Norwegian University of Life Sciences
Faculty of Biosciences
Department of Animal and Aquacultural Sciences

Philosophiae Doctor (PhD)
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Rearing of Norwegian Red replacement heifers; Short and long term effects of dietary energy and protein levels on growth characteristics and adipose tissue gene expression

Oppdrett av NRF-kviger; kort- og langtids-effekter av ulikt energi- og proteininnhold i fôret på tilvekstkarakteristika og genekspresjon i fettvev

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List of papers

I. Salte, R., Storli, K. S., Wærp, H. K. L., Sommerseth, J. K., Prestløkken, E., Klemetsdal, G., 2018. Designing a replacement heifer rearing strategy: Effects of growth profile on performance of Norwegian Red heifers and cows. Journal of Dairy Science, Submitted.

II. Wærp, H. K. L., Waters, S., McCabe, M. S., Cormican, P., Salte, R., 2018. RNA-seq analysis of bovine adipose tissue in heifers fed diets differing in energy and protein content. PLoS ONE 13(9): e0201284. https://doi.org/10.1371/journal.pone.0201284

III. Wærp, H. K. L., Waters, S., McCabe, M. S., Cormican, P., Salte, R., 2018. Long-term effects of former diet, feed transition and pregnancy on adipose gene expression in dairy heifers. Plos ONE, submitted.

Abstract

This thesis is part of a larger research project aimed at assessing the impact of calf and youngstock development on dairy cow production and profitability. The potential impact of heifer growth on future milk yield has been known for many decades, but the mechanisms mediating such effects are still not fully elucidated. Over the last 15-20 years, an important role of adipose tissue in the regulation of whole-body metabolism and energy allocation has become clear. However, knowledge about this connection in growing cattle is incomplete. Thus, the main objective of this thesis was to investigate how different feeding during rearing affects growth profiles, and how these two also affect adipose tissue gene expression in Norwegian Red replacement heifers.

This was achieved by performing a controlled feeding experiment in which Norwegian Red heifers were subjected to different levels of dietary energy and protein from three months of age to confirmed pregnancy. Energy and protein supplies were regulated by roughage quality and amount, and an additional difference in protein supply was achieved by the use of two experimental concentrates differing in protein content. After confirmed pregnancy, all heifers were fed the same, roughage-based diet. The experimental feeding lead to different growth profiles as assessed by body weights, body condition scores and withers heights. Subcutaneous adipose tissue biopsies were harvested from the experimental heifers at 12 months of age and at month seven of pregnancy. RNA was extracted from these biopsies and subjected to next-generation RNA sequencing to assess adipose gene expression differences between treatment groups as well as within-treatment changes occurring between 12 months of age and at month seven of pregnancy

Our results showed that Norwegian Red heifers can obtain a rapid pre-conception growth of >900 g/ day on roughage-based rations. When combined with low-energy feeding and a moderate growth post-conception, they may calve in at an adequate body condition score and body weight at 22 months of age without negative consequences for total milk yield over three lactations. Growth characteristics and adipose gene expression were strongly affected by dietary energy level, while dietary protein above current recommendations had minor effects. However, total ration characteristics was the most important determinant of overall outcome.

The nutritionally induced transcriptomic changes observed in this study seem to be reversible to a large extent. Nonetheless, we found that growth rates, BCS development and adipose gene expression were all affected by the experimental diets even more than 6 months after their termination. Two immune-related genes, *ENSBTAG00000007075* and *ENSBTAG00000048049* maintained the same pattern of differential expression from the experimental feeding period to seven months into pregnancy and may suggest that some kind of developmental programming has occurred in our heifers. Gene expression changes which seemed to have occurred from the experimental feeding period to month seven of pregnancy for all heifers, and thus might be induced by pregnancy, were dominated by genes and pathways related to inflammatory and immunologic functions.

We identified Transforming growth factor 1 (TGFB1) as a key regulator and *CFD*, the gene coding for adipsin, as candidates for further studies on pregnancy function and preservation. The genes coding for uncoupling protein 2 (*UCP2*), superoxide dismutase (*SOD*) and catalase (*CAT*) may act as indicators of nutritional status and oxidative stress, while PI3K, RICTOR and MAP4K seem to be key regulators of gene expression pertaining to metabolic function.

This is the first study to examine the total transcriptome of adipose tissue in the Norwegian Red. The results presented in this thesis are of both practical and theoretical relevance as they identify important focus points for dairy farmers when planning their management strategies, give further insights to adipose tissue metabolic regulation through gene expression, and provides candidate genes and systems for further studies to improve the performance of the Norwegian Red breed.

Sammendrag

Denne avhandlingen er en del av et større prosjekt, «the impact of calf and youngstock development on dairy cow production and profitability». Det overordnede målet for prosjektet var å skaffe ny kunnskap om fôringsstrategier for rekrutteringskviger til melkeproduksjon, utviklet med tanke på høy ytelse og lønnsomhet. At en kviges fremtidige ytelse kan påvirkes av tilveksten under oppdrettsperioden, har vært kjent i mange tiår, men mekanismene som ligger bak er i stor grad ukjent. I løpet av de siste 15-20 årene har fettvevets betydning for kroppens energiallokering og metabolske regulering blitt stadig tydeligere, men hos storfe har kunnskap om denne forbindelsen vært mangelfull. Hovedmålet med denne avhandlingen har derfor vært å belyse hvordan ulik fôring i oppdrettet påvirker tilvekstprofiler og hvordan disse to igjen påvirker genuttrykket i fettvev hos kviger av Norsk Rødt Fe. Dette ble gjort gjennom et kontrollert fôringsforsøk, hvor NRF-kviger fikk tildelt fôr med ulikt innhold av energi og protein fra tre måneders alder til bekreftet drektighet. Energi- og proteintilgang ble styrt via grovfôret, og en ytterligere forskjell i protein ble oppnådd ved bruk av to forsøkskraftfôr med ulikt proteininnhold. Etter bekreftet drektighet ble alle kviger tildelt det samme fôret. Forsøksfôringen ga ulike tilvekstprofiler, som ble registrert via kroppsvekt, hold og mankehøyde. Subkutane fettvevsbiopsier ble tatt ut fra kvigene ved 12 måneders alder og ved syv måneders drektighet. RNA ble ekstrahert fra biopsiene og sekvensert via neste-generasjons RNA-sekvensering for å vurdere forskjellene i genekspresjon mellom de ulike fôringsgruppene, samt utviklingen innad i hver gruppe mellom 12 måneders alder og syv måneders drektighet.

Våre resultater viste at NRF-kviger er i stand til å nå en tilvekst på >900 g/ dag på grovfôrbaserte rasjoner. Når kombinert med lavt energiinnhold i fôret og derved moderat tilvekst i drektigheten, kunne de kalve inn ved 22 måneders alder uten at dette gikk ut over total melkeytelse over 3 laktasjoner. Tilvekst og genekspresjon i fettvev ble sterkt påvirket av energitilførsel, mens proteintilførsel utover gjeldende angitt minimumsbehov kun hadde små effekter. Likevel var det rasjonens totale sammensetning som hadde mest å si for det totale resultatet.

De ernæringsrelaterte forskjellene i genuttrykk som ble observert i denne studien, ser i stor grad ut til å være reversible. Ikke desto mindre var både tilvekst, hold og

genuttrykk i fettvev fremdeles påvirket av forsøksfôringen mer enn seks måneder etter dens avslutning. To immunrelaterte gener, *ENSBTAG00000007075* and *ENSBTAG00000048049,* beholdt samme, ulike genekspresjonsmønster mellom forsøksgrupper fra 12 måneders alder til syvende drektighetsmåned. Dette kan antyde at en form for utviklingsmessig programmering har funnet sted. Endringer i genuttrykk mellom 12 måneders alder og syv måneders drektighet, som var felles for alle forsøksgrupper var trolig indusert av drektigheten. Disse endringene var dominert av gener og pathways relatert til inflammatoriske og immunologiske funksjoner.

Vi identifiserte TGFB1 som en nøkkel-regulator og *CFD,* genet som koder for adipsin, som kandidater for videre studier på endringer i og opprettholdelse av drektighetsfunksjoner. Genene som koder for uncuopling protein 2 (*UCP2*), superoxid dismutase (*SOD*) og katalase (*CAT*) kan ha potensielle roller som indikatorer for ernæringsstatus og oksidativt stress. PI3K, RICTOR og MAP4K ble funnet å være blant de mest sentrale regulatorene av genekspresjon relatert til metabolisme.

Dette er den første studien som undersøker hele fettvevstranskriptomet hos NRF. Resultatene som presenteres i denne avhandlingen kan være av både praktisk og teoretisk relevans. ettersom de har identifisert viktige fokuspunkter for melkebønders oppdrettsstrategier, har utvidet vår innsikt i metabolsk regulering i fettvev via genekspresjon, og har identifisert gener og systemer som er potensielle kandidater for videre studier på Norsk Rødt fe.

Abbreviations

AAT	-	amino acids available for absorption from the small intestine
ADG	-	Average daily gain
BW	-	Body weight
BCS	-	Body condition score
CP	-	Crude Protein
DEG	-	Differentially expressed gene(s)
DM(I)	-	Dry matter (intake)
ECM	-	Energy-corrected milk
NEG	-	Net energy for growth
PBV	-	Protein balance in rumen
RNAseq	-	Next generation RNA sequencing
WH	-	Withers height

1. Background

1.1 Background for main project, “The heifer project”

Dairy production is one of the largest animal productions in Norway. Still, the number of dairy cows and dairy herds is steadily decreasing, while the number of cows per herd and milk yield per cow is increasing. In 2017, the number of dairy herds in Norway was 7918 and there were on average 26.7 cows per herd, while average milk yield (energy-corrected milk, ECM) per cow was 8116 kg (1). Thus, the Norwegian dairy farms are getting bigger and fewer, and today approximately 20 % of Norwegian farms are dairy farms. The trends towards bigger herds and higher yields are brought about by an increased demand for efficiency. Today’s dairy farmer needs to maximize milk yield and optimize management and feeding in order to maintain profitability. To this end, there is a need for evidence-based management strategies.

This thesis is part of a larger research project, “The impact of calf and youngstock development on dairy cow health, production and profitability.” The project was divided into three work packages. Work package one investigated the impact of immune status, feeding and management of pre-weaning calves on calf diarrhea and growth. Work package two focused on the effects of pre- and postpubertal growth rates and nutrition on development and subsequent milk production. Work package three focused on economic analyses and modelling of rearing strategies to determine the most profitable way to rear Norwegian Red heifers when lifetime production is taken into account.

This thesis is a part of work package two, which focused on nutrition and growth profiles during pre- and postpubertal rearing and effects on milk production. The main hypotheses for this work package were:

1. When feeding a high energy level to calves from birth to three months of age a high protein supply will stimulate lean tissue growth and prevent fattening.
2. Rearing heifers for a rapid rate of body weight gain with a sufficient supply of metabolizable protein in the prepubertal period will have no negative effects on mammary gland development and subsequent lactational performance

3. Heifers fed for a rapid weight gain (>850 g/day) before insemination and a moderate gain (500-550 g/ day) during pregnancy, will obtain an optimal body condition score at calving (3.5-3.75) without negative effects on subsequent lactational performance.

1.2. Background for the thesis

Replacement heifer rearing is one of the major costs of the dairy farmer, in Norway representing approximately 30% of the running costs (2). Therefore, it is of paramount importance to farm profits to optimize the rearing strategy from birth to first calving in order to maximize returns on investments.

Norwegian recommendations for replacement heifer rearing has been based on heifer studies performed abroad, many of which were performed 20-30 years ago. Based on these studies it was stated that too high prepubertal growth rates were detrimental to mammary development and that there seemed to exist an optimum prepubertal growth rate at which milk yield in first lactation was maximized (3). Genetic potential for milk yield and growth are positively correlated (3). Therefore, this optimum growth rate is expected to change as breeding for higher milk yields will increase the genetic growth potential as well. However, no growth and production studies of this type have ever been performed on the Norwegian Red breed, and several generations of genetic progress has passed since the execution of the studies on which today's growth recommendations are based. Thus, we do not know much about the optimum growth curves of today's Norwegian Red heifers. Furthermore, the optimum growth curves for maximum milk yield is not necessarily the optimum growth curves for maximum profit. Therefore, the overall primary objective of the project was to obtain new knowledge on feeding strategies in dairy calves and heifers designed to achieve high production efficiency and profitability under Norwegian conditions.

Norwegian heifers are getting fatter each year, and in 2017, more than 70 % of heifer carcasses were devalued because of excessive fatness (4). The negative effects of over-conditioning at calving on health, fertility and production are well documented (5, 6).

Less is known about the long-term effects of over-conditioning during heifer development. However, associations has been shown both between fatness and nutritionally induced high growth rates (7), and between high prepubertal growth rates and reduced milk yield (8). Therefore, it has been suggested that any negative effect of high growth rates on milk yield is a result of general over-conditioning (9). This theory is supported by results stating that milk yield and growth capacity have a positive genetic correlation, and that only nutritionally induced high growth rates will negatively affect milk yield (3). Further, a study by Silva et al. showed that when given the same diet, milk yield of heifers was only affected by BCS at breeding and not by growth rate (10).

Another theory suggests that nutritionally induced fatness during growth may partly be caused by an inadequate level of protein in the diet. A suggested means to this end has been to ensure a sufficient supply of metabolizable protein in the diet as it has been found that when provided sufficient amounts of metabolizable protein a prepubertal growth rate of 800-900 g/d caused no negative effects (9).

As evident from the preceding paragraphs, degree of heifer fatness may have consequences both for feed efficiency during rearing and for later production performance. Although many heifer rearing studies over the years have focused on the effects of plane of nutrition and growth on future milk yield, very few heifer rearing studies have focused mainly on adiposity during rearing as a potential explanatory variable for later performance. With today's increased knowledge about the importance of adipose tissue in regulation of whole-body metabolism, an understanding of the effects of adiposity and the underlying regulatory mechanisms seems more important than ever. This is especially true in ruminants, in which adipose tissue is the main site for both lipid synthesis, storage and mobilization (in monogastrics the liver is the main organ for lipogenesis) (11). The emergence of high-throughput methods such as next-generation RNA sequencing (RNAseq), makes it possible to investigate tissue metabolism on a global scale, and may provide biomarkers for further studies on feed utilization, health and optimal growth.

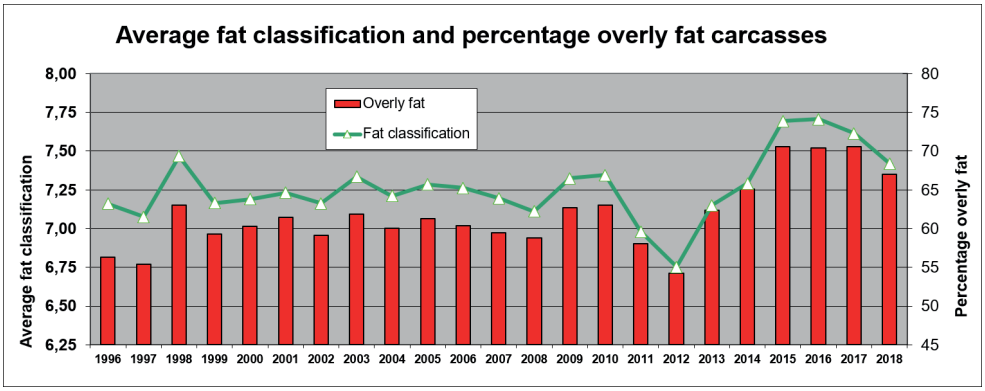

Figure 1. Average carcass fat classification for Norwegian Red heifers per year (Green line). Red bars indicate percentage of overly fat carcasses per year, showing how, with the exception of 2012, 8 of the last 10 years are the years with the highest degree of fatness in heifer carcasses. The decline in 2018, is probably due to severe draught and poor grass yields during summer 2018, leading to increased and premature slaughtering of heifers in many herds. From https://www.animalia.no/no/kjott--egg/klassifisering/klassifisering-av-storfe/, accessed oct 28, 2018.

