Bt)	60	2.0
TSHr	Thyroid stimulating hormone receptor	Thyroidal TSH sensing & TH secretion	Thyroid, hypothalamus, adipose tissues	(L)GGGAGTGAGGAGATGGTGTG; (R)GAGGATGACCAGGACGAAGA	NM_001009410.1 (Oa)	60	2.0
THRA	Thyroid hormone receptor alpha	Target gene activation or repression	All (-thyroid)	(L)CCTCTTCTCTCCTCCCTCTC; (R)TTGTCCGCTCTTAGTTCTCC	NM_001100919.1 (Oa)	60	1.9
THRB	Thyroid hormone receptor beta	Target gene activation or repression	All (-thyroid)	(L)GAAGCTCGTGGGAATGTCT; (R)GCCTTTGCACTTCTTCTCCT	NM_001190391.1 (Oa)	57	2.2
TRHr	Thyrotropin-releasing hormone receptor	Pituitary TRH sensing & TSH secretion	Pituitary	(L)GGGTCTATGGCTACGTTGGA; (R)GAGAAACTGGGCTTTGATGG	NM_001009407.1 (Oa)	60	2.1
SLC16A2	Solute carrier organic anion transporter 16A2	TH membrane transporter	All	(L)GCCTCCATACCAGCTCCTTC; (R)CCAGGATGACGAGAGATGGC	XM_004022631.1 (Oa)	60	2.0
NCOR1	Nuclear receptor co-repressor 1	Supress target gene transcription	All	(L)TCAGCAGGCTCCATCTCTCT; (R)GGGCTCTGACCAGTAAACCC	XM_004012724.1 (Oa)	60	1.8
HDAC3	Histone deacetylase 3	Supress target gene transcription	All	(L)CACTGCTGGTAGAAGAGGCC; (R)TCTGATTCTCGATGCGGGTG	XM_004008901.1 (Oa)	60	2.0

Figures

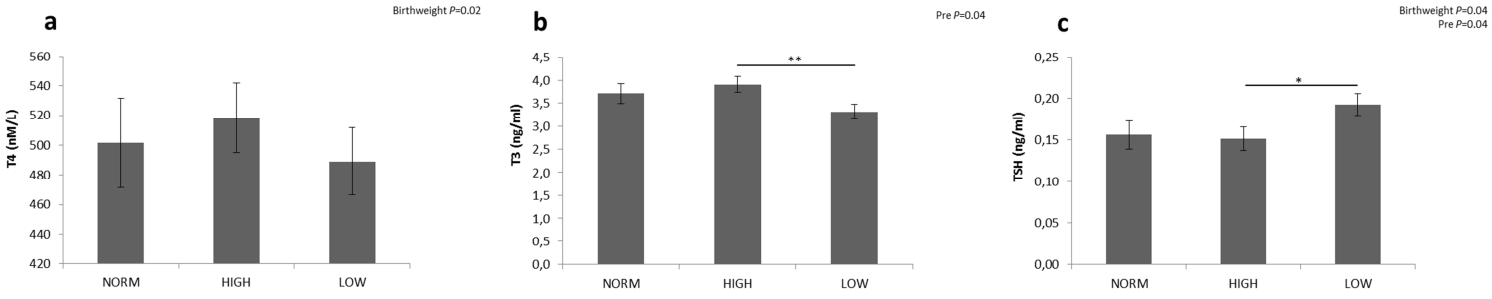


Figure 1 Basal serum concentrations of total T4 (nM), total T3 (ng/ml) and TSH (ng/ml) from 1-day-old lambs, of twin-pregnant ewes exposed to diets fulfilling 100% (NORM) or 150% required energy and 110% protein (HIGH) and 50% required energy and protein (LOW). Data are presented as least square means±SEM. Experimental design and dietary treatments have been fully described in the legend to Figure 2. Significant differences are *P≤0.05, **P≤0.01, or ***P≤0.001.

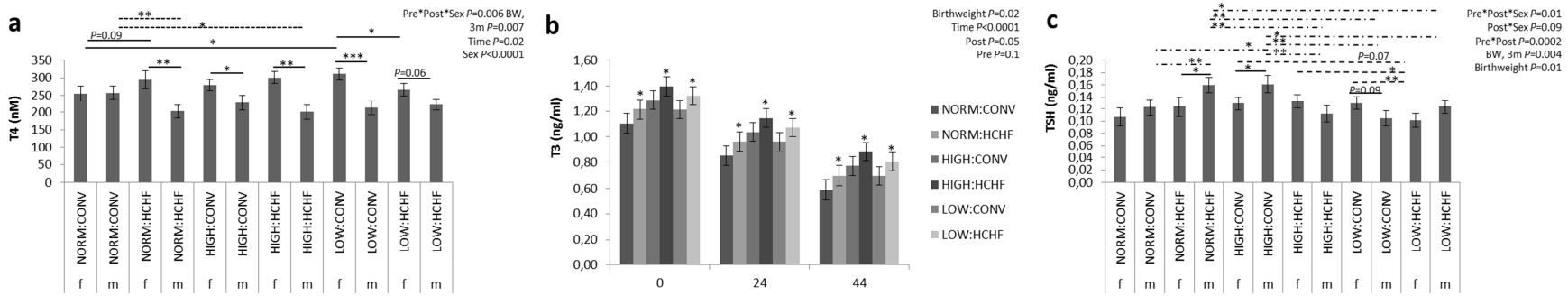


Figure 2 LSmeans±SEM of serum total T4 (nM), total T3 (ng/ml) and TSH (ng/ml) in 6-month-old lambs as affected by nutrition received in late fetal life (Pre) and the first 6 months of postnatal life (Post). Samples are from 0 hour- (basal), 24 hours- and 44 hours- fasted lambs, effect of time is not shown for T4 and TSH as the change was equal in all groups. Response over time can be seen in Supplementary Figure 1. Lambs were from twin-pregnant ewes, exposed to diets fulfilling 100% (NORM) or 150% required energy/110% protein (HIGH) or 50% required energy and protein (LOW). Twin offspring were assigned each their experimental diet from 3 days to 6 months postpartum; a conventional diet of milk replacer and hay until 8 weeks, followed by hay only, adjusted for moderate growth rates of app. 225 g/day (CONV) or an obesogenic high carbohydrate, high fat diet (HCHF) based on cream-milk replacer mix and rolled maize supplement. Twins were assigned postnatal treatments, ensuring best possible uniform gender distribution (first priority) and birth weight distribution (second priority). Thus, the 6 treatment groups in present experiment were: NORM:CONV, NORM:HCHF, HIGH:CONV, HIGH:HCHF, LOW:CONV and LOW:HCHF. MBW (3m): metabolic body weight at 3 months. *P≤0.05, **P≤0.01, or ***P≤0.001.

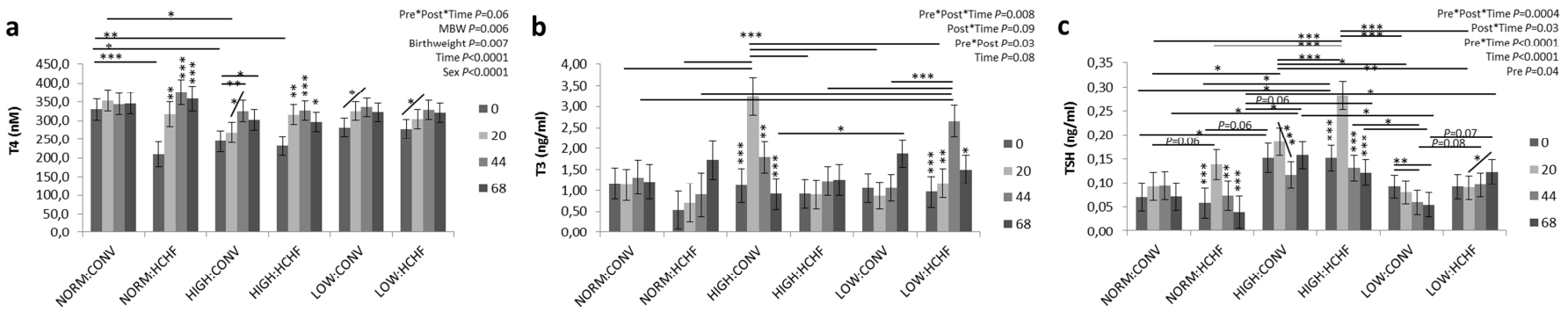


Figure 3 LSmeans±SEM of serum total T4 (nM), total T3 (ng/ml) and TSH (ng/ml) in 2½-year-old sheep, with a history of adequate nutrition (NORM), overnutrition (HIGH) or undernutrition (LOW) in late gestation, combined with a conventional (CONV) or a High-Carbohydrate-High-Fat (HCHF) diet for the first 6 postnatal months, yielding 6 experimental groups: NORM:CONV, NORM:HCHF, HIGH:CONV, HIGH:HCHF, LOW:CONV and LOW:HCHF. Samples were obtained from 0 hour- (basal), 20 hours-, 40 hours- and 68 hours- fasted sheep. Experimental design and dietary treatments have been fully described in the legend to Figure 2. Pre: prenatal diet; Post: postnatal diet; MBW: metabolic body weight. *P≤0.05, **P≤0.01, or ***P≤0.001.

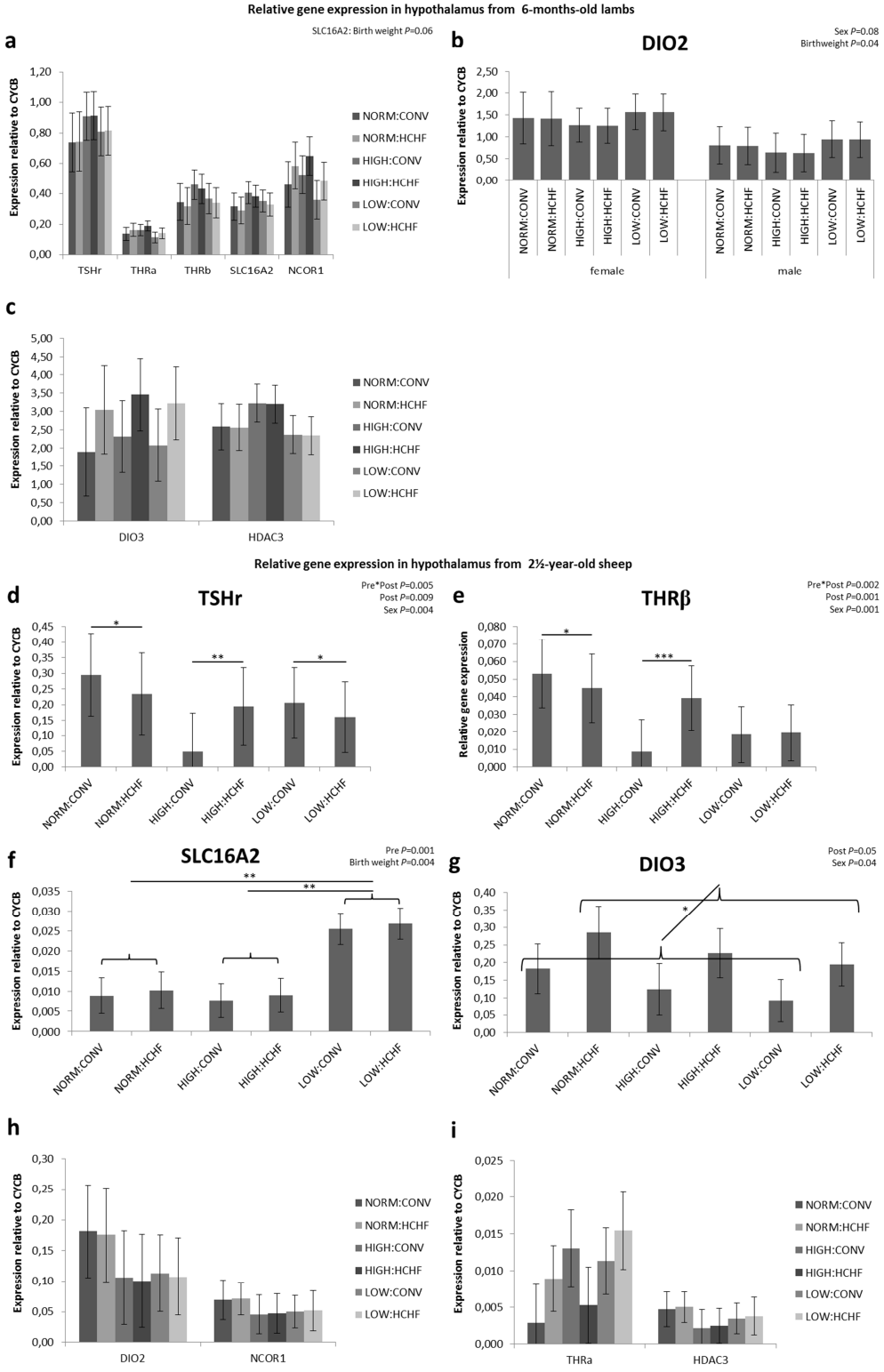


Figure 4 LSmeans±SEM of relative gene expression within the hypothalamus, for the genes *TSHr*, *THRa*, *THRb*, *SLC16A2*, *NCOR1*, *DIO2*, *DIO3* and *HDAC3* in 6-month-old lambs and 2½-year-old sheep, with a history of adequate nutrition (NORM), overnutrition (HIGH) or undernutrition (LOW) in late gestation, combined with a conventional (CONV) or a High-Carbohydrate-High-Fat (HCHF) diet for the first 6 postnatal months, yielding 6 experimental groups: NORM:CONV, NORM:HCHF, HIGH:CONV, HIGH:HCHF, LOW:CONV and LOW:HCHF. Experimental design and dietary treatments have been fully described in the legend to Figure 2. Pre: prenatal diet; Post: postnatal diet; MBW: metabolic body weight. *P≤0.05, **P≤0.01, or ***P≤0.001.

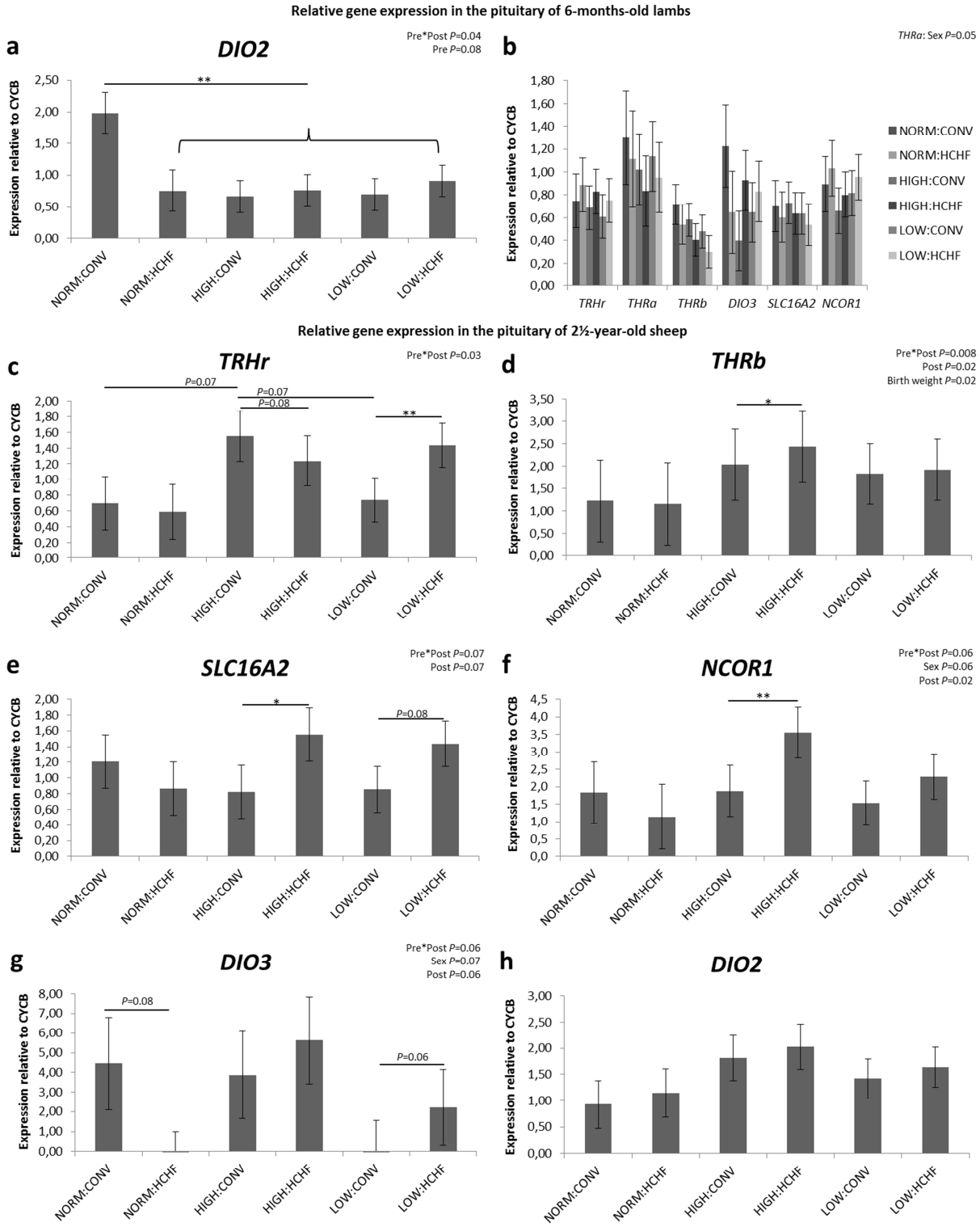


Figure 5 LSmeans±SEM of relative gene expression within the pituitary, for the genes *TRHr*, *THRa*, *THRb*, *DIO2*, *DIO3*, *SLC16A2* and *NCOR1* in 6-month-old lambs and 2½-year-old sheep, with a history of adequate nutrition (NORM), overnutrition (HIGH) or undernutrition (LOW) in late gestation, combined with a conventional (CONV) or a High-Carbohydrate-High-Fat (HCHF) diet for the first 6 postnatal months, yielding 6 experimental groups: NORM:CONV, NORM:HCHF, HIGH:CONV, HIGH:HCHF, LOW:CONV and LOW:HCHF. THRa was not sufficiently expressed in adult sheep and therefore omitted. Experimental design and dietary treatments have been fully described in the legend to Figure 2. Pre: prenatal diet; Post: postnatal diet; MBW: metabolic body weight. *P≤0.05, **P≤0.01, or ***P≤0.001.