All results presented in this thesis have been produced through one large station experiment, performed at the Animal Production Experimental Centre at the Norwegian University of Life Sciences: Eighty Norwegian Red heifers from the university herd were randomly assigned either to a high (HE) or low (LE) energy group, destined for a BW gain of 850 – 950 or 600 – 750 g/day from three months of age to confirmed pregnancy, respectively. Each of the energy groups were split into two protein groups, low (LP) or high (HP), to give four dietary treatment groups with 20 animals in each group, *viz* Low-protein high-energy (LPHE), high-protein high-energy (HPHE), low-protein low-energy (LPLE) and high-protein low-energy (HPLE). Target body weight at conception was 400 kg for all heifers. Thus, heifers on HE treatments were inseminated at an earlier age than LE heifers, because of their faster growth. After confirmed pregnancy (at day 28-42 of gestation), all heifers were group-fed the same low-energy, low protein diet *ad libitum.* The pregnancy diet was designed for a moderate growth of about 550 g/ d until calving to reach a target BW of 560 kg

and a maximum BCS of 3.75 at calving. Body weight, body condition score and wither height were registered through the rearing period. First-lactation cows should be fed an equal diet according to a standard lactation curve aiming for a 305 day yield of 7,500 kg ECM. In the second and third lactation, they should all be managed together with the rest of the university herd without any restrictions or claims other than the monitoring of milk yield and quality. Results from this experiment were used to partly fulfill the objectives of both WP2 and WP3, in terms of effects of growth responses to different feeding on subsequent milk production and profitability, as described by Kristin S. Storli and Jon Kristian Sommerseth as well as in this thesis (12, 13).

For the purpose of this thesis, and within the previously described station experiment, body weights, body condition scores and withers heights were recorded frequently throughout the study to assess body growth, conformation and composition. Additionally, subcutaneous adipose tissue biopsies from the tail base were harvested from animals at 12 months of age and at month 7 of pregnancy (20-24 months of age depending on treatment). RNA sequencing of the total adipose transcriptome was conducted to identify gene expression differences between the experimental groups at 12 months of age, between experimental groups at month 7 of pregnancy and between 12 months of age and month 7 of pregnancy within each group. Differentially expressed genes were analyzed through a pathway analyzer (Ingenuity pathway analyzer, IPA) to identify the most affected metabolic pathways, signal systems and active regulators represented by the differentially expressed genes.

2. Hypotheses and objectives of the thesis

The main objective of this thesis has been to investigate how different feeding during rearing affects growth profiles and adiposity and how these two also affect adipose tissue gene expression in Norwegian Red heifers.

The following hypotheses were stated for this PhD study:

1. Given a sufficient supply of metabolizable protein in the diet (120 % of current recommendations), a high growth rate (800-950 g/d) will not lead to unwanted fatness in growing heifers.
2. Heifers fed for a rapid growth rate before pregnancy and a moderate growth rate (~550 g/d) during pregnancy, will obtain an optimal body condition score (BCS ≤ 3.75) at calving.
3. a) Different levels of protein and energy in the diet will lead to differential expression of genes associated with energy metabolism in adipose tissue.

   b) Some of these differences will persist after the end of experimental feeding, indicating a genetic programming which may affect the heifers in adulthood.

Based on the hypotheses, the following objectives were defined:

- Investigate the effect of feeding regimes differing in protein and energy content on the growth profiles of Norwegian Red heifers.
- Examine whether different feeding regimes cause differences in the adipose transcriptome of the heifers, both during and after the experimental feeding period.
- Identify possible links between transcriptomic differences and dietary differences.
- Identify candidate genes/ biomarkers for further studies to improve efficiency, productivity and health in the Norwegian Red.

As implicated by the title, hypotheses and objectives, feeding, growth characteristics and gene expression in adipose tissue were the original focus points

of this PhD work. Paper I was originally a manuscript focusing on nutrition and growth responses, being the first of two companion papers where the second part would focus on production performance. Over the course of time, a decision was made to merge these manuscripts into one, thereby omitting some results from the rearing period. Thus, growth and nutrition results are partly presented in Paper I and partly in the Supplementary results section at the end of the thesis.

3. Theoretical introduction

3.1 A brief general overview of dairy replacement heifer research

The interest for heifer nutrition and growth started already in the 1940's, when Herman and Ragsdale performed a study on nutritional effects on heifer growth (14). They found that a rapid growth rate during rearing caused a decrease in milk yield for the three first lactations. Their results were supported by those of Swanson in 1960 (15), and in 1969, Sinha and Tucker found that mammary tissue grew at a faster rate than other body tissues during the prepubertal period from about two to nine months of age (16). This brought about an era of studies on heifer growth and subsequent milk yield. A large fraction of the performed studies were focused on pre-pubertal mammary development and for the most part, researchers seemed to find associations between rapid prepubertal growth, reduced mammary development and reduced milk yield in first lactation (3, 10, 16-34). Although the results were somewhat contradictive, the overall conclusion was that too high growth rates in the critical prepubertal phase must be avoided, a doctrine which lingered well into the new millennium. More recent studies have shed new light on the dogma of "the critical period" in heifer rearing and at least partly explains the discrepancies in resultant milk yields from studies on different prepubertal growth rates (27, 28). Feeding strategies and growth rates during different rearing phases seem to have different effects on growth and future milk yield. Therefore, the following sections will describe shortly the effects of different feeding strategies during different rearing phases.

3.1.1. Calf period

From birth and until about three months of age, there seem to be an overall positive effect of a high plane of nutrition. The amount of high-quality (i.e. > 50 g IgG/ l) colostrum fed during the first 24 hours after birth, has long been recognized as crucial to calf health and resistance towards infectious diseases (35, 36). However, a high colostrum intake also has a positive effect on first lactation milk yield (37, 38). In a study by Faber et al., calves fed four liters instead of two liters of colostrum during

their first meal displayed faster growth and lower veterinary costs during rearing, as well as a 550 kg increase in energy-corrected milk (ECM) yield during first lactation (38).

Over the years, the economics of feeding whole milk to calves have been questioned, as milk constitutes the main income of a dairy farm. As a result, many farms still practice restricted milk or milk replacer feeding supplemented by liberal amounts of concentrate. When given a total amount of milk and concentrates equaling *ad lib* feeding of one of them (the calf leaves remains of either milk or concentrates), the ratio between milk and concentrate in the ration has little effect on overall growth rate through the first months of life (39). However, if one also looks at later results on health and milk yield, liberal milk feeding, and preferably whole milk rather than milk replacer, in the calf period has positive effects. Soberon (2012) found that by increasing milk replacer intake in pre-weaning heifer calves, there was an effect of +100 kg milk in first lactation per 100 g increase in daily gain in the pre-weaning phase (40). Rincker (2011) also found an increased first lactation milk yield in heifers fed increased amounts of energy and protein from both milk replacer and concentrate during the first 42 days of life (41). Moallem et al. (2010) found that giving whole milk instead of milk replacer increased first lactation yields by 10% (42). Collectively, these results indicate that some extra hours and litres of colostrum and milk spent on young calves is an investment with high return.

Although an effect of early nutrition on subsequent production seems evident, the underlying mechanisms or proximate causes of this effect are not known. One possibility is simply that sufficient colostrum and nutrients early in life gives the heifer a head start with good health and immunity to prevent from diseases and a rapid growth which brings on a positive cycle of a large body size and increased feed intake capacity relative to age at later stages. However, besides immunoglobulins, colostrum (and to a small degree whole milk) is also rich in growth factors such as insulin-like growth factor 1 (IGF1), insulin and transforming growth factor β (TGFβ) which are shown to improve the growth of the gastrointestinal tract (43, 44). Several other bioactive compounds have been detected in colostrum, but their specific effects are as yet unknown. Based on piglet studies, Bartol et al. have proposed “the lactocrine hypothesis”, stating that bioactive components in colostrum and milk play an

important role in postnatal programming of reproductive tissue development. (45, 46). We already know that mammary development is also dependent on reproductive hormones and growth factors, and especially on IGF1 (17-19). Thus, it is logical to assume that IGF1 and other signal molecules in milk may affect the development of mammary tissue as well as the reproductive tract.

The general positive effect of a high plane of nutrition during the calf period, regardless of energy source, might also be mediated by endocrine mechanisms. The hypothalamic-pituitary-gonadal axis has been studied for decades for its involvement in body development and onset of puberty (47, 48). The endocrine activity of this axis is affected by feed intake and nutritional status, mediated through changes in circulating leptin, IGF1, insulin, adiponectin, kisspeptin and other hormones (49, 50). Lately, hormones pertaining to this signaling pathway has been shown to have an increased gene expression in the adipose tissue of young Holstein calves fed a high-energy diet (51). Therefore, it is hypothesized that nutrition at very young ages may strongly affect the reproductive programming and development of cattle.

3.1.2. Prepubertal period

A negative impact of high growth rates in the prepubertal period (about 3-9 months of age) on the development of mammary parenchyma has been postulated in a series of studies (16, 29, 34, 52, 53). However, Daniels et al. (2009, (21)) and Meyer et al. (2006, (27, 28)) showed that prepubertal development of mammary parenchyma was not affected by plane of nutrition directly, but seemed to be a function of time: As puberty is induced by body size rather than age in heifers, a high prepubertal growth rate leaves less time for rapid allometric growth of the mammary tissue before it is slowed down at onset of puberty (54). This makes fast-growing heifers enter pregnancy with a smaller and less cell-rich mammary parenchyma than slower-growing and older ones. Size and cell numbers of the mammary gland is shown to affect the milk yield potential of an animal, and therefore a decreased number of mammary cells at puberty may cause a decreased milk yield later on (55, 56). However, other factors may have just as important effects on actual milk yield. The udder also has a well-developed ability to make new milk producing cells during the last part of gestation and the first part of

lactation (56, 57). Furthermore, milk production is not dependent on mammary cell numbers alone, but also on the activity of these cells and a well-regulated nutrient allocation (55, 58). An increase in mammary cell numbers caused by feeding and appropriate growth rate in the prepubertal phase is therefore likely to have a positive effect on later milk production capacity, but this effect may be overruled by other factors. Furthermore, in dairy breeds milk yield is a heavily weighted breeding goal. In contrast to nutritionally induced high growth rates, genetically induced high growth rates are positively correlated to high milk yield (3). This is probably because large animals have a high feed intake capacity, which is a driver for milk yield, and they will also inherently display higher growth rates than smaller animals in order to reach maturity at the same age. Therefore, unless corrected for within the breeding goals, all dairy breeds will eventually drift towards faster growth and larger animals along with the desire for higher milk yields. This effect may also affect the optimum growth rate of heifers, which will increase for every generation. Therefore, the results of early investigations may be hard to reproduce and easy to reject if attempting to perform the exact same studies and growth rates on heifers of today.

3.1.3. Postpubertal period and pregnancy

During gestation, the mammary tissue once again enters a period of allometric growth. However, growth and plane of nutrition seem to have no effect on the growth of mammary tissue during this phase (59, 60), and thus any growth effects during this period on performance during first lactation is probably caused by other factors. Pregnancy is the last growth phase before calving, and it is likely that main effects of growth rate in this period are mediated directly through body weight and BCS at calving, both known to have an effect on lactation performance (61-64). Lacasse et al. reported that a high gestational growth rate brought about by *ad lib* feeding had no effect on milk yield, but a negative effect on health and reproduction (60). On the other side, Macdonald et al. found a positive effect of a moderately high postpubertal growth on first lactation milk yield (65). To try and explain these contrasting results one may look into the details of the studies: Lacasse et al. fed the heifers on a postpubertal high plane of nutrition to an ADG of 840-950 g/ day until 14 days pre-calving, and they

were also the ones calving in at the highest BCS scores (about 4.1 on a 1-5 scale). Macdonald et al. fed their high-energy heifers to an ADG of about 600 g/ day and terminated the high-energy feeding 2 months before calving. At the termination of experimental feeding, the Holstein heifers on high and low plane of nutrition in the Macdonald study only scored around 3 BCS points at a 1-10 scale, and thus all were leaner than their counterparts in the Lacasse study. In both studies, heifers fed a high plane of nutrition calved in at body weights 40-60 kg above those fed more restrictively. Thus, the main differences were absolute growth rate and BCS at calving, and the two studies reported different outcomes on lactation performance. This example goes to show how the effect of growth rates is probably not a question of "high" or "low", but of optimums and what they lead to in terms of body weight and body composition.

3.2. Dietary energy and protein

Energy intake is the main driver for growth (66). A high energy intake leads to a high daily gain, and therefore a higher body weight (BW) at a younger age. However, the composition of deposited tissue and thus the resultant body composition will differ depending on both the amount and the sources of dietary energy available to the heifer (7, 67, 68). Heifers with a high energy intake will deposit fat at an earlier age and to a greater degree than less well-fed counterparts, and will thus present at an increased body fat content at any given body weight (69). This effect may be modulated by dietary protein content (52). Fat and carbohydrates yield energy available for the energy-demanding reactions of maintenance, anabolism and activity, and any surplus of these feed constituents will be deposited as glycogen and fat. However, the metabolic machinery of mammals is only able to make some of the 20 amino acids (AA) needed for protein synthesis from these sources, and only in the presence of a nitrogen (N) source. They are therefore dependent on dietary sources of essential amino acids in order to deposit lean tissue. Ruminants are special in this respect, as they are not dependent on a diet containing amino acids, but can do well and even maintain a moderate production level on an amino acid-free diet with urea and ammonium salts as the only N sources (70). This is because of the capacity of rumen

microbes to produce protein from non-protein N , which subsequently becomes available to the cow when rumen digesta flows into the small intestine to be digested and absorbed (70). However, the amino acid profile of microbial protein is not perfectly matched to that of ruminant needs (71). To mitigate this, most ruminant diets for production purposes contain additional protein sources as soybean meal, rapeseed meal and/or maize gluten meal. Parts of this protein is bypass-protein; protein which is undegradable to rumen microbes and which will pass unchanged from the rumen to the small intestine to yield essential AA available for absorbtion (71, 72).