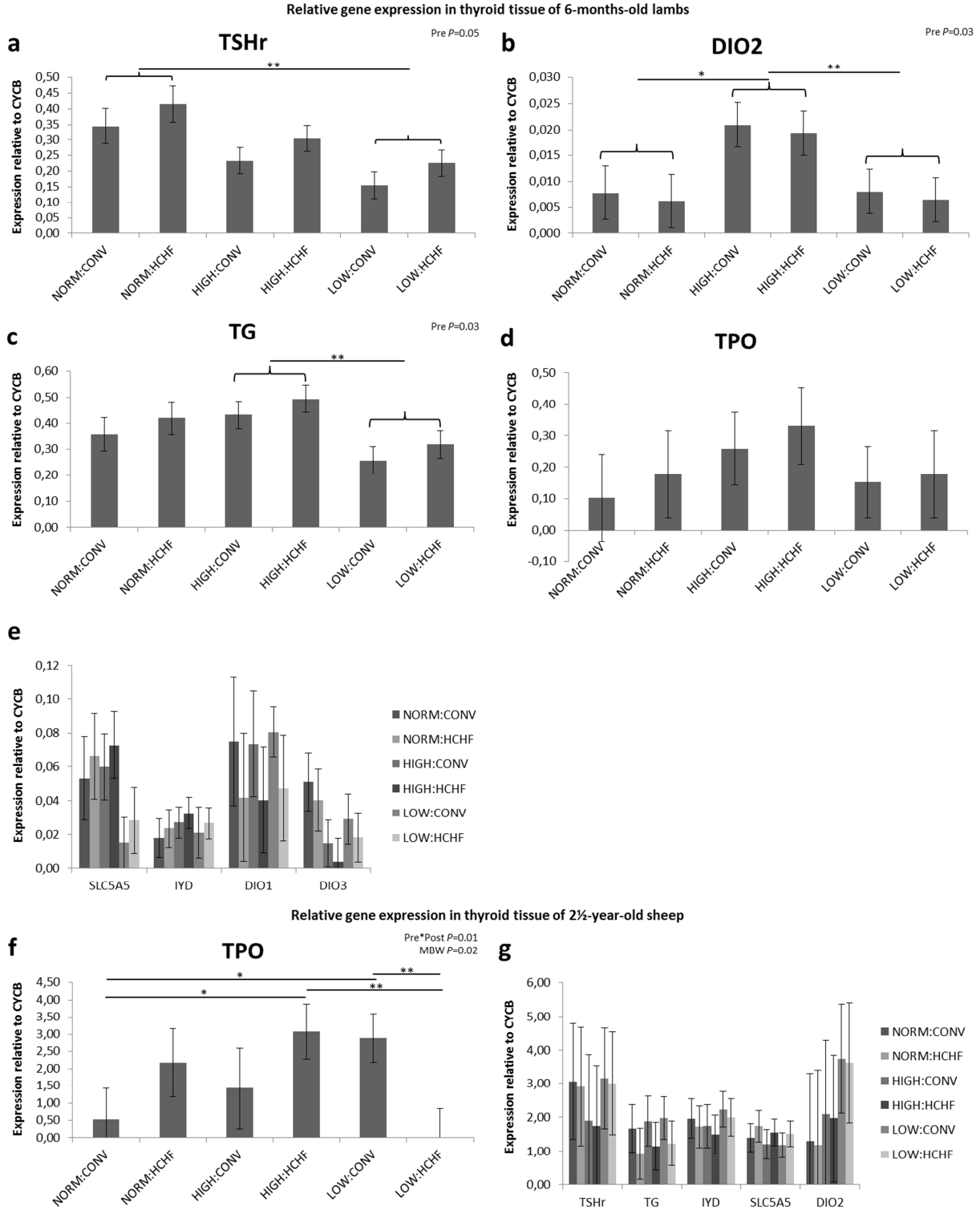


Figure 6 LSmeans±SEM of relative gene expression within the thyroid, for the genes T*SHr*, *DIO1*, *DIO2*, *DIO3*, *TG*, *TPO*, *IYD* and *SLC5A5* in 6-month-old lambs and 2½-year-old sheep, with a history of adequate nutrition (NORM), overnutrition (HIGH) or undernutrition (LOW) in late gestation, combined with a conventional (CONV) or a High-Carbohydrate-High-Fat (HCHF) diet for the first 6 postnatal months, yielding 6 experimental groups: NORM:CONV, NORM:HCHF, HIGH:CONV, HIGH:HCHF, LOW:CONV and LOW:HCHF. DIO1 and DIO3 was not successfully quantified in adult sheep and therefore omitted. Experimental design and dietary treatments have been fully described in the legend to Figure 2. Pre: prenatal diet; Post: postnatal diet; MBW: metabolic body weight. *P≤0.05, **P≤0.01, or ***P≤0.001.

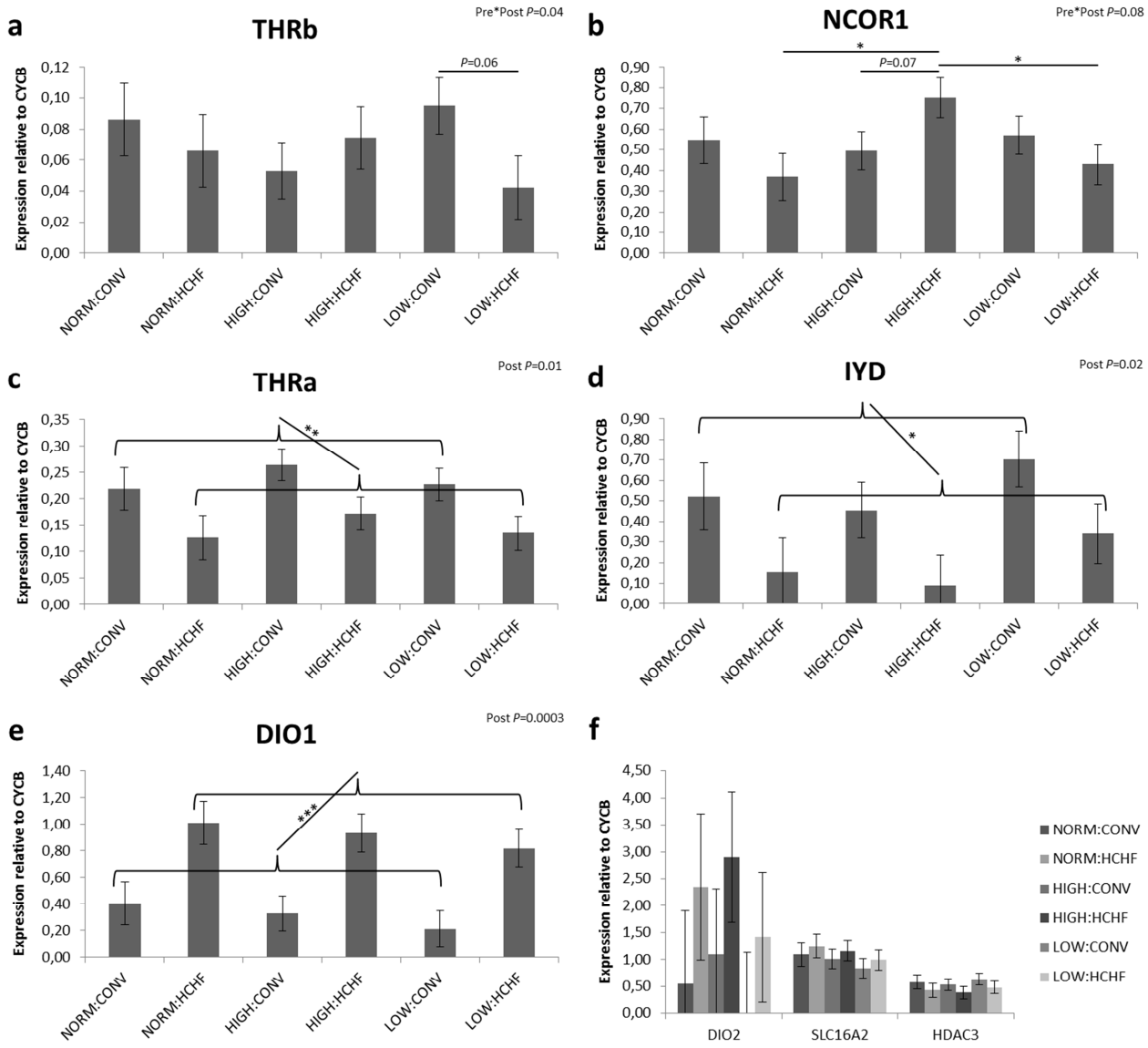


Figure 7 LSmeans±SEM of relative gene expression in liver tissue, for the genes *THRa*, *THRb*, *DIO1*, *DIO2*, *SLC16A2*, *IYD*, *NCOR1* and *HDAC3* in 6-month-old lambs, with a history of adequate nutrition (NORM), overnutrition (HIGH) or undernutrition (LOW) in late gestation, combined with a conventional (CONV) or a High-Carbohydrate-High-Fat (HCHF) diet for the first 6 postnatal months, yielding 6 experimental groups: NORM:CONV, NORM:HCHF, HIGH:CONV, HIGH:HCHF, LOW:CONV and LOW:HCHF. Experimental design and dietary treatments have been fully described in the legend to Figure 2. Pre: prenatal diet; Post: postnatal diet; MBW: metabolic body weight. *P≤0.05, **P≤0.01, or ***P≤0.001.