Protein content is probably the most complex variable to evaluate in ruminant feed. Because of the extensive protein degradation and production by rumen microbial proteins, what is fed (the ration crude protein (CP) content) and what is available to the heifer (metabolizable protein/ amino acids available for absorption) is not the same. The protein available for absorption in the small intestine of the animal is a combination of microbial protein produced in the rumen, and dietary rumen undegradable protein (RUP). The latter will pass through the rumen and enter the small intestine in the same form as it was fed (hence the phrase by-pass protein). Through the 80's and 90's, this knowledge was brought into practice by the development of systems and models to calculate the available metabolizable protein in a diet, such as the AAT/ PBV system (73, 74), the Cornell net carbohydrate and protein system (75), and the DVE/ OEB system (76). These systems are more complex, but have the advantage that they are able to predict animal response to a diet with much higher precision than CP measurements because they estimate the true amount of amino acids absorbed in the small intestine Nevertheless, crude protein content has been, and still is, an important feed evaluation variable because it is easy to comprehend, it is easy to compare across feedstuffs and the overall CP content of a ration is usually roughly reflective of the amount of protein available to the animal.

The effect of dietary protein on heifer growth and subsequent performance has been repeatedly investigated. Lammers and Heinrichs (2000) found that heifers fed a diet containing 64 g CP per Mcal metabolizable feed energy (as calculated by the NRC system) displayed higher feed efficiency and thus increased growth compared to heifers fed a diet with 46g CP / Mcal. The increased efficiency led to faster growth, which again gave a higher intake capacity and a further increase in growth in high-

protein heifers fed ad libitum (24). An elevated dietary protein content up to about 16 % CP of dry matter intake (DMI) has been shown to increase structural growth and feed efficiency while minimizing BCS increase in growing heifers (52, 77). Bagg et al. reported a quadratic response of dietary protein on growth, indicating there exists an optimum level where protein input gives the highest return in terms of lean growth and feed efficiency. This optimum probably varies with age or size of the heifer as well as level of energy in the diet (77). As evident from the paragraph above, this optimum will also vary with the relative proportions of rumen degradable- and undegradable protein in the diet, and thus cannot be described merely in terms of CP in total ration (78). This is well illustrated through the study of Tomlinson et al., who found that increasing the ratio of RUP to dietary CP in total mixed rations designed to meet the protein requirements given by NRC for heifers at about 250 kg increased feed efficiency, body weight gain and hip height. CP levels were 11.8-12.7 (in % of DM), and % RUP of CP was 28-55. DMI was decreased from RUP percentages above 40, probably because of a negative protein-to energy balance in the rumen, with too little rumen degradable protein available for the rumen microbes to maintain a high production of microbial protein.

In summary, increased dietary energy fed to heifers will increase ADG and BCS and, above a certain optimum level it may cause negative effects on future milk yield. The negative effects of increased dietary energy on fatness during growth may be modulated by increasing the availability of metabolizable protein in the diet. However, increased dietary protein in heifer rearing has also been shown to cause decreased milk yields (79), and as ruminant rations tend to increase in cost with increasing protein content, the investment may not be worth the cost.

3.2.1. Dietary energy and protein for growing cattle within the NorFôr system

NorFôr is the commonly used feed evaluation and planning system in the Nordic countries (80). It is a semi-mechanistic static model which calculates animal requirements and total feed ration characteristics based on animal and feed input. For growing animals, animal input is type of animal (breed, sex), activity level (tie-stall or loose-housing), BW and ADG. The system is mainly an evaluation system for total feed rations rather than single feedstuffs, meaning it relies on the analyzed characteristics

of single feeds, but also takes into account the interaction between diet composition, feeding level and animal characteristics (80).

In NorFor, dietary energy for growing cattle is expressed as net energy for growth (NEG). The calculation of NEG is based on Dutch and French systems, and in its simplest form, it can be described as metabolizable energy (ME) * joint efficiency of ME utilization for maintenance and growth (80-83). As NorFor is a feed ration evaluation system, in practice NEG is usually defined as NEG per kg DM at a given level of DMI (80).

Protein evaluation in NorFor is based on the AAT/PBV system, and its principles are shown in figure 2 (73, 74). The accounted sources for calculation of amino acids available for absorption in the small intestine are rumen undegradable dietary protein, microbial protein synthesized in the rumen and endogenous protein. The endogenous protein originates from gastrointestinal epithelial sloughing, and pancreatic and biliary excretions. Out of the endogenous protein originating cranially to the duodenum, about 60 % is digested and reabsorbed in the small intestine (80). Additional endogenous protein N may become available to the animal through recycling of NPN from microbial degradation in the large intestine, but all in all, the endogenous protein represents a net loss of body protein (80, 84). This loss is increased with increasing dry matter intake, and thus fast-growing animals on a high feeding level have higher dietary protein requirements (80).

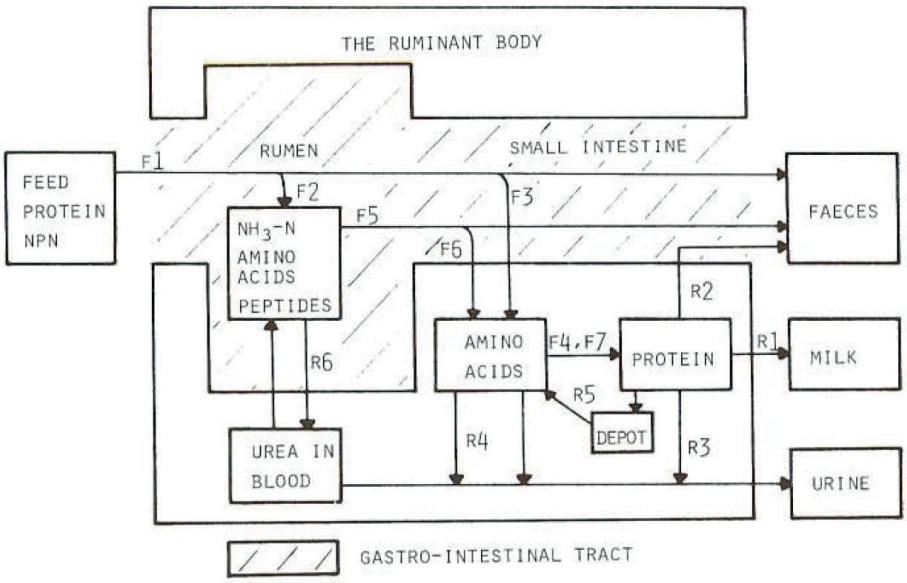

Figure 2. Nitrogen metabolism in ruminants as described through the AAT/PBV system. F's represents feed factors, R's represent animal requirements. F1= Crude protein (CP, N * 6.25) in feed; F2= Rumen degradation of feed protein; F3= Small intestine digestibility of non-degraded feed protein; F4= Utilization of non-degraded feed protein; F5= Microbial protein synthesis; F6= Small intestine digestibility of microbial protein; F7= Utilization of microbial protein; R1= Protein in Milk; R2= Endogenous faecal nitrogen; R3= Endogenous urinary nitrogen; R4= Gluconeogenesis; R5= Mobilization and deposition; R6= Urea recycled back to rumen. From Madsen et al. (73).

3.3. Growth profiles

Heifer rearing has traditionally been subdivided into calve period, prepubertal, postpubertal and/or pregnancy phases based on the effects of nutrition and growth in the different phases on mammary development and subsequent milk yield. Thus, most heifer growth studies have also been divided into at least two rearing phases with different growth rates. Therefore, the plethora of performed heifer studies not only gives information about effect of growth rates, but also on the timing of different growth rates, i.e. different growth trajectories. This is both an advantage and a

drawback: It gives information about a larger range of rearing strategies, but it also makes it more difficult to compare results from different experiments. Out of the heifer growth experiments which report effects on actual milk yield and not just mammary development (which is shown by Meyer et al. to be independent of growth rate and adiposity (27, 28)), three were designed as factorials where heifers were first subjected to different prepubertal growth rates, and subsequently either randomly distributed to new or continued feed treatments (MacDonald) or transitioned to a common diet for all animals (Van Amburgh and Lacasse) in a postpubertal phase (60, 65, 85). From these three studies, we find that Van Amburgh found a negative effect on milk yield from a prepubertal ADG of 940 g, but when corrected for weight at calving, there was no effect. Lacasse et al. found no effect of neither pre- or postpubertal ADG in the range from 720-950 g. Macdonald et al. found an effect of postpubertal ADG, but not of prepubertal ADG, and when corrected for weight at calving the only finding was a negative effect of prepubertal growth of 800 g/ day, which was the high prepubertal treatment in their study. However, a somewhat reduced ADG and weight at calving is commonly observed in pasture-based dairy systems like the one used in the study of Macdonald et al., when compared to more intensive feeding systems (86) . It is noteworthy that in the Van Amburgh-study, heifers subjected to the high prepubertal growth treatment were the smallest at calving, while in the Macdonald-study, they were the largest. When considering the results from both actual and corrected BW, it seems that rapid prepubertal gain may have a negative effect on the milk yield of heifers when their calving size is the same or smaller than their counterparts. Overall, neither pre- nor postpubertal daily gains or the combination of them seem to have conclusive effects on milk yield per se. First lactation milk yield seems to be mostly affected by BW at calving which is the result of total growth rate and age, and in later lactations, when mature size is achieved for all animals these effects seems to be evened out. This is probably because heifer who are small at calving, needs to allocate some energy towards body growth as well as milk production during first lactation. In studies where BCS was recorded and heifers were at a BCS ≤ 3.8 at calving, no negative effects of high postpubertal or pregnancy gains were observed (65, 85). In the study of Lacasse et al. the heifers with the highest postpubertal and gestational growth rate reached a BCS of about 4.1 two weeks before calving. Still no negative effects on milk yield were found, but negative effects on health and reproduction were observed (60).

3.3.1. Compensatory growth

Compensatory growth has been defined as "a physiological process whereby an organism accelerates its growth after a period of restricted development, usually due to restricted feed intake" (87). The capacity for compensatory growth is limited in very young (un-weaned) animals, and in animals nearing maturity (87). Thus, compensatory growth may only occur within a specific time frame of the life of an animal (87). The degree of compensatory response will vary with the duration of restricted feeding and refeeding, age and size of the animal. Generally, the compensatory response is highest during the first few weeks after a limited period of moderately restricted feeding (87). This is probably because during this period, GH levels are still elevated after the restricted feeding, but GH sensitivity is increased by an increase in receptors mediating the lipolytic effect of GH and the growth stimulatory effects of IGF-1 (87). After some weeks of liberal feeding, GH levels may decline causing a larger fraction of gained weight to be fat. With increasing adiposity, leptin secretion from adipose tissue is increased, causing decreased feed intake(88).

Gene expression in muscle tissue of growing cattle subjected to feed-restriction and alimentation has been studied. In correspondence with the gene expression differences found in adipose tissue of heifers fed high- or low energy diets or in heifers transitioned from a high-energy to a low-energy diet, the main differences were pertaining to mitochondrial function and energy metabolism (89, 90).

Compensatory growth may be exploited in cattle rearing, by subjecting heifers to a stair-step growth and feeding regime, with repeated periods of restricted and liberal feeding to keep total feed efficiency at a maximum (91-93). Such feeding regimes has also been found to increase the lactation potential of dairy heifers (91-93) , but so far it has not been routinely used by Norwegian dairy farmers. This may be because it is a heifer rearing strategy which demands commitment and effort in management practices to achieve the desired results. Nevertheless, in Norway there is a tradition for letting heifers out on free range mountain or forest pastures during summer, and during these periods the ADG may be decreased (94). Thus, most Norwegian farmers have the possibility to include at least one period of compensatory growth after first grazing season in their heifer feeding regimes without much extra efforts. However, in

animals nearing maturity, even compensatory growth may be a large fraction of fat, and therefore, high body weight gains during the last months before first calving should be avoided, regardless of whether the growth has been compensatory or constant (87).

3.4. Adiposity and growth

The importance of adipose tissue in the regulation of dairy cow metabolism is well established, although as yet not fully understood (95-113). Several studies have also confirmed the importance of an appropriate BCS at calving in order to achieve a high milk yield and to reduce the risk of disease (5, 6, 114-117). There is reason to believe that a heifer, not subjected to the extreme metabolic challenges of a lactating cow, is more robust and can tolerate a wider range of BCS without negative consequences. However, only a limited amount of research exists on which to draw conclusions, and many questions are still unsolved.

The BCS of a growing heifer depends on several factors, including growth rate, genotype, feed characteristics and age. At early ages, body growth is dominated by the deposition of lean tissue. With time, a gradually larger fraction of gained weight will be fat and the body lipid content increases relative to BW (118). Traditionally, body fat in growing cattle has been calculated as a constant fraction of BW, with degree of maturity (i.e. actual BW relative to expected mature BW) as the only source of variation (80, 119, 120). However, studies show that the relationship is not that simple and that body fat content at a given BW may vary widely depending on genetics, nutrition and growth profile (60, 121, 122). Compensatory growth may add to this difference, as it can lead to an increased lean mass gain during the first weeks of realimentation, but fat deposition will increase again after a while and may then surpass that of counterparts fed a constant ration (87). Thus, body fat percentage during development may be nutritionally altered, but further research is needed to assess the optimum BCS at a given point of development as well as the minimum and maximum BCS to avoid negative consequences within heifer rearing.

At high planes of nutrition, feed efficiency is reduced. Nutritionally induced rapid weight gain leads to a larger fraction of fat in gained mass. Because of a low water content and the high caloric content of triglycerides, the deposition of one kg fat demands about six times as much energy as the deposition of one kg lean mass (123). In cattle reared for slaughter, the deposition of excess fat is a cost with negative returns, as the fat is costly to make in terms of feed input. Additionally, in Norway the farmer will be punished by reduced carcass prices when animals are too fat.

3.4.1. Effect of former diet and BCS

An effect of former diet and BCS on feed intake in a subsequent period is found for cows in transition between pregnancy and lactation (124), as well as for cattle in other periods of life and within both beef and dairy production (60, 61, 85, 125, 126).

In 1998, Van Amburgh et al. performed a study to evaluate the effect of three different growth rates and two different protein sources in the prepubertal phase on subsequent milk production. They found that the fastest-growing heifers had the lowest yield, but also found that this lower yield to a large extent was explained by post-treatment factors such as weight and BCS at calving. Heifers were bred at similar body weights, but the heifers fed most intensively during the prepubertal period grew the slowest postpubertally and thus calved in at a lower body weight than their counterparts (85). This effect has also been observed by Lacasse in 1993 (60). This indicates that growth rate during a certain rearing phase is dependent on feeding level in the former phase, and this should be taken into account when making rearing strategies.

3.5. Next-generation RNA sequencing

3.5.1. Principle

Every protein of every cell is encoded by one or several genes. The full gene set (genome) of an individual is conserved in the nuclei of all its cells. The genome contains genes coding for all the proteins needed in the body, as well as a large fraction of non-coding DNA out of which some is transcribed into RNA with regulatory

functions (non-coding RNA) (127). Only some of all protein-coding genes are actively being transcribed into mRNA and further translated into proteins in a given cell at any given time point. This is the basis for the ability of multicellular organisms to be organized in different tissues with different functions, and it is also a part of an individual's ability to adapt to different environments (homeostasis) and to the varying demands of different life stages (homeorhesis). RNA sequencing is based on this principle; that no cell ever makes use of its full genome.