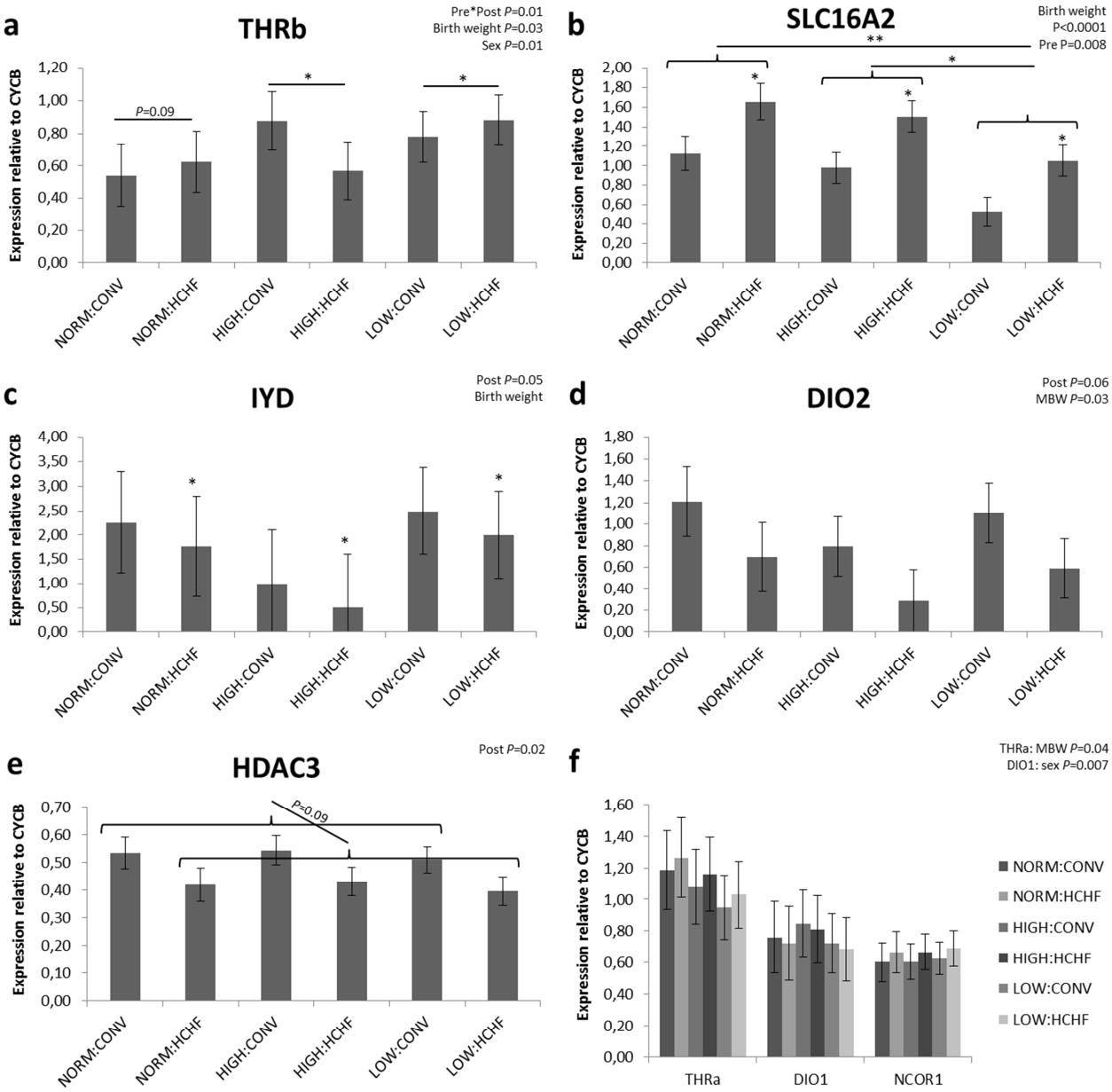


Figure 8 LSmeans±SEM of relative gene expression in liver tissue, for the genes *THRa*, *THRb*, *DIO1*, *DIO2*, *SLC16A2*, *IYD*, *NCOR1* and *HDAC3* in 2½-year-old lambs, with a history of adequate nutrition (NORM), overnutrition (HIGH) or undernutrition (LOW) in late gestation, combined with a conventional (CONV) or a High-Carbohydrate-High-Fat (HCHF) diet for the first 6 postnatal months, yielding 6 experimental groups: NORM:CONV, NORM:HCHF, HIGH:CONV, HIGH:HCHF, LOW:CONV and LOW:HCHF. Experimental design and dietary treatments have been fully described in the legend to Figure 2. Pre: prenatal diet; Post: postnatal diet; MBW: metabolic body weight. *P≤0.05, **P≤0.01, or ***P≤0.001.

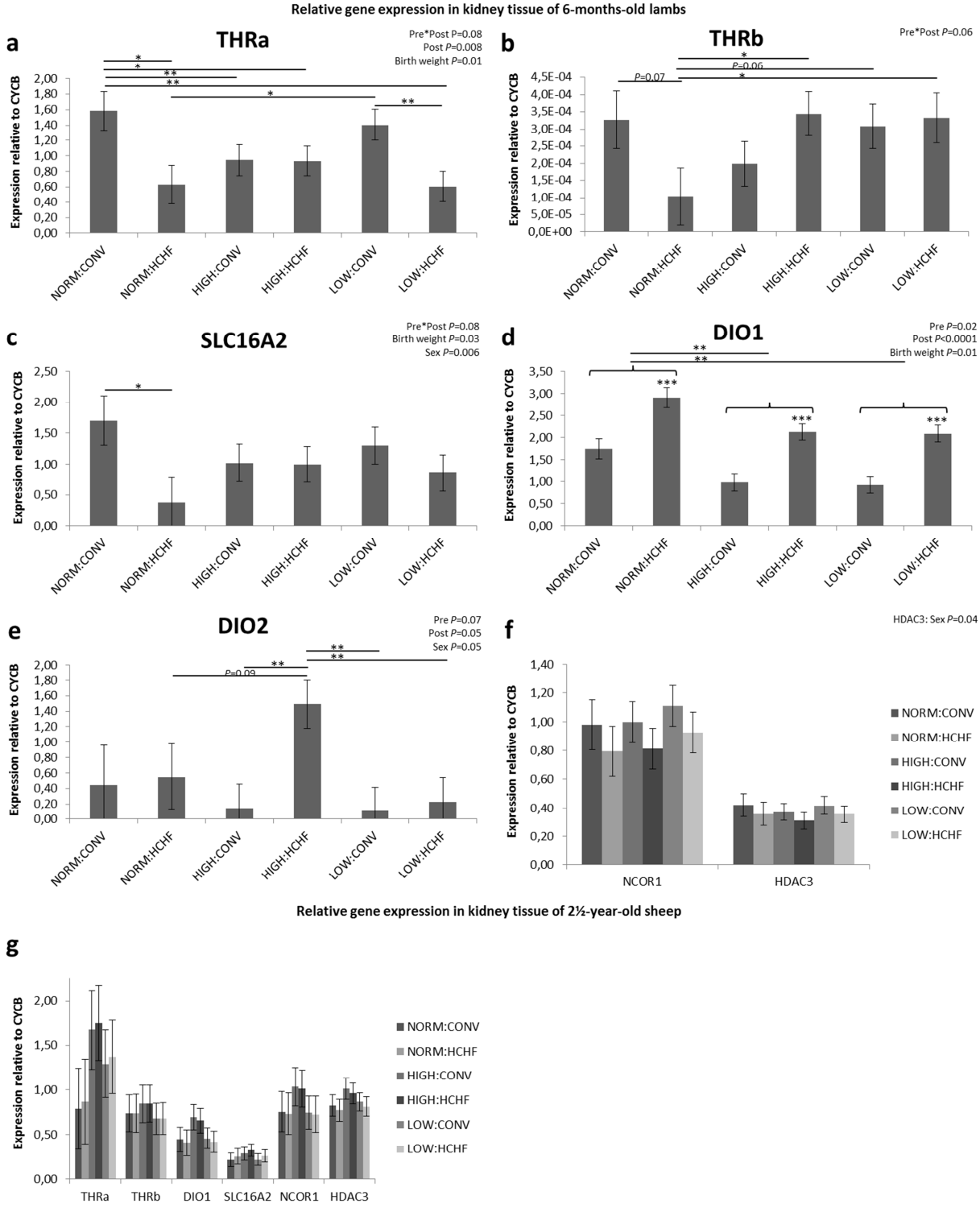


Figure 9 LSmeans±SEM of relative gene expression in kidney tissue, for the genes *THRa*, *THRb*, *DIO1*, *DIO2*, *SLC16A2*, *NCOR1* and *HDAC3* in 6-month-old lambs and 2½-year-old sheep, with a history of adequate nutrition (NORM), overnutrition (HIGH) or undernutrition (LOW) in late gestation, combined with a conventional (CONV) or a High-Carbohydrate-High-Fat (HCHF) diet for the first 6 postnatal months, yielding 6 experimental groups: NORM:CONV, NORM:HCHF, HIGH:CONV, HIGH:HCHF, LOW:CONV and LOW:HCHF. DIO2 was not successfully quantified in adult sheep and therefore omitted. Experimental design and dietary treatments have been fully described in the legend to Figure 2. Pre: prenatal diet; Post: postnatal diet; MBW: metabolic body weight. *P≤0.05, **P≤0.01, or ***P≤0.001.

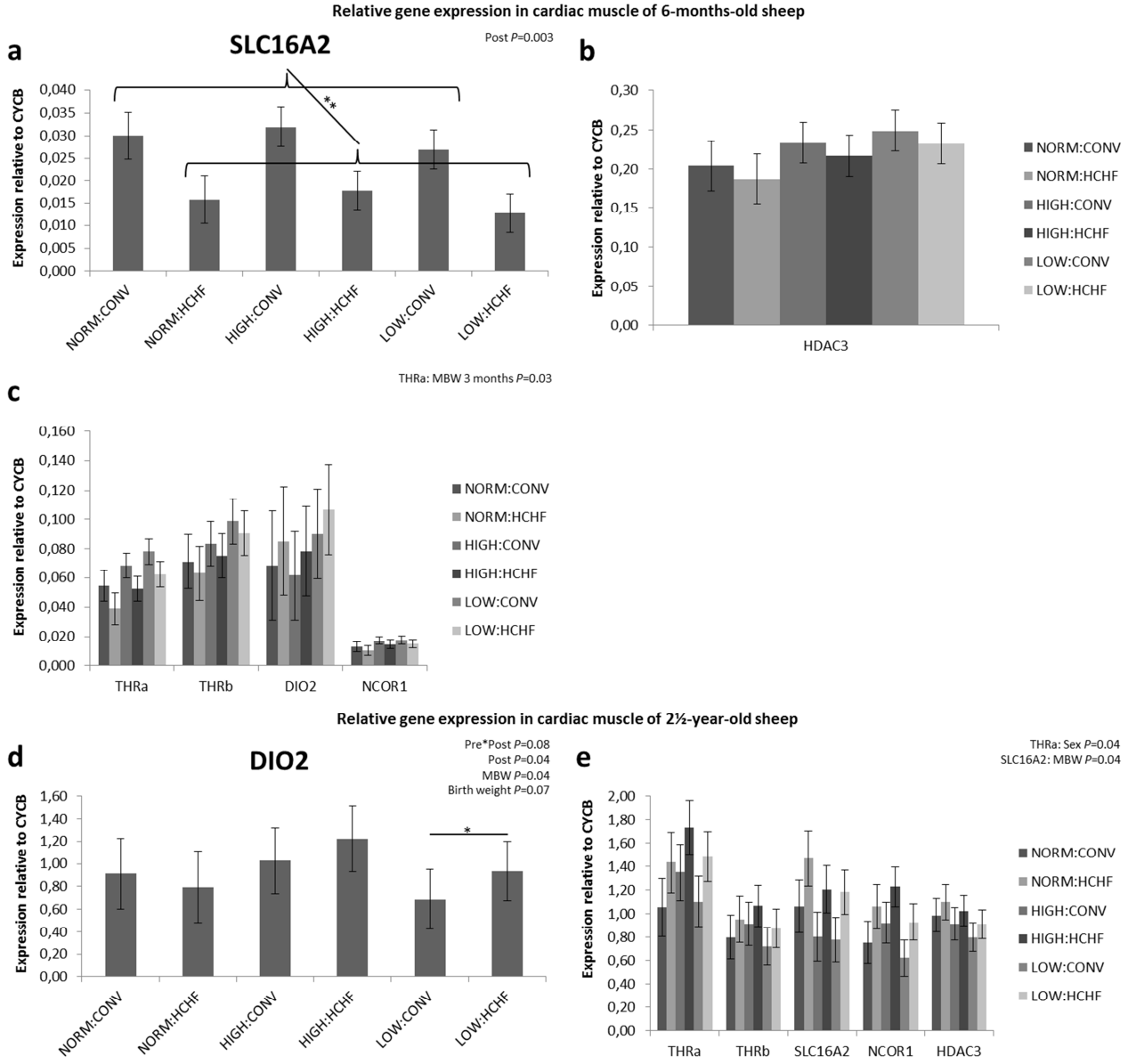


Figure 10 LSmeans±SEM of relative gene expression in cardiac muscle, for the genes *THRa*, *THRb*, *DIO2*, *SLC16A2*, *NCOR1* and *HDAC3* in 6-month-old lambs and 2½-year-old sheep, with a history of adequate nutrition (NORM), overnutrition (HIGH) or undernutrition (LOW) in late gestation, combined with a conventional (CONV) or a High-Carbohydrate-High-Fat (HCHF) diet for the first 6 postnatal months, yielding 6 experimental groups: NORM:CONV, NORM:HCHF, HIGH:CONV, HIGH:HCHF, LOW:CONV and LOW:HCHF. Experimental design and dietary treatments have been fully described in the legend to Figure 2. Pre: prenatal diet; Post: postnatal diet; MBW: metabolic body weight. *P≤0.05, **P≤0.01, or ***P≤0.001.