When a cell or tissue has an increased need for a specific protein, it may upregulate the transcription rate of the gene coding for this protein into mRNA. A summary of transcription and translation processes are shown in figure 3. Newly produced mRNA is subsequently transported out of the nucleus and into the cytoplasm, where it is translated into its encoded protein by ribosomes. Thus, an increased amount of mRNA representing a specific gene, usually indicates an increased need and upregulated activity for this specific protein and its function in the cell. However, it is important to note that protein-coding genes and their products may be subject to a vast array of post-transcriptional and post-translational modifications (128, 129). Therefore, upregulation of a gene may have several different outcomes, which must be taken into account when interpreting such information.

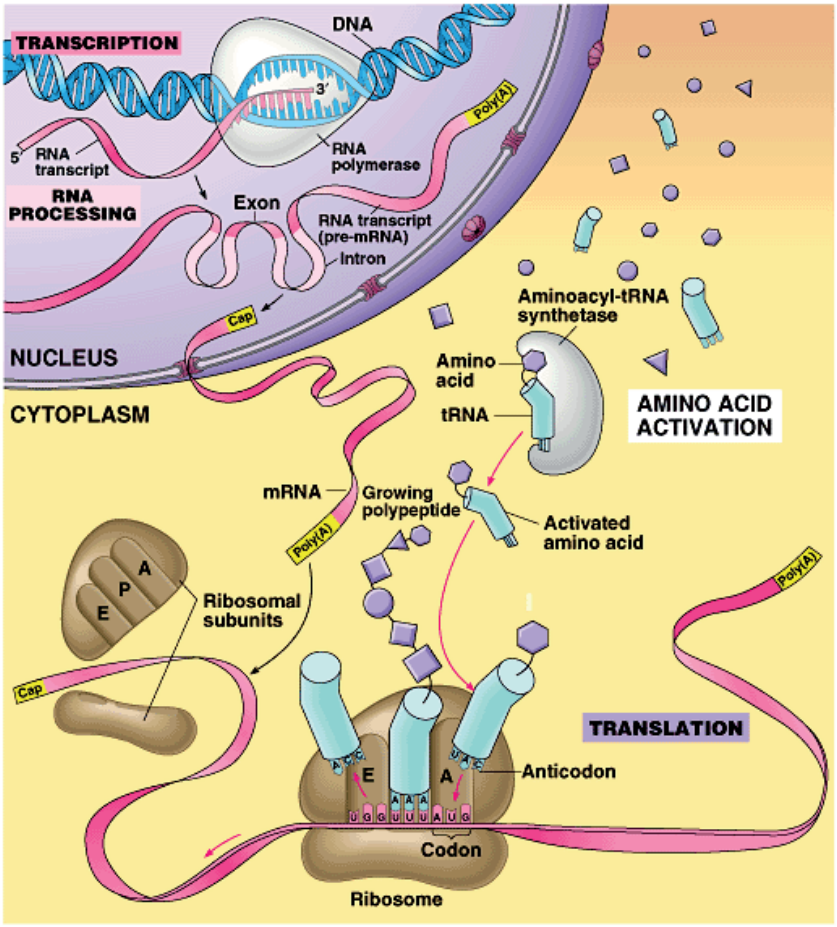

Figure 3. Transcription and translation in eukaryotic cells. (Accessed through https://slideplayer.com/slide/9797361/, Dec 12, 2018).

3.5.2. Methodology

For successful RNA sequencing, one wants the purest possible RNA extracted from the tissue of interest. In recent years, several extraction kits have become commercially available, specifically tailored for extraction of RNA from different tissues and for different purposes. The amount and quality of the extracted RNA is then assessed in terms of nanograms/µl sample and RNA integrity number (RIN) value, respectively.

RNA is a stable molecule, but it is rapidly digested to shorter molecules in the presence of RNAse enzymes, which are present in all tissues. Therefore, a quality control and assessment of degree of degradation is critical. The RIN value is based on size distribution of RNA fragments in each sample, measured after automated electrophoretic separation of fragments (130). If the quality of the RNA has been found satisfying, the samples may be prepared for sequencing. The preparation depends on the chosen extraction kit and sequencing platform but usually includes:

1. Random cutting of the RNA into smaller fragments by RNAse enzymes.
2. Production of complementary DNA (cDNA) strands.
3. Priming /Labelling of cDNA strings
4. PCR amplification of cDNA strands to produce detectable amounts of each strand.
5. Quality control of cDNA libraries
6. RNA sequencing.
7. Quality control and pre-processing of raw data.
8. Alignment and annotation to known species genome (if available).
9. Analysis. From this point the analysis may take many paths, depending on experimental design and research objective, e.g. gene expression, alternative splicing events, variant calling for detection of single nucleotide polymorphisms (SNPs), insertions and deletions (INDELs). The range of available bioinformatics tools to perform different analyses and to visualize results is vast and it is a field of rapid development.

An overview of the process from point three to six is given in figure 4.

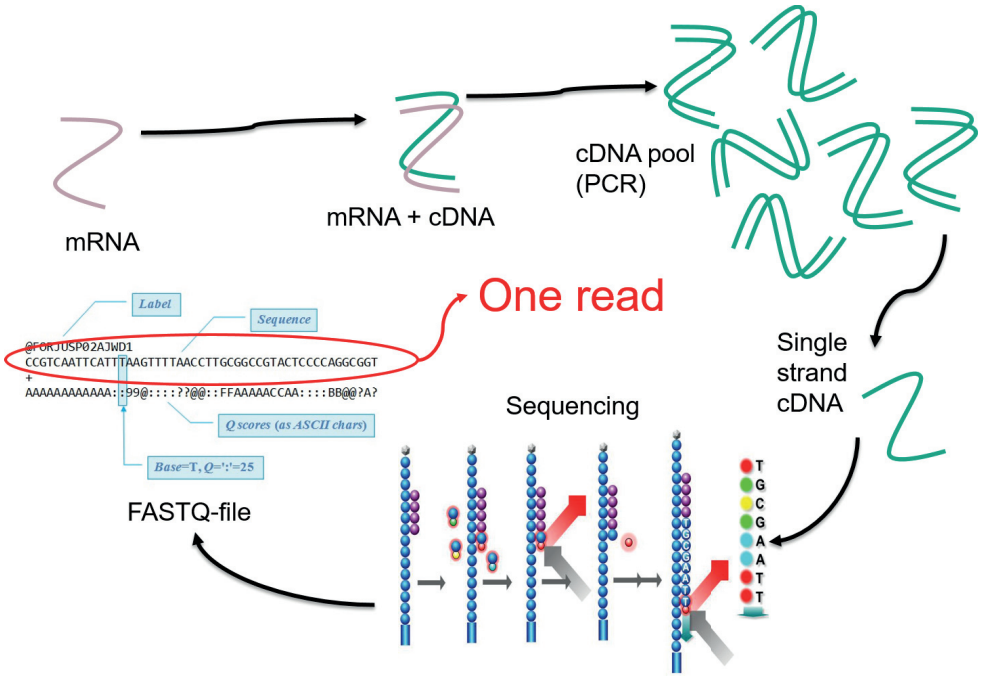

Figure 4. An overview of RNA-seq workflow. Fragmented RNA strands from sample tissue are used as templates for synthesis of complementary DNA strands (cDNA). cDNA is prepared and labelled (not shown), denatured into single strand DNA and put into the sequencer. cDNA strands are sequenced base by base by addition of complementary single bases with fluorescent terminal ends. Fluorescent signals are registered, the fluorescent ends are cleaved off, and new fluorescent single bases are added in a repetitive manner until required read length (usually 50-300 bases) is achieved. Sequence raw data are stored in FASTQ files of the format shown in picture.

4. Summary of papers

Paper I

Designing a replacement heifer rearing strategy: Effects of growth profile on performance of Norwegian Red heifers and cows

The main objective of this study was to develop an optimal replacement heifer rearing strategy for today's modern NR heifer. This was done by contrasting groups of heifers with diets differing in energy and protein content and consequently different growth profiles during both the pre- and postpubertal periods until confirmed pregnancy, and similar feeding which resulted in dissimilar growth profiles during pregnancy. Secondly, we evaluated the effects of the different diets and growth profiles on lifetime performance, i.e. through 3 lactations, of the same animals.

Main results:

- NorFors Optifor software is a precise tool for designing total rations for pre-planned growth rates in Norwegian Red heifers.
- Energy is the main driver for growth, while protein allowance above minimum requirements only have a small effect.
- Pre-pregnancy dietary energy level had an impact on pregnancy growth rates.
- Heifers fed high-energy diets calved in at a younger age (22 versus 26 mo) and a lower body weight (535 versus 565 kg) than heifers fed low-energy diets, but at the same body condition score.
- Heifers fed high-energy diets had a numerically lower milk yield during first lactation than heifers fed low-energy diets, but over the course of three lactations, this effect was evened out.
- Over three lactations, total milk yields were similar across treatments, although heifers fed low-protein diets had the numerically highest total milk yields.

Main conclusion:

- Norwegian Red heifers may be fed for an ADG of up to 900 g in the pre- and postpubertal phase combined with a moderate ADG through pregnancy without negative effects on subsequent lifetime production.

Paper II

RNA-seq analysis of bovine adipose tissue in heifers fed diets differing in energy and protein content

The objective of this study was to investigate the effects of diets differing in protein and energy density and consequently different growth rates on the full adipose transcriptome of growing dairy heifers, and to identify gene networks related to critical metabolic pathways which can be targeted in future studies. Subcutaneous adipose tissue biopsies were harvested from 24 heifers fed four diets differing in energy and protein content (n=6) and thus subjected to different growth profiles. mRNA was extracted from the biopsies and sequenced by high-throughput sequencing. Resulting lists of expressed genes were analyzed to find differentially expressed genes (DEG) between treatment groups. The DEG were subsequently run through a pathway analyzer to find the main pathways affected by the DEG.

Main results:

- Dietary energy content has a massive impact on the bovine adipose tissue transcriptome regulating several aspects of energy metabolism.
- The size of the effect is modulated by dietary protein content.
- We identified possible biomarkers for early-stage obesity (*GSTT1*, *GSTT3, GSTM1, MGST1, MGST3*, *SOD2, CAT*) and glucose load (*CRYM)* in cattle.

Main conclusion:

- Bovine adipose tissue is highly transcriptionally active, displaying gene expression patterns reflective of differences in dietary energy and protein content and expected changes in metabolism. Because of this and its easy access, subcutaneous adipose tissue is well suited for further nutrigenomic studies.

Paper III

Long-term effects of former diet, feed transition and pregnancy on adipose gene expression in dairy heifers.

The main objectives of this study were to look for post-treatment gene expression differences between Norwegian Red heifers fed former diets differing in energy and protein density and to examine the long-term gene expression changes occurring in the adipose tissue of heifers subjected to a feed transition and pregnancy. 48 subcutaneous adipose tissue biopsies from 24 Norwegian Red heifers were harvested at two time points: At 12 months of age, while the heifers were fed four experimental diets differing in energy and protein content, and again at month seven of pregnancy, when heifers were 20-24 months old and had been offered the same diet *ad lib* for six months. mRNA was extracted from the biopsies and sequenced by next-generation sequencing. Resulting lists of expressed genes were analyzed to find differentially expressed genes (DEG) between treatment groups during pregnancy, as well as gene expression changes between 12 months of age and month seven of pregnancy within each treatment group. The DEG were subsequently run through a pathway analyzer to find the main pathways affected by the DEG.

Main results:

- Adipose gene expression is still affected by experimental diets containing different levels of energy and protein even 6 months after the termination of different feeding regimes and transition to the same diet.
- At 12 months, *ENSBTAG00000007075* was differentially expressed between LPHE and LPLE heifers, and *ENSBTAG00000048049* was differentially expressed between HPHE and HPLE heifers during the experimental feeding. These genes maintained the same gene expression differences post-treatment, indicating a possible immunologic programming.
- Most nutritionally induced gene expression changes in adipose tissue are reversible.

- Pregnancy seem to cause gene expression changes related to complement activation and increased leukotriene synthesis in the adipose tissue of NR heifers.
- We have identified candidate genes and key gene regulators which should be studied further with regards to pregnancy preservation (TGFB1, *CFD*) and metabolic regulation and efficiency (*UCP2,* PI3K, RICTOR, MAP4K4,) in dairy cattle.

Main conclusions:

Adipose tissue gene expression is influenced by both current and former diet, and also by pregnancy. Our results indicate a possibility for nutritionally induced developmental programming to occur even after three months of age. This finding should be studied further.

5. Discussion

As this thesis in its entirety is based on one large experiment, some of the overall discussions are already made in the Papers. Still, and as mentioned in the introduction, Paper I through time developed into a more comprehensive paper than first planned. Therefore, a few points which did not make the cut to be included in Paper I are discussed in this section, as well as the fulfillment of objectives and hypotheses.

5.1. Growth responses to dietary energy and protein

As shown in Paper I, in this study we found that energy was the main driver for growth, while dietary protein above current recommendations had a small positive effect on lean growth. The different energy levels in the rations led to significantly different BW between HE and LE heifers already from four months of age and throughout the experimental feeding period. Body condition scores were different between energy groups from the start of BCS assessments at six months and throughout the experimental feeding period. The divergence in BW from four months of age is even earlier than that found by Chelikani et al. (2003), who also described significant differences in body size and composition between high- and low-energy fed heifers as early as at 8 months of age (121). Chelikani et al. also found significant differences in WH at puberty between heifers fed different levels of energy and protein. For the purpose of Paper I, difference in WH at puberty was not tested. However, at onset of puberty WH was 105.1 cm for LPHE, 105.7 cm for HPHE, 111.7 cm for LPLE and 109.8 cm for HPLE heifers. Differences in WH at insemination and at calving were tested statistically and were found to be significant throughout pregnancy and until calving, with the LE heifers being the highest. When compared at similar ages, the HE heifers became significantly higher at the withers than LE heifers at 7 months, and this difference persisted until the HE heifers were transitioned to the pregnancy feed at 12 months of age. These observations suggest that frame growth, as represented by WH, is affected by both energy supply (HE heifers were tallest when compared at similar ages) and of age (LE heifers were tallest when compared at the same physiological stages, LE heifers being older than HE heifers). As evident from table 4 and 5 in Paper I and table 1 in supplementary results, BW/WH ratio was lower

for LE heifers at any point after six months regardless of being compared at similar age or physiological stage. This draws a picture of LE heifers being leaner and taller relative to BW through all rearing phases, until all groups converged at a similar BCS at calving.

The evaluation of dietary protein for ruminants is not straightforward. In our experiment, all the HE heifers (both HPHE and LPHE) received the most protein when given in terms of CP and PBV, both in grams per kg DM and in total intake per day, as well as in total AAT intake per day. However, when given in terms of grams AAT per kg DM, AAT/NEG or AAT balance (80), HPHE and HPLE were the high-protein groups. An implication of the way the NorFor system estimates AAT, is that negative PBV values will yield overestimated AAT values (73, 74, 80). This therefore applies to the LE heifers, but as the overestimation applies to both LE groups, the difference between them is assumed to be approximately correct.

When comparing treatment groups at similar age instead of physiological stages, a small but significant difference in BW emerged between HPLE and LPLE from 5 months of age and between HPHE and LPHE from 9 months of age. The HPLE heifers were 15 kg heavier and HPHE heifers were 7 kg heavier than their LP counterparts at 12 months of age (Supplementary table 1, page 56-57). At this age, BCS was identical between protein treatments, while the HPHE heifers were significantly higher at the withers than the LPHE heifers. Wither heights became significantly different between LPLE and HPLE heifers at 15 months of age, at about the same BW as the HE heifers were at 12 months. This may indicate that the BW difference between protein treatments within energy groups was brought about by a larger frame size, not by increased fatness. The younger age at which BW became significantly different between protein groups within the LE treatments than within the HE treatments may reflect the relatively larger proportion of concentrates in the ration and thus bigger differences in grams AAT/ day relative to total feed intake for LE heifers compared to HE heifers: AAT intake in grams per day was significantly different between the LE protein treatments from 4 to 11 months of age, while for the HE heifers, the difference was significant from 7 months of age and throughout the experimental feeding period. This could indicate that the difference in AAT intake per day became significant 1-2 months before BW became significantly different between different protein groups

within each energy treatment. Therefore, it seems that AAT intake per day may have had a small but significant positive effect on structural growth. A similar effect of protein supply on lean growth has also been described by others (7, 121, 131). However, a Danish study indicate a negative effect of high-protein feeding in the rearing period on subsequent milk yields (79). This finding is partially supported by our study as the HP heifers gave numerically less milk than the LP heifers, regardless of energy level. Furthermore, the low nitrogen efficiency of cattle is decreased even further with increasing levels of protein in the diet (80, 84). Therefore, high-protein diets will lead to increased nitrogen excretion. This is unwanted in agriculture because of environmental concerns (132). Thus, supplying growing heifers with dietary protein above current recommendations may not be worth the extra cost, neither economically nor environmentally.

Despite a significant positive effect of metabolizable protein on structural growth, this was not sufficient to prevent the fattening of HPHE heifers, as they achieved the same BCS as the LPHE heifers throughout the experimental feeding period. Thus, the hypothesis that an increased supply of metabolizable protein given to fast-growing heifers will prevent fattening has not been confirmed.

5.2. The relationship between nutrition, growth and adipose tissue gene expression

When this PhD work was initiated, no full transcriptome of bovine adipose tissue had ever been fully sequenced. Therefore, we did not know what results to expect, but we hoped for a few genes about which to say something sensible.

-We got more than we bargained for!

The observation of a massive up- and down-regulation of genes which made biological sense when feeding regimes were considered suggested that adipose tissue gene expression is indeed tightly connected to nutrition and physiological state.

Not many RNAseq studies have been performed on tissues of growing cattle, but the gene expression differences described between heifers on high- or low-energy diets in our study, have some things in common with the findings of Keogh et al (2016) and English et al. (2018) (51, 89). In a study published in 2016, Keogh et al. describe gene expression differences in muscle tissue of steers subjected to a feed restriction and subsequent re-alimentation versus steers constantly fed *ad lib*. The study of English et al. investigates the adipose tissue gene expression differences in pre-weaned calves on two different dietary energy levels. As for our study, diets of either high or low energy elicited expression changes mainly in pathways related to energy metabolism, such as oxidative phosphorylation, TCA cycle and gluconeogenesis (51, 89). Interestingly, the direction of muscle gene expression differences related to oxidative phosphorylation and the TCA cycle were opposite of those observed in adipose tissue when considering feeding level (89) In our study, these pathways were upregulated in LPHE heifers compared to LPLE heifers at 12 months, and they were also upregulated in HE heifers (both groups) at 12 months when compared to themselves at month seven of pregnancy, when they were fed a diet of a much lower energy content. This is in concordance with the study of English et al. in which calves fed a high-energy diet displayed an upregulation of genes related to mitochondrial energy production and the TCA cycle in their adipose tissue (51). In the study of Keogh et al. however, steers subjected to a feed restriction displayed a muscle tissue upregulation of genes pertaining to oxidative phosphorylation and the TCA cycle when compared to steers fed ad lib. When the feed-restricted steers had been re-alimented for 15 days, genes related to these pathways were downregulated when compared to ad lib-fed animals (89). This illustrates how gene expression can be differentially regulated to help accommodate the energy needs and availability of different tissues. In the muscle of restrictively fed steers, an upregulation of genes related to the TCA cycle and mitochondrial ATP production is probably reflective of increased catabolism for energy purposes and possibly an increase in mitochondrial numbers to maximize cellular energy efficiency when energy is scarce (89, 133). In the adipose tissue of heifers on a high plane of nutrition, an upregulation of genes related to oxidative phosphorylation and the TCA cycle is probably a response to the need to handle and deposit excess energy as lipids, through an increase in production of ATP and lipid precursors for anabolic purposes. The upregulation of these pathways in the muscle

tissue of feed-restricted steers and in the adipose tissue of heifers and calves fed a high-energy diet is probably brought about by different regulatory and signaling pathways, and an investigation of upstream gene regulators for these scenarios could possibly yield valuable insights to the regulation of whole-body metabolism in growing cattle.

Amino acid metabolic pathways have also been found to be affected by dietary energy level in all three of these studies, especially for the branched-chain amino acids leucine, isoleucine and valine. These amino acids are mainly degraded in peripheral tissue (134). Their plasma levels are associated with obesity and insulin resistance in humans and are regulated by degradation in adipose tissue (134). In adipose tissue, gene expression related to degradation of BCAAs seem to be upregulated by high-energy feeding (51), (Paper II and III). In muscle however, the isoleucine degradation pathway was downregulated in re-alimented animals 15 days after transition from a restricted feeding regime relative to animals constantly fed *ad lib* (89). This may be a part of the regulatory mechanisms which maintain an increased feed efficiency during the first weeks of compensatory growth, or it may indicate that adipose tissue BCAA degradation is mainly upregulated as a consequence of fatness instead of diet.

Based on these three RNAseq studies on muscle and adipose tissue of growing cattle subjected to different planes of nutrition, it seems that we can draw out the following:

- Different dietary energy levels are able to cause massive changes in gene expression related to energy metabolic pathways such as oxidative phosphorylation, glycolysis, and the TCA cycle, both in muscle and adipose tissue. The direction of expression changes seems to be tissue-specific.
- Most nutritionally induced gene expression changes are reversible. However, in the subsequent feeding period after a feed challenge, the interaction between former and current diet may elicit differential expression of other genes.
- Dietary energy levels also affect gene expression related to amino acid metabolism in both muscle tissue and adipose tissue. Especially genes pertaining to branched-chain amino acid degradation seem to be affected, which is in line with a known role of these amino acids in the regulation of energy metabolism and adaptation to different planes of nutrition (134-136).

A major question which arose from Paper II was: Why did we find so massive gene expression differences between LPHE and LPLE heifers, and only small differences between HPHE and HPLE heifers? Hopefully, a full explanation will be found in the future, but for now, a possible explanation may lie in the results of Paper III: When looking at figure 4-7 in Paper III, it becomes clear that the gene expression changes occurring in the HPLE heifers from 12 months of age to month seven of pregnancy had more in common with the gene expression changes of the HE groups than those of the LPLE heifers. As stated in Paper I, the LPLE heifers grew the slowest and received the diet with the lowest PBV, which was negative. In parameters such as ADG, PBV, CP and AAT, the HPLE heifers were more similar to the LPLE heifers than HE heifers, but they still represented an intermediate level between the LPLE and the HE heifers both in nutrition and performance. Therefore, it is imaginable that the HPLE heifers were physiologically more similar to the HE heifers than the LPLE heifers were, and that this is what we see reflected in both figure 4 and 5 in Paper II, and figure 4-7 in Paper III.

Storli et al. stated the optimum pre- and postpubertal growth rate of NR heifers to be 879 and 830 g/ day, respectively (137). Growth rates above or below the identified optimum are expected to cause decreased milk yields, but the mechanisms behind these effects are still unknown, and are probably multifactorial. Lifetime milk production was similar between LE and HE heifers, and thus it is tempting to state that the temporary BCS of 4.0 in the HE heifers had no lasting negative effects. However, the growth rates of heifers in our study were above the indicated optimum for the HE heifers, and below optimum for the LE heifers. Therefore, and despite calving in at adequate BW, BCS and age, both energy treatments might have shown suboptimal milk yields, but for different and unknown reasons. For the LE heifers, it is difficult to pinpoint plausible explanations for a suboptimal milk yield caused by their moderate growth curve. The HE heifers on the other hand, would have had a too high growth rate, and as shown in Paper I, this led to an excessive fatness represented by a BCS of 4.0 at 12 months (Supplementary table 1). The gene expression differences between LPHE and LPLE heifers at 12 months showed a massive shift in mitochondrial function, with the pathway named mitochondrial dysfunction ranged among the most affected pathways. As cited from IPA, the pathway analyzer used: “Mitochondria are the

primary consumers of oxygen in a cell and contain a multitude of redox carriers that are capable of transferring single electrons to oxygen. This results in the formation of the reactive oxygen species (ROS) superoxide. Mitochondria also contain an extensive antioxidant defense system to detoxify ROS, which would otherwise cause oxidative damage to cellular components. Thus, in structurally and functionally intact mitochondria, there is little net ROS production. Mitochondrial dysfunction occurs when the ROS-mediated oxidative stress overpowers the antioxidant defense system. The factors for triggering oxidative stress can be genetic defects, environmental factors like radiation and toxins and metabolic fluctuations." Therefore, we cannot rule out that the HE heifers were in fact slightly overfed, leading to a premature and increased fat deposition, resulting in an increase in adipocyte metabolism to a degree of overload. During pregnancy, when compared to LE heifers, the HE heifers displayed a reduced growth response to the feed ration, a downregulation of genes pertaining to energy metabolism, and an upregulation of the gene coding for uncoupling protein 2. Considering that such differences between feeding groups still exists more than 6 months after the termination of experimental feeding, and only 2 months before calving, it is not difficult to imagine that the experimental feeding may have caused metabolic differences which also followed the heifers into adulthood and may have affected nutrient allocation and milk yield. However, the rejection or confirmation of such theories will demand further studies.

5.3. Post-treatment effects of diets differing in energy and protein content

Our study did confirm the hypothesis that heifers fed for a rapid growth rate before pregnancy and a moderate growth rate (500-550 g/d) during pregnancy, can obtain an optimal body condition score (BCS ≤ 3.75) at calving. However, the HE and LE treatments did not follow the same trajectories towards their calving BCS. The rapid growth and steady BCS of LE heifers during pregnancy, may indicate there are several possible routes to a well-functioning dairy cow: The LE heifers deposited 35 kg more BW than the HE heifers during pregnancy. Could this increased growth potential have been exploited to inseminate the LE heifers at about 365 kg and still make them calve

in at the same BW and BCS as the HE heifers, at about 24 months of age instead of 26? Further studies would be required to test this hypothesis, and our study cannot predict what effect such a strategy might have on future milk yield.

In Paper I, the BW at calving was estimated mostly based on weight registrations done after calving, for practical reasons. However, as a cow loses 40-80 kg BW (blood, calf, embryonic membranes and fetal fluid) at the day of calving, which are not immediately replaced, the weights at late pregnancy were actually even higher than those given in Paper I. Running the Model given in Paper I based on all pregnancy weights instead of lactation weights, the estimated weights the day before calving were 559, 554, 601 and 595 kg for LPHE, HPHE, LPLE and HPLE heifers, respectively. Thus, differences between the treatments were about the same, but it changes the picture when it comes to which group who grew as planned: With these calving weights, the case was not the HE heifers growing slower than expected during pregnancy, it was the LE heifers growing faster. This effect, which mostly resembles a very late compensatory growth, has also been observed in other heifer growth trials (7, 85, 138). However, several issues separates the observed pregnancy growth of our LE heifers from that usually observed during compensatory growth studies: Compensatory growth is expected to occur immediately after a change from restricted to unrestricted feeding, with a gradually increasing growth rate until a maximum is achieved about 30 days after realimentation (87). In our study, the LE heifers did not reach their maximum growth rate until about 100 days of gestation, 60 days after the feed change. Another study also observed increased post-treatment growth rates in heifers initially fed low-energy rations, but this was reported as a classical compensatory growth, mainly occurring during the period directly after the feed change (85).The initial decrease in growth rate in all our experimental groups after change to pregnancy feeding may be partly explained by the fact that the heifers were moved from tie stalls to a loose-house barn in connection with the feed change. This may temporarily have increased the activity level and decreased the feed intake of the animals. If this is the case, this may also explain why the immediate decrease in ADG after feed transition is biggest in the LE heifers: With a similar BW at feed transition, but a higher percentage of lean body tissue, the LE heifers would have had a higher maintenance requirement than the HE

heifers at the time of change, as the maintenance requirement of adipose tissue is close to zero, and the requirement of muscle is relatively high (139).

Classical compensatory growth is also expected to have a higher fraction of lean tissue accretion than that of normal growth. The opposite was found in our study. While the HE heifers decreased their BCS with about 0.25 points, the BCS of LE heifers remained constant at 3.75. This means that a larger fraction of the pregnancy weight gain in the LE heifers was fat. However, the assumption of compensatory body gain to be leaner than normal, may not always be correct. Most studies examine compensatory growth in a period of 3-16 weeks after realimentation, not for more than half a year, as was the case for this study. Hornick et al. report that the final body composition in compensatory animals depends on the duration of the refeeding period. This is because compensatory growth will be dominated by protein deposition during the early phase of about 0-10 weeks. Thereafter, an increasing fraction of the body gain will be fat. Hornick also claims that in animals with a limited muscle growth potential such as dairy breeds, or in animals near to maturity, fat gain may dominate the compensatory growth from the start (87).

An overall project hypothesis was that heifers could be fed to a rapid prepubertal growth and a moderate postpubertal growth, to calve in at a young age without negative effects on milk yield. This has been confirmed. However, it is important to note that the HE heifers reached a BCS of almost 4, which is well above the recommended level, before starting to lose BCS points during pregnancy. This reduction of BCS was probably very important to the subsequent milk yield and health of HE heifers, and a feeding regime aiming for a flat, high ADG throughout the whole rearing period is not recommended.

5.4. Reliability of RNAseq results and their interpretation

Next generation RNA sequencing is a relatively new method which is still in rapid progress. Thus, no gold standards regarding the raw data treatment and statistical analysis of RNAseq data have yet been set. This is a challenge, as the production, analysis and interpretation of RNAseq data is not straightforward, and there are many potential obstacles to the successful and reliable reporting of RNAseq results. Firstly,

there is the absolute dependency of adequate sample quality and library preparation to achieve reliable read counts. Secondly, gene expression levels may be presented in many different forms and it is important to be aware that not all of them are suited for direct comparisons: The first, and the basis of the data, is the read counts, that is the number of cDNA strings mapping to a given gene in a given sample. This number may vary from zero to several hundred thousand. However, these read counts are not suited for direct comparison, neither between samples nor within one sample. The first is because the library size (total number of reads) of each sample also varies, due to variance in tissue sample size, in successfulness of RNA extraction, and because of the PCR the cDNA libraries are subjected to to make up detectable amounts of the gene fragments present. The latter is because within any sample long genes will have a tendency to achieve higher read counts than short ones. This is caused by the random fragmenting of RNA strings during sample preparation: Random fragmenting means fragments will approach some average fragment length, and thus long genes will generally be split into more fragments than short genes and will therefore make out a larger fraction of the total read counts in a sample. To correct for this, relative read abundance of a gene within a sample is usually given by Fragments Per Kilobase of Exon Per Million Fragments Mapped (FPKM), Reads Per Kilobase of Exon Per Million Fragments Mapped (RPKM) or transcripts per million (TPM). Subsequently, the data must be normalized before gene expression levels can be compared between samples. The choice of normalization method may vary between softwares, and so may the outcome. Studies have been performed to identify the best methods for different data types (140, 141). Once the data have been properly treated and are ready for analysis, one is faced with the challenges associated with statistical testing on a multitude of factors (thousands of genes in several samples of different origin /experimental treatments, often multifactorial). This requires a correction for multiple testing, in order to avoid a large number of false positive results. The common strategy to this end is to evaluate the results by false discovery rate (FDR) as found by the Benjamini-Hochberg procedure (142). There is also a vast range of free software available for statistical analysis of RNAseq data. These may apply different algorithms for analysis, different normalization methods and even different statistical distribution patterns. Thus, making the right choice of software for different experimental designs and research questions is important, but not always easy. The fact that new algorithms and

associated software is usually launched and presented in a publication written by the developers themselves further complicates the matter. New software is usually presented as "new and better", but the methods chosen to "prove" this statement are, consciously or unconsciously, chosen because they are well suited to present the new method as superior to others, as there are no standard methods yet defined to compare RNAseq software tools and algorithms (140).