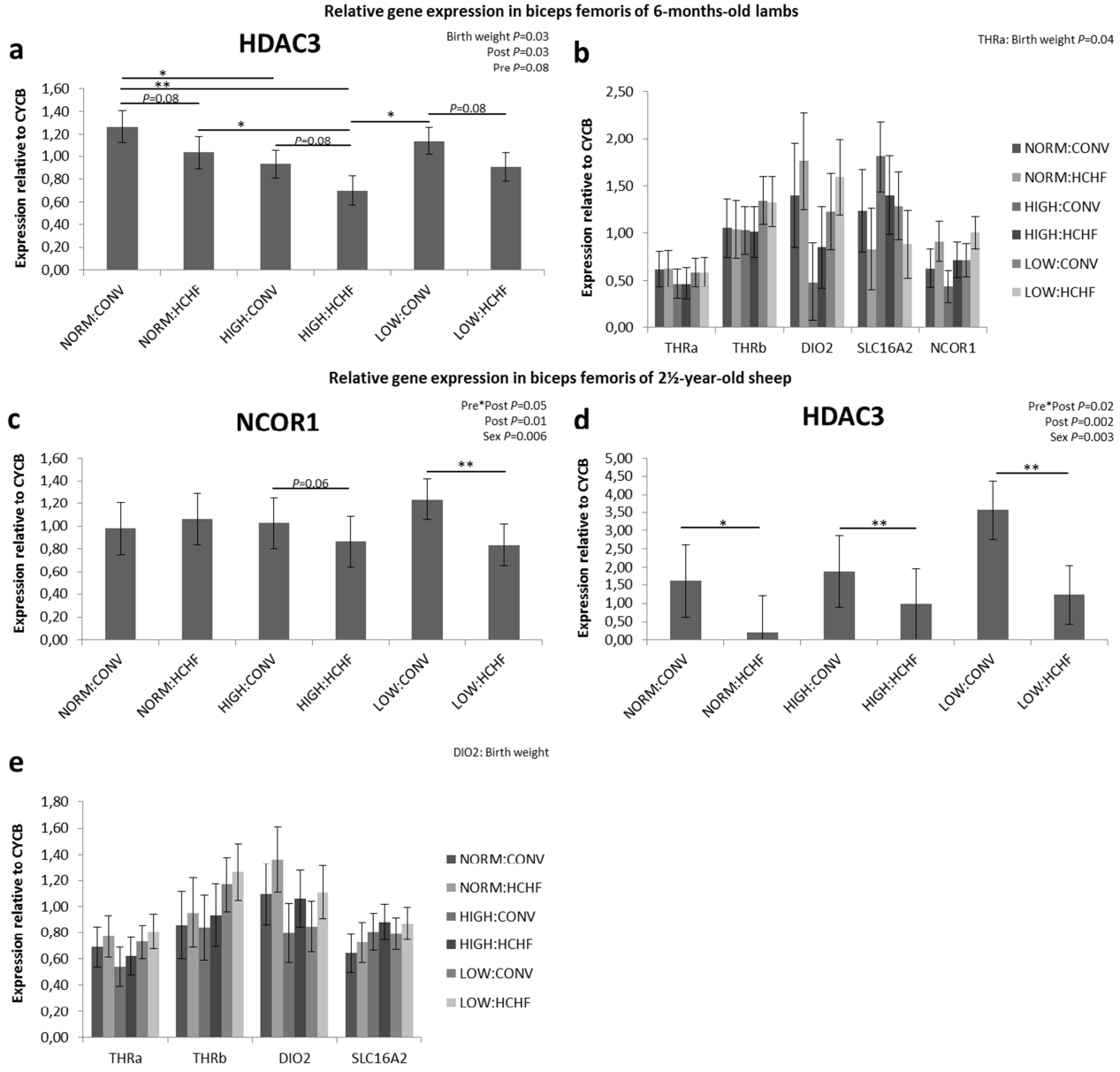


Figure 11 LSmeans±SEM of relative gene expression in biceps femoris muscle tissue, for the genes *THRa*, *THRb*, *DIO2*, *SLC16A2*, *NCOR1* and *HDAC3* in 6-month-old lambs and 2½-year-old sheep, with a history of adequate nutrition (NORM), overnutrition (HIGH) or undernutrition (LOW) in late gestation, combined with a conventional (CONV) or a High-Carbohydrate-High-Fat (HCHF) diet for the first 6 postnatal months, yielding 6 experimental groups: NORM:CONV, NORM:HCHF, HIGH:CONV, HIGH:HCHF, LOW:CONV and LOW:HCHF. Experimental design and dietary treatments have been fully described in the legend to Figure 2. Pre: prenatal diet; Post: postnatal diet; MBW: metabolic body weight. *P≤0.05, **P≤0.01, or ***P≤0.001.

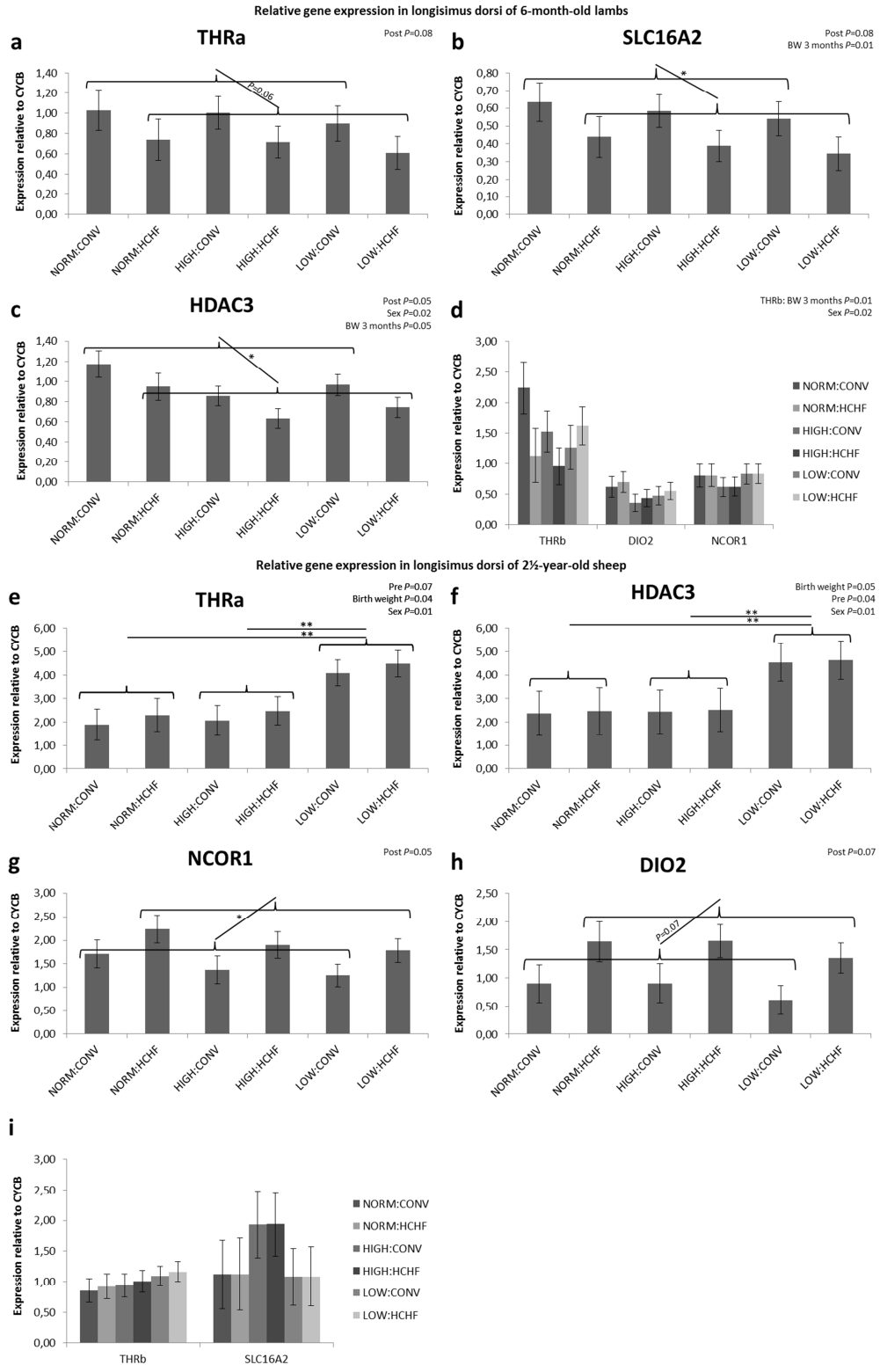


Figure 12 LSmeans±SEM of relative gene expression in longisimus dorsi muscle tissue, for the genes *THRa*, *THRb*, *DIO2*, *SLC16A2*, *NCOR1* and *HDAC3* in 6-month-old lambs and 2½-year-old sheep, with a history of adequate nutrition (NORM), overnutrition (HIGH) or undernutrition (LOW) in late gestation, combined with a conventional (CONV) or a High-Carbohydrate-High-Fat (HCHF) diet for the first 6 postnatal months, yielding 6 experimental groups: NORM:CONV, NORM:HCHF, HIGH:CONV, HIGH:HCHF, LOW:CONV and LOW:HCHF. Experimental design and dietary treatments have been fully described in the legend to Figure 2. Pre: prenatal diet; Post: postnatal diet; MBW: metabolic body weight. *P≤0.05, **P≤0.01, or ***P≤0.001.

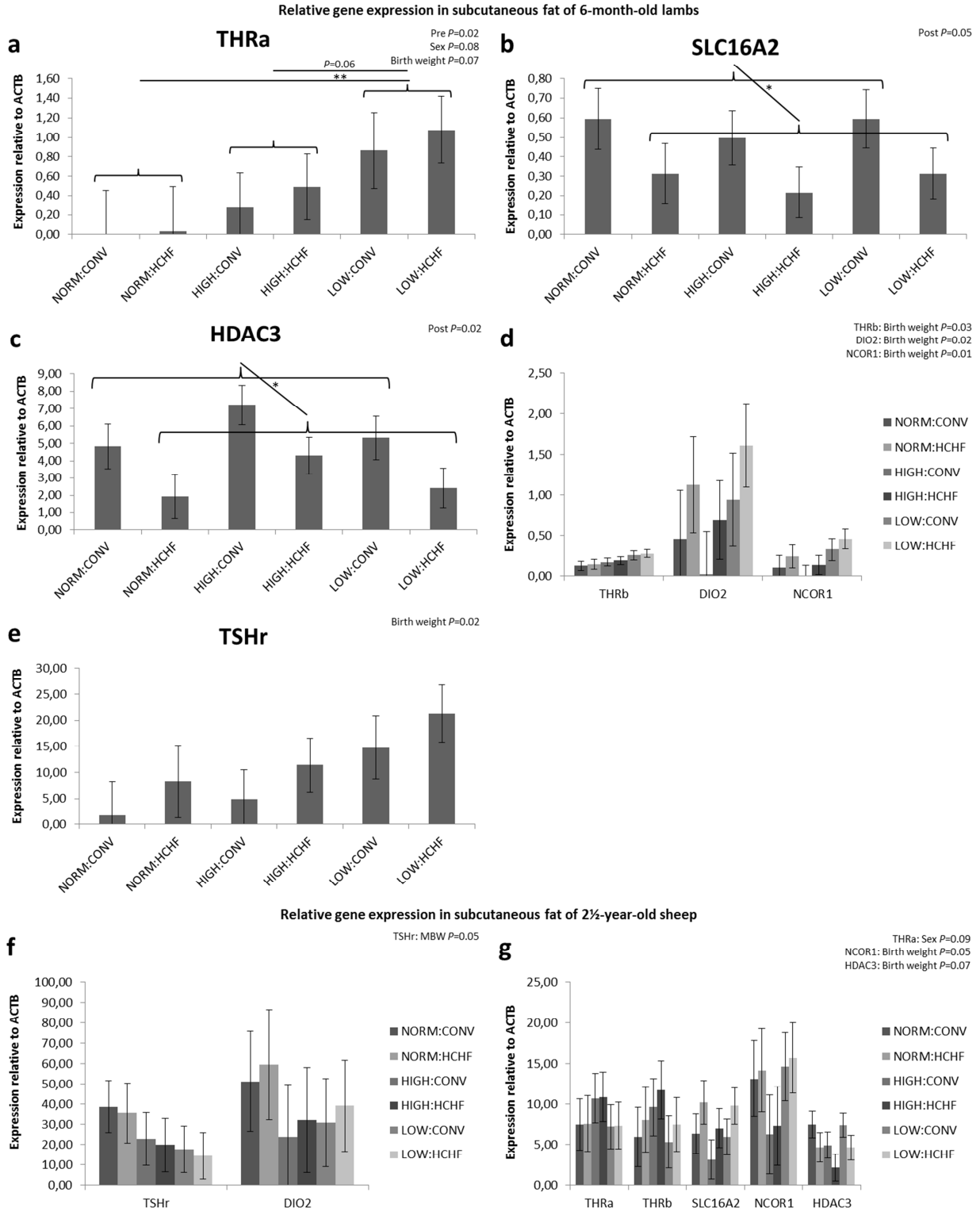


Figure 13 LSmeans±SEM of relative gene expression in subcutaneous fat, for the genes *THRa*, *THRb*, *TSHr*, *DIO2*, *SLC16A2*, *NCOR1* and *HDAC3* in 6-month-old lambs and 2½-year-old sheep, with a history of adequate nutrition (NORM), overnutrition (HIGH) or undernutrition (LOW) in late gestation, combined with a conventional (CONV) or a High-Carbohydrate-High-Fat (HCHF) diet for the first 6 postnatal months, yielding 6 experimental groups: NORM:CONV, NORM:HCHF, HIGH:CONV, HIGH:HCHF, LOW:CONV and LOW:HCHF. Experimental design and dietary treatments have been fully described in the legend to Figure 2. Pre: prenatal diet; Post: postnatal diet; MBW: metabolic body weight. *P≤0.05, **P≤0.01, or ***P≤0.001.

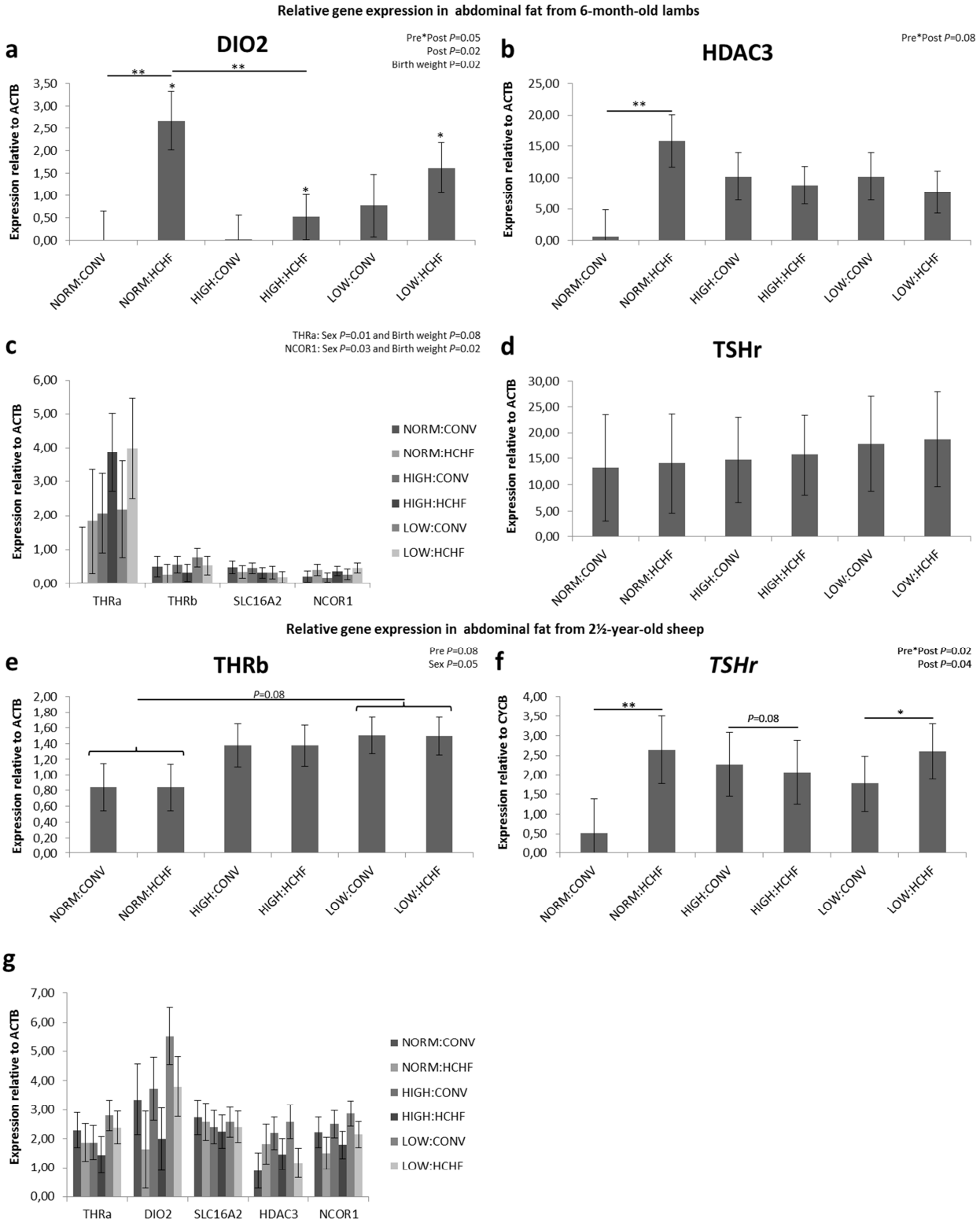


Figure 14 LSmeans±SEM of relative gene expression in abdominal fat, for the genes *THRa*, *THRb*, *TSHr*, *DIO2*, *SLC16A2*, *NCOR1* and *HDAC3* in 6-month-old lambs and 2½-year-old sheep, with a history of adequate nutrition (NORM), overnutrition (HIGH) or undernutrition (LOW) in late gestation, combined with a conventional (CONV) or a High-Carbohydrate-High-Fat (HCHF) diet for the first 6 postnatal months, yielding 6 experimental groups: NORM:CONV, NORM:HCHF, HIGH:CONV, HIGH:HCHF, LOW:CONV and LOW:HCHF. Experimental design and dietary treatments have been fully described in the legend to Figure 2. Pre: prenatal diet; Post: postnatal diet; MBW: metabolic body weight. *P≤0.05, **P≤0.01, or ***P≤0.001.

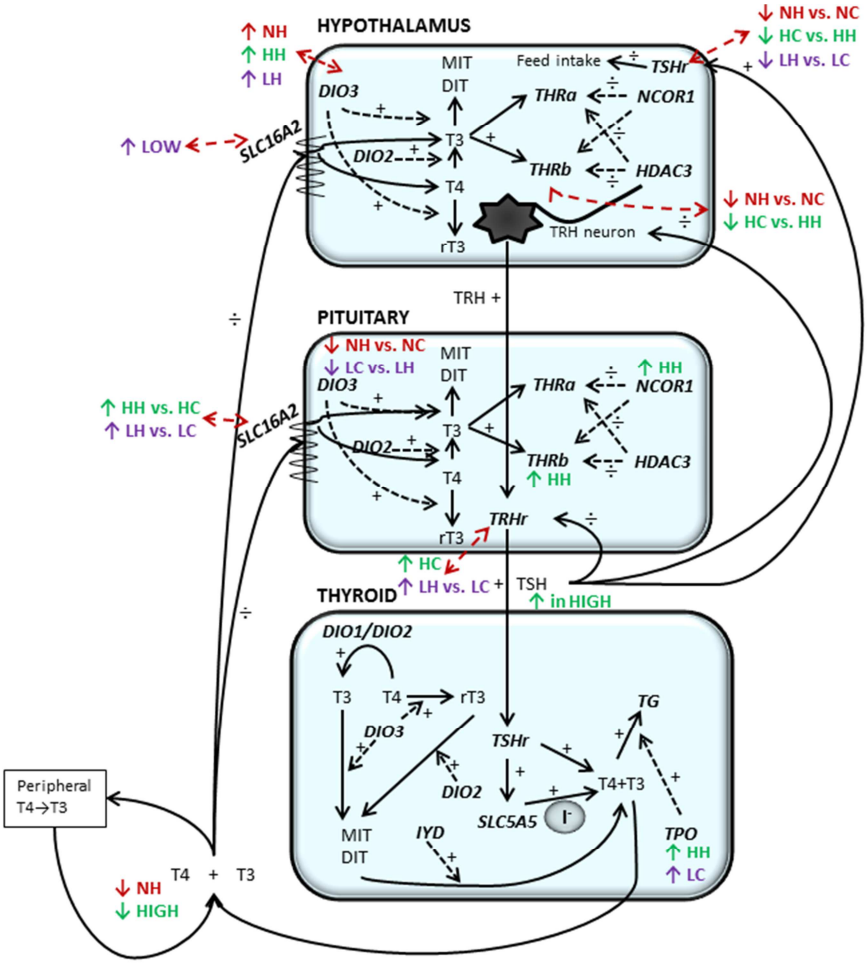


Figure 15 Summary of long-term effects of prenatal- undernutrition or overnutrition combined with conventional postnatal nutrition or early-life overnutrition on the HPT axis of 2½ year old sheep (male and female). Experimental design and dietary treatments have been fully described in the legend to Figure 2.Included effects are $P<0.1$