RNAseq usually results in a plethora of differentially expressed genes. Therefore, it is both possible and necessary to analyze the resulting lists of genes as a whole, rather than to investigate the genes one by one. Pathway analyzing software are developed for this purpose, and there are both freely available versions (KEGG, DAVID) and commercial ones (Ingenuity pathway analysis; IPA).

The pathway analyzers may differ in terms of details and available functions, but the main principle applied to find significantly enriched pathways is usually the same: A significantly over-represented pathway means a pathway showing more DEG than expected by chance, given the number of genes belonging to the pathway. The pathway analyzers are comprehensive, continuously updated with new knowledge and represent a way to analyze and make deductions about amounts of information which greatly surpasses that which is possible to handle manually. Still, it is important to remember that they are also just software tools and may only give results based on already known and imputed information. For example, many genes have a known role in several signaling or metabolic pathways, and in a pathway analyzer such genes will be connected to all of them. If an RNAseq study result yield many such differentially expressed genes, several of these pathways will be returned as significantly affected by the study design, even if only one of the pathways was actually involved. In these cases it is up to the researcher to evaluate the biological relevance and probability of the pathway analyzer output. Finally, a transcriptional upregulation of a gene does not necessarily equal an increased amount or function of the encoded protein. Both the resultant mRNA and protein may be subject to a vast array of post-transcriptional and post-translational modifications. With all this in mind, it might seem to some that RNAseq may hardly yield valuable information at all. Still, one must assume that evolution has caused a change in gene expression to usually be associated with an increased need for the encoded protein. The rapidly increasing amount of published

RNAseq data matched with hormonal and metabolic assays, tissue function analyses or performance data support this assumption. The sequencing quality control consortium (SEQC) has also published studies on the reliability of different sequencing platforms, and found that for differential gene expression studies like ours, when preprocessing and bioinformatics is adequately performed, accuracy and reproducibility is overall good across all platforms studied (143).

5.5. Epigenetics and Metabolic programming

It is raised above question that the feeding regime which an animal is subjected to, may have not only concurrent effects, but also short- and long-term effects. However, these effects are still not fully understood, and especially not the long-term effects. The advent of study fields such as metabolic programming and epigenetics have drastically changed our view of the potential plasticity and metabolic "memory" held by developing animals (144-148). This phenomenon seems to be very influential during embryonic and fetal development, but it also seems to linger at least into the postnatal phase, which in dairy cattle is most evident through the positive effects of a high plane of nutrition during the early calf period on future milk yield. Within our study, the observation of two immunologically related genes maintaining a treatment-specific direction of expression though time, conception and a feed transition (Paper III), may indicate that some gene expression programming may occur even after three months of age.

Early theories mostly sought for explanations for such long-term developmental effects in endocrine and metabolic pathways directly (18, 149, 150). However, our increasing knowledge about gene expression and how it can be finely tuned with both long- and short-term regulatory mechanisms, makes it more and more likely that this is where we may find the full explanations for how nutrition may elicit significant effects even years after an impact. Epigenetics are defined as heritable changes in gene expression not caused by changes in the DNA code itself (151). The term developmental programming is broader and involves all early-life interventions which may cause lasting effects. When affecting gene expression, such changes are brought about by

three known main mechanisms: DNA methylation, histone modification and noncoding RNAs (ncRNA) (151). DNA methylation involves the covalent binding of a methyl group to a cytosine base, thereby inhibiting the binding of transcription factors (152). Histones are proteins involved in the wrapping of DNA into chromatin. They can be modified post-translationally through a very large range of chemical reactions, thereby changing the density of chromatin and thus availability of nearby genes. Non-coding RNAs belong to many different classes, and may interfere with gene expression at several levels, both pre- and post-transcriptionally. Non-coding RNAs may even modify other RNAs, thereby giving rise to the term "epitranscriptomics" (153). Knowledge about developmental programming further underpins the importance of the rearing phase in bringing forth robust and high-yielding dairy cows, but it also complicates matters: With today's knowledge, we need to acknowledge that the feeding of a pregnant heifer may not only affect the future performance of the heifer, it may also affect the future performance of her offspring (146)!

5.6. Nutrigenomics and Systems biology as tools for development of dairy heifer rearing strategies

If epigenetics and developmental programming studies have been an eye-opener towards the potential for long-term developmental effects caused by nutrition and environment, the emergence of nutrigenomics and other integrated –omics techniques may be the door-opener which drastically increases our possibilities to explain these effects on a regulatory and molecular level. A study aimed at assessing the effect of pre-weaning nutrition on adipose gene expression related to onset of puberty in Holstein bull calves have already been performed with promising results, and there are no reason to believe such a study on heifers would yield a less interesting outcome (51). Within dairy cow nutrigenomics, the subject most thoroughly studies thus far is probably the transitioning from pregnancy to lactation, a period of utmost importance to the success of a lactating cow (96, 154-157). These studies apply systems biology methods to assess gene expression, hormone levels and metabolic markers during the pre- and peripartal period as a way to gather information about the regulatory mechanisms by which the nutritional success of a dairy cow is achieved. What if earlier

rearing periods have just as big an impact as the immediate prepartal period on metabolic function, nutrient allocation, health and milk yield? Results from calf nutrition studies in particular, but also heifer rearing studies suggest this might be the case. If we can use high-throughput methods and a systems biology approach to identify and describe the mechanisms and pathways mediating long-term effects of early nutrition, this will be a first step towards control of these functions, as such insights would profoundly improve the basis for well-grounded and successful intervention and decision-making in dairy heifer rearing.

5.7. Some general considerations

The Norwegian dairy cow and heifer population is getting fatter. Slaughtered Norwegian Red heifers and young cows become fatter each year, and in 2017, more than 70% of the carcasses were devalued because of fatness (4). Even though it is likely that a larger proportion of the fattest animals will be culled, the trend is clear, and the increase in fat slaughtered animals could also reflect a tendency towards increased fatness in the Norwegian heifer population in general. This is most likely caused by the ever-increasing demand for efficiency, bringing about a call for high growth rates and leading to heifers being offered energy dense rations, causing some of the animals to be overfed. This trend represents major losses in several ways. When heifers are slaughtered because of fatness, directly or indirectly, it represents an economical loss to the farmer in several ways: As the deposition of one kg body fat demands several times as much energy as that of one kg lean body mass, fat heifers represent a waste of feed (123). When considering that fat carcasses have a lower slaughter value than those with an appropriate amount of fat, it further underpins the inexpedient nature of such a management. Furthermore, if the fattest heifers are being slaughtered, it indicates an association between level of fatness and unwanted traits, such as disease or failure to conceive. This relationship has been shown in several studies (92).

Additionally, the slaughtering of young fat animals will cause a decrease in the number of animals available for breeding selection and genetic progress, representing a

potential economic loss in the long run. This loss may be augmented by the possibility that the heifers becoming fat actually hold traits one would want to preserve in the herd: when group-fed an energy-dense ration, heifers with large feed intake capacity, well-functioning digestion and an efficient metabolism are the ones most likely to become obese. If a heifer has been fed a more energy-dense ration than required for her maximum growth rate, leading to energy excess in order to fulfill the heifers' requirement for rumen filling and satiety, does the problem really lie with the heifer? If one imagines a heifer with a low mature BW, a consequently low maximum growth rate, a high feed intake capacity and an efficient metabolism, she is one likely to become fat when group-fed a ration which other, less efficient heifers will thrive on. However, culling such animals because of fatness or (assumed) inefficiency, actually means indirectly putting a selection pressure towards less efficient feed utilization, when instead, measures should have been taken in management practices. Our study confirms that NR heifers are able to obtain growth rates and BCS above the recommended maximum even on roughage-based diets with very small amounts of concentrates. The LE heifers, fed a roughage even higher in NDF content than the HE heifers, had an NDFi intake per kg BW (unpublished) above that of their HE contemporaries from the start of the experimental feeding until 8 months of age, and they still mostly ate up all of their allotted, restricted rations. So, how fast can the NR grow if fed very high-structure roughage *ad lib?* As it seems the NR may be even more adaptable to different feeding regimes and have a higher intake capacity than expected, future rearing and feeding studies on the NR breed should focus not only on what feeding level and growth profile is the best for animal output, but also on what input (e.g. feedstuffs) is considered the best when regarding environmental impacts and feedstuffs easily available to Norwegian farmers.

According to Sommerseth (2018), the rearing of HE heifers was more profitable than that of LE heifers, when lifetime production was taken into account (13). However, the rearing costs saved were mainly related to housing and labour. Therefore, if heifers are reared extensively at farms with limited area available for the production of high-energy silage, but with large rangeland areas available (which is the case for a lot of Norwegian farms), it might be beneficial to rear heifers extensively and according to our "low-energy regime" after all and save the best grasslands for the lactating cows.

6. Conclusions

From this study on nutrition, growth and adipose gene expression in Norwegian Red heifers, we have drawn the following conclusions:

- Feeding heifers different energy levels pre-pregnancy, has profound effects on growth rate and BCS, while metabolizable protein above current recommendations only has minor effects.
- Regardless of dietary protein levels, Norwegian Red heifers fed a diet with sufficient energy to sustain an ADG of 900 g will deposit excessive fat and may achieve a BCS above the recommended 3.75 at breeding.
- Norwegian red heifers have the ability to maintain a rapid growth on roughage-based diets and may be fed for an ADG of up to 900 g/ day before pregnancy and a moderate ADG during pregnancy to calve in at 22 months without negative effects on subsequent milk yield over 3 lactations.
- Adipose tissue gene expression is strongly affected by and highly reflective of plane of nutrition, but the effect is modulated by total ration characteristics and dietary protein level.
- Pre-conception diets and growth may affect feed utilization, growth rate and adipose gene expression for more than six months after cessation of the diet.
- Most nutritionally induced gene expression changes in subcutaneous adipose tissue are reversible. However, some gene expression differences linger for more than 6 months after cessation of different feeding and may be indicative of genetic programming occurring after 3 months of age.

7. Future perspectives

This study represents the first analysis of a global transcriptome from the Norwegian Red and is a first step towards new knowledge about the links between nutrition, genotype and phenotype in this breed. The bovine genome is fully annotated, and the breeding of Norwegian Red is already based on genomic selection. Thus, knowledge about the genetics of the NR is already considerable, and the heifers used in this experiment must be assumed to be representative of the general population. The raw RNAseq data from this study are uploaded and freely available at GEO (accession no GSE79347). It represents a large potential to harvest more new knowledge. If efforts are made to increase the level of integration between genetics, transcriptomics, nutrition and performance data this can make the Norwegian Red breed progress even further both genetically and by management. Below, I will give two examples. However, possibilities are almost endless, and are mainly limited by our own imagination.

Studies which uses RNAseq and genomic prediction to identify genes and gene networks associated with feed efficiency in cattle have already been performed, but not in the Norwegian Red (158, 159). As our study data include growth, feed intake and a full adipose tissue transcriptome, such a study may be undertaken with already existing data and may yield valuable information about feed efficiency genetics.

Within heifer rearing, the mature size of the animal is central, as it is associated with growth capacity, feed intake capacity and milk yield. The optimum growth rate of an animal is likely to be genetically determined, linked to mature size, and to differ between individuals. However, the mature size of an individual cannot be foreseen. This is a major hindrance towards informed decision-making in practical rearing management rearing. With the increasing number of automated milking systems with cow weights in Norway, a new source of data on mature weights in the Norwegian cow population is emerging. In the future, these data may be used for genomic predictions of individual mature body weight, information which may drastically improve decision-making related to heifer rearing on herd level.

8. References

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9. Supplementary results

Table 1. Body weight (BW), Body condition score (BCS) and withers height (WH) through the experimental feeding period, by age, and WH at onset of puberty, insemination and late pregnancy.. LPHE = Low-protein, high-energy; HPHE = High-protein, high-energy; LPLE = Low-protein, low-energy; and HPLE = High-protein, low-energy treatments. Results with different superscripts within a row differ. Results for HE groups at 15 months are not shown, as these heifers were transitioned to a low-energy, low-protein pregnancy feed by that time. Standard errors (SE) in parentheses.

		Treatment group			
Age, months	variable	LPHE	HPHE	LPLE	HPLE
6	BW	194 (0.8)^{a}	194 (0.8)^{a}	172 (0.8)^{b}	178 (0.8)^{c}
	BCS	3.70 (0.03)^{a}	3.65 (0.03)^{a}	3.46 (0.02)^{b}	3.49 (0.03)^{c}
	WH	93.1 (0.33)^{a}	92.4 (0.33)^{ab}	92.4 (0.31)^{ab}	91.6 (0.32)^{b}
9	BW	280 (0.7)^{a}	283 (0.7)^{b}	235 (0.7)^{c}	246 (0.7)^{d}
	BCS	3.87 (0.02)^{a}	3.87 (0.01)^{a}	3.55 (0.01)^{b}	3.57 (0.01)^{b}
	WH	105.1 (0.17)^{a}	105.7 (0.17)^{a}	103.0 (0.16)^{b}	103.2 (0.16)^{b}

		Treatment group			
Age, months	variable	LPHE	HPHE	LPLE	HPLE
12	BW	363 (0.9)[a]	371 (1.1)[b]	296 (0.8)[c]	311 (0.8)[d]
	BCS	3.99 (0.02)[a]	3.95 (0.02)[a]	3.63 (0.01)[b]	3.63 (0.01)[b]
	WH	114.3 (0.17)[a]	115.3 (0.17)[b]	111.7 (0.17)[c]	112.4 (0.16)[c]
15	BW	-	-	354 (1.3)[a]	373 (1.2)[b]
	BCS	-	-	3.68 (0.01)[a]	3.68 (0.01)[a]
	WH	-	-	118.3 (0.21)[a]	119.1 (0.20)[b]
Onset of puberty	WH	105.1 (0.17)	105.7 (0.17)	111.7 (0.17)	109.8 (0.15)
Insemination	WH	120.1 (0.59)[ac]	119.4 (0.62)[a]	123.2 (0.59)[b]	121.8 (0.71)[c]
Late pregnancy (day 266)	WH	129.1 (0.47)[a]	129.4 (0.43)[a]	130.6 (0.7)[b]	130.8 (0.34)[b]

Table 2. Ration characteristics at 6, 9, 12 and 15 months of age. Standard errors (SE) are given in parentheses. LPHE = Low-protein, high-energy; HPHE = High-protein, high-energy; LPLE = Low-protein, low-energy; and HPLE = High-protein, low-energy treatments. Results with different superscripts within a row differ. Results for HE groups at 15 months are not shown, as these heifers were transitioned to a low-energy, low-protein pregnancy feed by that time. g/ kg DM for NDF, CP and AAT are found by dividing G/day with DMI, and thus have no SE.

		Treatment			
Age	Feed variable	LPHE	HPHE	LPLE	HPLE
6	DMI (kg/d)	5.28 (0.03)[a]	5.26 (0.03)[a]	4.73 (0.03)[b]	4.80 (0,03)[b]
	NEG (MJ/d)	30.4 (0.15)[a]	30.3 (0.14)[a]	24.6 (0.14)[b]	24.7 (0.14)[b]
	(MJ/kg DM)	5.82 (0.02)[a]	5.82 (0.02)[a]	5.30 (0.01)[b]	5.30 (0.01)[b]
	NDF (g/d)	2470 (19)[a]	2463 (19)[a]	2540 (18)[a]	2586 (18)[a]
	(g/kg DM)	468	468	537	540
	CP (g/d)	754 (4.8)[a]	816 (4.6)[b]	529 (4.3)[c]	604 (4.4)[d]
	(g/ kg DM)	143	155	112	126
	AAT (g/d)	487 (3.5)[a]	514 (3.4)[a]	415 (3.1)[b]	451 (3.2)[c]
	(g/kg DM)	92	98	88	94
	PBV (g/d)	32 (2.5)[a]	65 (2.4)[a]	-89 (2.2)[b]	-50 (2.2)[b]
	(g/kg DM)	7.0 (0.41)[a]	14.0 (0.40)[a]	-17.0 (0.37)[b]	-8.4 (0.37)[b]
	AAT/NEG (g/MJ)	22.9 (0.34)[a]	24.6 (0.34)[a]	28.7 (0.32)[b]	29.7 (0.33)[b]

		Treatment			
Age	Feed variable	LPHE	HPHE	LPLE	HPLE
9	DMI (kg/d)	6.96 (0.03)[a]	7.02 (0.03)[a]	5.80 (0.02)[b]	5.90 (0.02)[b]
	NEG (g/d)	40.0 (0.12)[a]	40.2 (0.12)[a]	30.9 (0.11)[b]	31.0 (0.11)[b]
	(MJ/kg DM)	5.80 (0.01)[a]	5.76 (0.01)[a]	5.38 (0.01)[b]	5.31 (0.01)[b]
	NDF (g/d)	3415 (16)[a]	3451 (16)[a]	3232 (15)[a]	3305 (14)[a]
	(g/kg DM)	491	491	557	561
	CP (g/d)	959 (3.5)[a]	1035 (3.5)[b]	649 (3.3)[c]	723 (3.2)[d]
	(g/kg DM)	138	147	112	123
	AAT (g/d)	605 (2.6)[a]	645 (2.6)[b]	478 (2.5)[c]	512 (2.4)[d]
	(g/kg DM)	87	92	82	87
	PBV (g/d)	54 (2.0)[a]	86 (2.0)[a]	-70 (1.9)[b]	-29 (1.9)[b]
	(g/kg DM)	8.8 (0.28)[a]	13.9 (0.28)[a]	-11.6 (0.27)[b]	-4.4 (0.26)[b]
	AAT/NEG	22.9 (0.27)[a]	23.2 (0.27)[a]	29.1 (0.27)[b]	27.5 (0.26)[b]

		Treatment			
Age	Feed variable	LPHE	HPHE	LPLE	HPLE
12	DMI (kg/d)	8.10 (0.03)[a]	8.25 (0.04)[a]	6.52 (0.03)[b]	6.61 (0.03)[b]
	NEG (g/d)	47.4 (0.14)[a]	48.1 (0.16)[a]	36.3 (0.11)[b]	36.6 (0.11)[b]
	(MJ/kg DM)	5.85 (0.01)[a]	5.83 (0.01)[ab]	5.55 (0.01)[bc]	5.51 (0.01)[c]
	NDF (g/d)	4073 (20)[a]	4158 (21)[a]	3704 (18)[b]	3772 (18)[b]
	(g/kg DM)	502	504	567	570
	CP (g/d)	1088 (4.7)[a]	1171 (5.0)[b]	717 (4.2)[c]	792 (4.0)[d]
	(g/kg DM)	134	142	110	120
	AAT (g/d)	667 (3.5)[a]	707 (3.8)[b]	509 (3.1)[c]	539 (3.0)[c]
	(g/kg DM)	82	86	71	82
	PBV (g/d)	83 (2.5)[a]	117 (2.7)[a]	-52 (2.2)[b]	-6 (2.2)[b]
	(g/kg DM)	11.0 (0.32)[a]	15.6 (0.34)[a]	-8.1 (0.28)[b]	-1.0 (0.26)[b]
	AAT/NEG	21.3 (0.30)[a]	21.9 (0.32)[a]	27.5 (0.30)[b]	25.7 (0.29)[b]

		Treatment			
Age	Feed variable	LPHE	HPHE	LPLE	HPLE
15	DMI (kg/d)	-	-	7.08 (0.04)[a]	7.15 (0.04)[a]
	NEG (g/d)	-	-	40.7 (0.16)[a]	41.8 (0.15)[a]
	(MJ/kg DM)			5.71 (0.01)[a]	5.80 (0.01)[a]
	NDF (g/d)	-	-	4112 (23)[a]	4156 (22)[a]
	(g/kg DM)			580	581
	CP (g/d)	-	-	757 (5.5)[a]	830 (5.3)[b]
	(g/kg DM)			107	116
	AAT (g/d)	-	-	527 (3.8)[a]	555 (3.7)[a]
	(g/kg DM)			74	78
	PBV (g/d)	-	-	-45 (2.6)[a]	2 (2.5)[a]
	(g/kg DM)			-6.4 (0.37)[a]	0.2 (0.35)[a]
	AAT/NEG	-	-	28.0 (0.45)[a]	30.5 (0.44)[b]

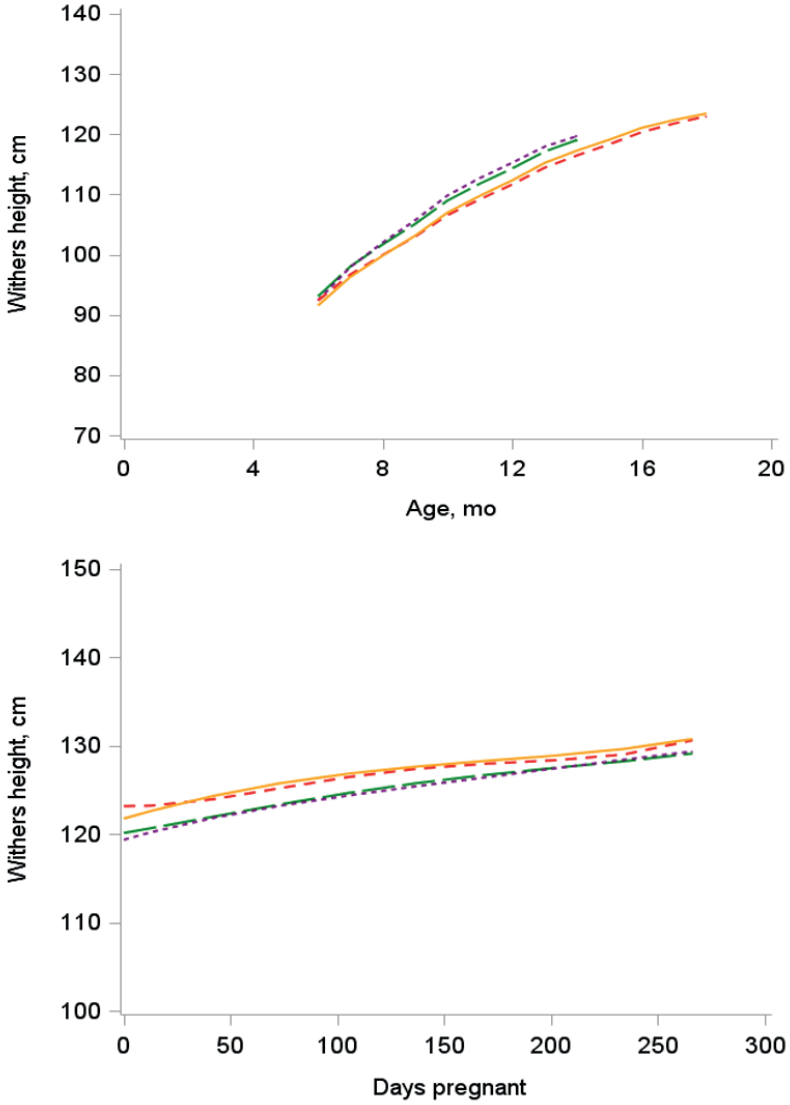

Figure 1. Development in withers height through experimental period (top) and pregnancy (bottom) for low-protein, high-energy (long-dashed line, green); high-protein, high-energy (dotted line, purple); low-protein, low-energy (short-dashed line; red); and high-protein, low-energy (solid line, orange) treatments.

Paper I

Designing a replacement heifer rearing strategy: Effects of growth profile on performance of Norwegian Red heifers and cows

R. Salte, K. S. Storli, H. K. L. Wærp, J. K. Sommerseth, E. Prestløkken, and G. Klemetsdal [1]

Department of Animal and Aquacultural Sciences, Faculty of Biosciences, Norwegian University of Life Sciences, PO Box 5003, 1432 Ås, Norway

[1]Corresponding author: gunnar.klemetsdal@nmbu.no

ABSTRACT

Our main objective was to develop an optimal replacement heifer rearing strategy for today's Norwegian Red. Eighty heifers born into the university herd, 40 each from year classes 2010 and 2011, were randomly assigned either to a high or low energy group planned for a body weight gain of 850 – 1,000 or 600 – 750 g/d from 3 mo of age to confirmed pregnancy, respectively. Each energy group was further subdivided into 2 protein groups. From confirmed pregnancy to first calving all animals were fed the same diet, adjusted to sustain an average daily gain of 450 – 550 g/d and reached a post-calving weight of 530 – 570 kg with a body condition score slightly less than 3.75 at calving. Statistical analyses were carried out by one-way analysis of variance or longitudinal models. We have shown that it is possible to rear Norwegian Red heifers for a rapid weight gain from 3 mo of age to successful insemination combined with a moderate average daily gain throughout pregnancy without negative effect on subsequent performance over 3 lactations. Thus, we recommend reducing age at first calving from the current national average of around 26 mo of age by 4 mo to 22 mo of age. This rearing practice would improve energy efficiency. Furthermore, there are indications that protein supply above requirement does not pay off, whereas the opposite might constitute a problem. Results further suggest that the TINE Optifôr Ungdyr client of NorFor is a highly efficient tool to achieve a predefined, desired growth rate.

Key words: longitudinal model, preconception, pregnancy, feeding, lifetime yield

INTRODUCTION

Replacement heifer rearing is a high-cost enterprise involving capital, land and labor, and it represents a major cost to the dairy farmer (Heinrichs, 1993; Gabler et al., 2000). Rearing should thus be evidence-based and designed to maximize lifetime performance of the animals (Brickell et al., 2009). A means to this end would be a heifer growth profile that allows animals to reach puberty and sexual maturity at an early age and then to be bred and calve without compromising future production. Developing such a model implies decisions on at what age heifers should reach puberty, and when to breed them to attain a desired age at first calving (**AFC**). Puberty is a function of weight rather than age of the animal (Wathes et al., 2014), and heifers of the dual-purpose Norwegian Red (**NR**) breed reach puberty at around 280 kg (Tine Rådgiving, 2017). Thus, to reach puberty and sexual maturity for them to conceive and go through pregnancy without adverse side effects at an early age heifer calves must grow fast. What defines fast, or a high ADG, seems, however, to vary between breeds (Hohenboken et al., 1995). In addition, comes the continuous genetic improvement of economically important traits attained through years of selection and breeding, with size, and thus growth, increasing as a correlated response to selection for milk yield (Geno, 2016).

Utilizing data from Hohenboken et al. (1995) from the mid - 1980s, Sejrsen et al. (2000) demonstrated a quadratic response in milk yield to increased post-weaning, prepubertal growth rate and suggested there exists an optimal prepubertal ADG for first lactation yield, 650 g/d at that time, which they assumed would increase with genetic potential for milk production. Later, a meta-analysis by Zanton and Heinrichs (2005) showed evidence of a similar prepubertal curvilinear relationship based on data from the 1990s, with maximum first lactation yield occurring at 799 g/d. Our recent field study suggested there exists an optimal and dynamic postpubertal ADG, currently some 830 g/d, which would increase

from selection over time (Storli et al., 2017). This would be consistent with the increased growth and development of the mammary parenchyma that occurs from puberty on (Capuco and Ellis, 2013). Findings also indicated that current national rearing practices do not meet the requirements of today's genetically improved NR heifer (Storli et al., 2017). Consequently, replacement heifer growth is too slow from birth to successful insemination, which again means that heifers on an average Norwegian dairy farm enter the milking herd at an unnecessarily high age. Based on this information we hypothesized that by improving the growth profile of the heifers we can reduce their unproductive period without compromising future lactation performance. Accordingly, our main objective was to develop an optimal replacement heifer rearing strategy for today's NR.

We approached this task by first contrasting groups of heifers with different planned growth profiles during both the pre- and postpubertal periods until confirmed pregnancy and intended similar growth profiles during pregnancy. Secondly, we evaluated the effects of the different growth profiles on lifetime ECM yield, i.e. through 3 lactations, of the same animals.

MATERIALS AND METHODS

Experimental Design

Eighty NR heifers born into the university herd, 40 each from year classes 2010 and 2011, BW at birth 37.1 kg (SD 4.3), were randomly assigned either to a high (**HE**) or low (**LE**) energy group planned for a BW gain of 850 – 1,000 or 600 – 750 g/d from 3 mo of age to confirmed pregnancy, respectively. Each energy group was further subdivided into 2 protein groups, one that was fed according to the protein requirement recommendation for the targeted growth rates using the TINE Optifôr Ungdyr client of NorFor, The Nordic feed

evaluation system (Volden, 2011). The other half was fed ~10 % higher CP level, to ensure that metabolizable protein supply would meet the requirements for rapidly growing bone and muscle and possibly promote lean tissue growth (Van Amburgh et al., 2001; Drackley, 2008). This gave 4 dietary treatments with 20 animals in each group designated high-energy low-protein (**HELP**), high-energy high-protein (**HEHP**), low-energy low-protein (**LELP**) and low-energy high-protein (**LEHP**). All heifers should further be successfully inseminated at a target BW of 400 kg. From confirmed pregnancy to first calving all animals should be fed the same diet, adjusted to sustain an ADG of 450 – 550 g/d, and they should reach a post-calving target weight of 560 kg with a BCS of maximum 3.75 at calving. First-lactation cows should be fed an equal diet according to a standard lactation curve aiming for a 305d yield of 7,500 kg ECM. In the second and third lactation, they should all be managed together with the rest of the university herd without any restrictions or claims other than the monitoring of milk yield and quality. Low fertility was the major reason for culling in first lactation, involving 5 and 6 from the LELP and LEHP groups, respectively, but only one each from the HE groups. All procedures were performed in compliance with the regulatory requirements that apply to the use of animals for scientific purposes in Norway and were approved by the National Animal Research Authority (FOTS id 2955, reference no 2010/203231).