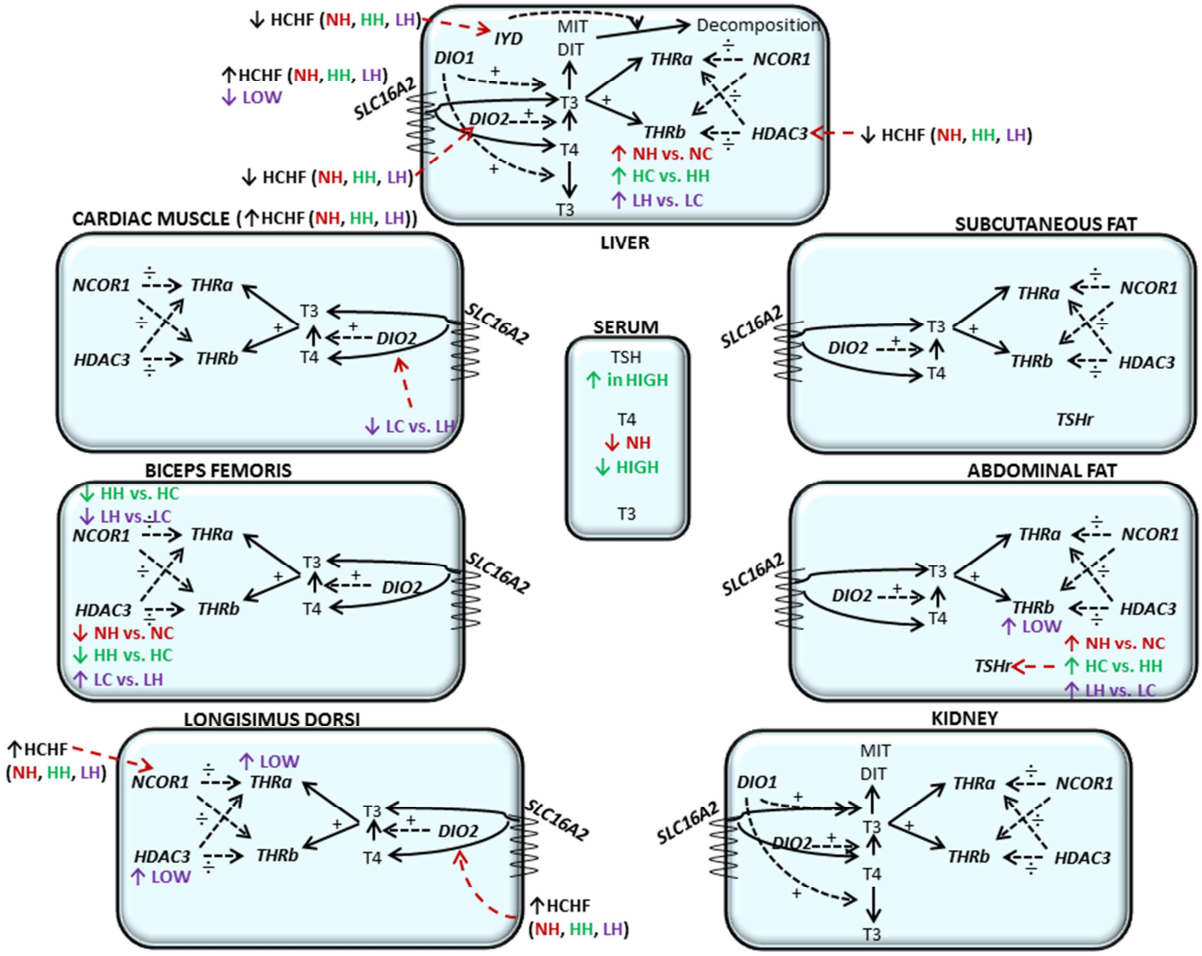
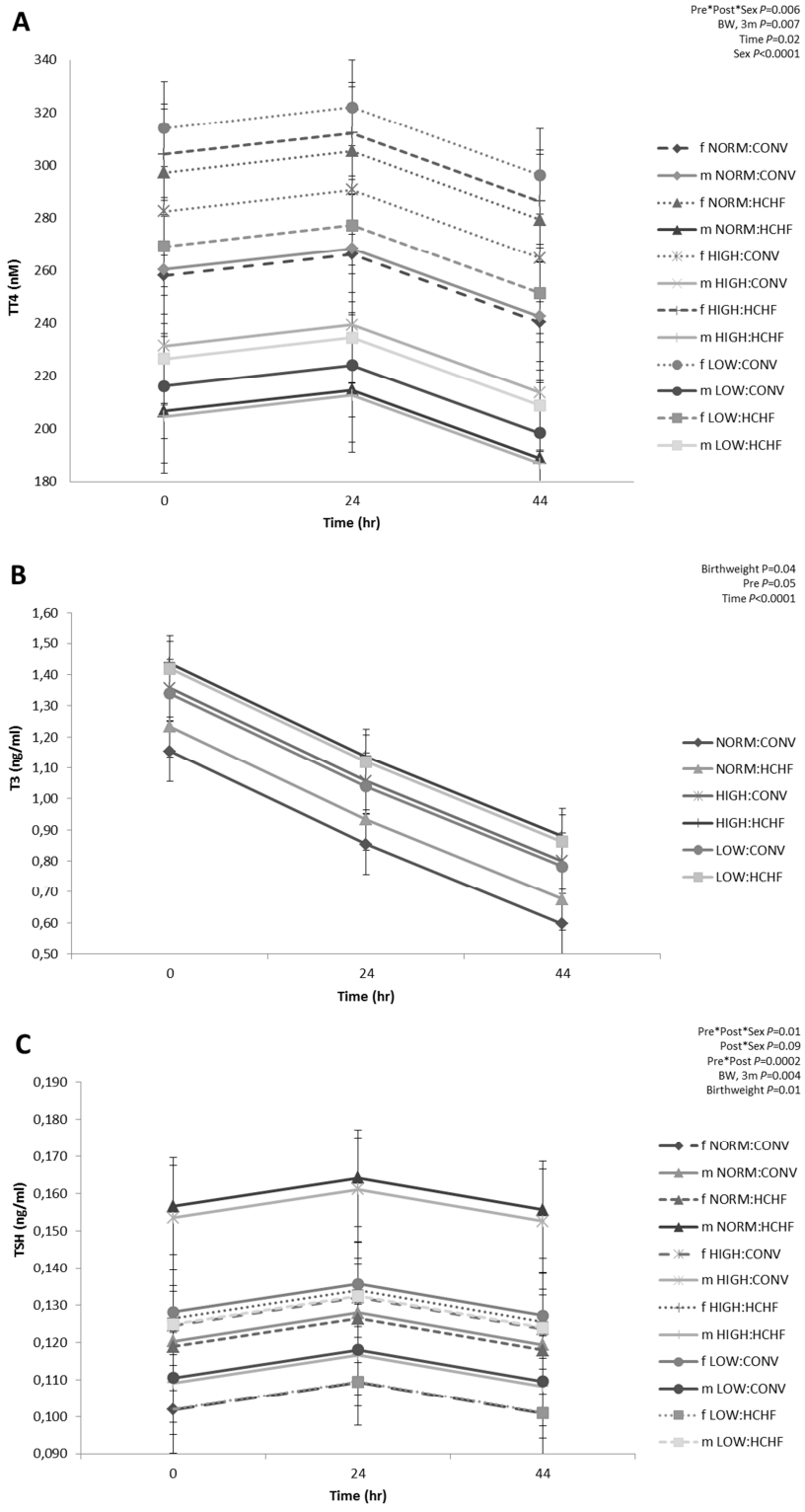


Figure 16 Summary of long-term effects of prenatal- undernutrition or overnutrition combined with conventional postnatal nutrition or early-life overnutrition on peripheral thyroid function of 2½ year old sheep (male and female). Experimental design and dietary treatments have been fully described in the legend to Figure 2.Included effects are P<0.1



Supplementary Figure 1 LSmeans±SEM of serum total T4 (nM), total T3 (ng/ml) and TSH (ng/ml) in 6-month-old lambs (male and female) with a history of adequate nutrition (NORM), overnutrition (HIGH) or undernutrition (LOW) in late gestation, combined with a conventional (CONV) or a High-Carbohydrate-High-Fat (HCHF) diet for the first 6 postnatal months, yielding 6 experimental groups: NORM:CONV, NORM:HCHF, HIGH:CONV, HIGH:HCHF, LOW:CONV and LOW:HCHF. Group differences have been illustrated in Figure 2 and experimental design and dietary treatments have been fully described in the legend to Figure 2. Pre: prenatal diet; Post: postnatal diet; MBW: metabolic body weight.