Heifer Feeding

All heifer calves were fed the same diet from birth: 2 L good quality colostrum, preferably from the dam, within the first 2 h and another 4 L within 6 hours to ensure protective immune status (see e.g. Godden, 2008) and stimulate growth (Pakkanen and Aalto, 1997). Calves were held in separate pens for 10 days where they were offered (4 times a day) up to

6 L whole milk for 5 days, 50/50 whole milk/sour milk on day 6, and sour milk from day 7. Calves had free access to a commercial starter grain that contained 19.6% CP, 4.6% fat, 0.80% Ca, 0.45% P and 11.5% NDF (Formel Mysli Start, Felleskjøpet Agri, Lillestrøm, Norway), hay, and water. From day 10 they were moved to group housing. They were offered 7 L sour whole milk per day from automated feeder (Lely, Maassluis, The Netherlands), the starter grain (from the automated feeder) gradually increasing to a maximum of 1.3 kg/d at 49 d of age, and hay and silage *ad libitum*; they had free access to water at any time. Calves were weaned over a period of 2 wk from 35 to 49 d of age, after which also the starter grain was changed to Formel kalv (Felleskjøpet Agri) containing 19 % CP, 5.1 % fat, 1.0 % Ca, 0.7 % P and 21.3 % NDF. From start of the experiment at 3 mo of age until confirmed pregnancy, heifers were housed in a tie-stall barn and fed 1 of the 4 experimental diets. Fresh feed was offered twice daily. Individual feed intake was recorded a maximum of 4 d a wk. To avoid substitution differences between diets, all heifers were fed a fixed amount of 1 kg concentrate per d. To avoid hunger stress, LE animals were offered 0.5 kg extra wheat straw if the daily ration had been eaten before the evening routine.

HE heifers had *ad libitum* (> 10 %, or at least 1 kg ort) access to silage of adequate quality for the predetermined ADG, whereas the rations for LE heifers (consisting of the same silage, but with 10 -40 % wheat straw (on a DM basis)) were calculated with the Ungdyr client of TINE Optifôr and fed as restricted rations; the energy density and the protein level of the diets were adjusted with the roughage quality. To avoid hunger stress, LE animals were offered 0.5 kg extra wheat straw if the daily ration had been eaten before the evening routine.

The grass silages used were first, second and third cut from 11 fields of origin. The swards were dominated by timothy (*Phleum pratense*) and meadow fescue (*Festuca pratensis*) with varying concentrations of red clover (*Trifolium pratense*). The grass were added a formic

acid based additive and ensiled in big bales. Before the experimental feeding period started silage samples from the 11 fields of origin had been analyzed according to the NorFor package (Eurofins Agro, Moss, Norway) for chemical composition and fermentation quality by near-infrared spectrometry (NIR) The results were transferred electronically into TINE Optifôr and used for diet calculations. Wheat straw was analyzed in-house for DM concentration.

During the experimental period, weekly silage samples were stored at -20 °C. All samples were pooled, one subsample analyzed by near-infrared spectrometry (NIR - NorFor, Eurofins) and a second subsample analyzed chemically in-house for DM, CP (Expressed as Kjeldahl-nitrogen × 6.25), crude fat and NDF. Pooled concentrate and pooled wheat straw samples were only analyzed chemically in-house for DM, Ash, CP, crude fat, starch (only concentrates), and NDF. Dry matter in silage was determined by drying at 60ºC for 24 h and loss of volatiles during drying corrected as described in NorFor (Volden, 2011). Except for that, the analyses were conducted as follows: Dry matter by heating at 103 °C overnight, ash using ISO 5984 method (550 °C for a minimum of 4 h), Kjeldahl-N using Method 2001.11 (AOAC, 2002) according to Thiex et al. (2002) with Kjeltec 2400/2460 Auto Sampler System (Foss Analytical, Hilleroed, Denmark) and CP estimated as N × 6.25, total starch using AACCI Method 76-13.01 (Megazyme amyloglucosidase/α-amylase method), NDF with an ANKOM220 fiber analyzer (ANKOM Technology, Fairport, NY) according to Mertens (2002) using sodium sulfite and α-amylase but not corrected for residual ash, and finally, crude fat using an Accelerated Solvent Extractor (ASE200; Dionex, Sunnyvale, CA). In addition, fermentation products (FPF) and ammonia-N in fresh silage samples were analyzed by Eurofins.

Table 1 gives the average (SD) nutrient content of roughage and custom-made concentrates weighted by number of feed d on each experimental diet. Additional supply of vitamins and

minerals were given throughout the experiment. Note that all values of amino acids absorbed in the small intestine (metabolizable protein supply given as **AAT**), protein balance in rumen (**PBV**) and NE_L given in Table 1 are calculated values from Optifôr. The remaining LE roughage characteristics (Table 1) were calculated employing the 'make feed mixture' function in Optifôr entering data from the analyses of silage and wheat straw together with the amounts of these feed ingredients.

BW was recorded bi-weekly from 3 mo to successful insemination and then monthly until calving. BCS was recorded monthly, starting from 6 mo of age, and until calving. The Norwegian 1-5 scale BCS system is based on Edmonson et al. (1989) but calibrated for NR (Gillund et al., 1999), here with steps of 0.25; one BCS score unit is equivalent to ~ 60 kg.

To determine time of first estrus progesterone was measured by ELISA (Monobind inc., Lake Forest, CA, USA) in weekly serum samples taken from all animals from 220 kg BW until established cyclicity. First estrus was defined as the wk before the first sample with a progesterone level > 2 ng/ml, or the first wk of an established 3-wk cyclicity pattern. Breeding was initiated at 380 and 370 kg BW for the LE and HE groups, respectively, aiming for an average BW at successful insemination of 400 kg.

From confirmed pregnancy heifers were managed as the rest of the university herd and fed a roughage diet optimized to sustain an ADG of 450 – 550 g/d. This equaled an amount allowing free access to feed. To avoid competition for feed, there was at least 1 eating-place per animal at any time. No concentrate was offered, and feed intake was not measured. Heifers were housed in a deep-straw barn except for the compulsory 8 wk on pasture during the grazing season. A moderate growth on pasture was ensured by allocating all heifers to pastures of lower quality than the ones used for milking cows. During periods of draught, or early and late in the season when grass was scarce, heifers were fed the same roughage as

they were given indoors.

Detailed information on data recorded during the heifer-rearing period is given in Table 2.

Feeding of primiparous Cows

About 3 wk prior to expected calving, animals were moved to a free-stall barn. Cows were offered grass silages from different fields of origin as described earlier and blended in a Cormall multimix 30 M3 (Cormall, Sonderborg, Denmark) to give a total of 34 combinations aiming at a uniform nutrient content for both year classes during lactation. Animals were fed *ad libitum* (> 10 % orts) and silage intake was recorded automatically in bins with vertically moving gates, where automatic cow identification ensured each cow access (BioControl AS, Rakkestad, Norway). All animals were fed 0.5 kg/d commercial concentrate from 2 wk prior to calving and 1.0 kg/d in the last wk. After calving, they were given concentrates that met NorFor nutrient requirements optimized for a milk yield of 7,500 kg ECM using the TINE OptiFôr Ku client in NorFor (Volden, 2011) following an step-up scheme of 0.3 kg/d until a maximum of 7 to 9 kg/d depending on roughage quality and type of commercial concentrate. This level was kept until 120 DIM after which it was linearly reduced by about 33 g/d. Concentrate intake was recorded automatically.

Samples were taken from the silage combinations (weekly) and concentrates (weekly) used and stored at -20 °C. All silage samples were pooled, subsamples were analyzed by near-infrared spectroscopy (NIR-NorFor plus fermentation quality; Eurofins, Norway). Samples from each of the 4 concentrates were pooled. Subsamples of silages and concentrate were analyzed chemically as described for heifers. Nutrient composition, fermentation quality, and estimated feeding value of silage and concentrates are given as averages weighted by number of feed d in Table 3.

After calving BW was recorded twice daily, and BCS war recorded weekly. All cows were inseminated on the second observed estrus after calving. Milk yield data in first lactation was available daily, whereas fat, protein and lactose data were available on a monthly basis. In addition test-day ECM data from first, second and third lactation were extracted from the Norwegian Dairy Herd Recording System.

Detailed information on data recorded during the 3 lactations is given in Table 2.

NorFor Calculations

NorFor calculations (using the NorFor Development Tool, Volden, 2011) were performed separately for heifers and cows, but in both cases based on 3 sets of data. One set for feed contained roughage and concentrate values from Table 1 (heifers) and Table 3 (cows), respectively. A second set contained variables for each individual heifer per week; each record comprised BW at birth, BW at start and end of the experiment, BW per actual week, d in gestation per week, age at start of the experiment, age at the actual recording date, and growth rate per d in the actual week. The corresponding information for cows was on a daily basis; each record comprised the expected mature weight of 600 kg and an expected ADG of 0.1 kg/d, BW and BCS at calving, BW at the actual recording date (DIM), gestation d when pregnant during first lactation, as well as milk yield, fat and protein percentages per d and BCS change per d, where1 unit of BCS change was set to 60 kg BW. The third data set contained feed intake as DM per week for heifers, and per day for cows with one record per feed.

Because the length of the growth periods differed between energy levels, BW and DM intake of heifers were calculated separately per energy group (40 animals per group), whereas variables for cows (66 animals) included milk yield, protein and fat percentages,

BW, BCS, and finally DM intake of concentrate and silage. Predicted values per week (heifers) and per day (cows) were calculated with the following smoothing model (***Model 1***):

$$Y_{ij} = \mu + \beta_1 \cdot LEG_1 + \ldots + \beta_n \cdot LEG_n + \beta_{0i} \cdot LEG_0 + \ldots + \beta_{xi} \cdot LEG_x + e_{ij}$$

were Y_{ij} is 1 observation of each variable ; μ is the overall mean; $\beta_1, \ldots, \beta_n$ are fixed regression coefficients of 1st ,…, nth order Legendre Polynomials (***LEG***) for the average curve; $\beta_{0i}, \ldots, \beta_{xi}$ are random regression coefficients of orders 0 to x for individual animals deviating from the average curve, respectively,

assuming $\sim \boldsymbol{N}\left(\begin{pmatrix}\mathbf{0}\\ \vdots \\ \mathbf{0}\end{pmatrix}, \begin{pmatrix}\boldsymbol{\sigma^2_{\beta_0}} & \cdots & \boldsymbol{\sigma_{\beta_0\beta_x}} \\ \vdots & \ddots & \vdots \\ \boldsymbol{\sigma_{\beta_0\beta_x}} & \cdots & \boldsymbol{\sigma^2_{\beta_x}}\end{pmatrix}\right)$; where the diagonal contains the variances of the coefficients and the off-diagonal the co-variances. Finally, e is a random error term for the j-th measurement of $animal_i$. Heterogeneous residual variances were included for DMI of heifers and estimated for number of feed days per wk (1 - 4). The model complexity was based on the following principles: firstly, by determining the order of the fixed regression coefficients using F-tests, and then determining the number of random Legendre polynomials by use of the Bayesian Information Criterion (BIC). Legendre polynomials have frequently been used in animal breeding to model nonlinear relationships, e.g. how growth curves change with time (Schaeffer, 2016). All statistical analyses were carried out in SAS®.

As a final input into NorFor solely predicted values were used for BW and BCS, whereas for the remaining variables predicted values were inserted for missing values only.

Heifer Growth and BCS Development

The university herd consists of 2 breeding groups, a high-milk yield and a low-clinical mastitis group (Heringstad et al., 2007). To correct for the differences in production potential between groups, milk index (I) was included in all the following models.

Age of each experimental group was calculated at the start of the experimental feeding period (80 animals), at first estrus (79 animals), at successful insemination (79 animals) and at calving (66 animals) with a univariate model (***Model 2***):

$$Y_{ij} = \mu + T_i + \beta_I \cdot I + e_{ij}$$

where Y_{ij} is 1 observation of age (mo) either at the start of the experimental feeding period, at onset of puberty, at successful insemination or at calving; μ is the overall mean; T_i is the fixed effect of i-th treatment group (i = 1, 2, 3, 4; HELP, HEHP, LELP, and LEHP); β_I is the regression coefficient on the milk index of cows (I), and e is the random error term.

As opposed to age, individual BW at the start of the experimental feeding period was not known because calves were not necessarily weighed on the d they entered the trial. Thus, these weights had to be predicted. Likewise, we had to predict individual BW and BCS at first estrus, at successful insemination and at calving. To this end, we used Model 1 to fit a curve to BW and BCS records made for each individual animal during the relevant period and included an empty record at the date of either start, first estrus, successful insemination or calving. To calculate BW at the start of the experimental feeding period we utilized 589 BW records (after deleting 10 outliers) from birth to start of the experimental feeding period, i.e. from 0 to 3 mo of age; note that these weights were recorded before the experimental feeding period started. The data used to calculate BW and BCS at first estrus and at successful insemination were restricted to data recorded during the experimental period, i.e. 2,026 and 714 records, respectively (after deleting 15 and 8 outliers). Data used to calculate calving weight involved the last record before calving and records until

100 DIM, altogether 3,881 records (after deleting 17 outliers), whereas those used to calculate BCS at calving comprised 612 records from 200 days prior to calving until the first recording after calving (after deleting 10 outliers). In all cases, the fixed polynomials were either of order 3 or 4 and the order of the random regression up to 10 and 5, respectively, for BW and BCS at calving. The predicted BW and BCS from Model 1 then replaced age (mo) in Model 2 to obtain solutions for the 4 experimental groups.

Cumulative Intake of DM, NE_G and CP throughout the experimental Period

Utilizing DMI, CP or NE_G, each comprising 4,243 records (80 animals) over the experimental period and deleting 74, 76 and 88 records with residuals > 3 the following smoothing model was used to predict individual values per day for the 3 variables (**Model 3**):

$$Y_{ijkl} = \mu + \beta_1 \cdot LEG_1 + \ldots + \beta_n \cdot LEG_n + \beta_{0i} \cdot LEG_0 + \ldots + \beta_{xi} \cdot LEG_x + YS_j + f_k + e_{ijkl}$$

were Y_{ijkl} is 1 observation of either DMI, NE_G or CP; where μ; $\beta_1, \ldots, \beta_n$, *LEG, and* $\beta_{0i}, \ldots, \beta_{xi}$ and e are described in Model 1; YS_j is the j-th fixed year-season effect of test (j = 1, …, 5; wk 51 2010 – wk 23 2011, wk 24 2011 – wk 50 2011, wk 51 2011 – wk 23 2012, wk 24 2012 – 46 2012, wk 47 2012 – wk 39 2013), and f_k is the random effect of the 11 fields of origin of the fed roughage $\sim \boldsymbol{N(0, \sigma_f^2)}$**.** All variables had 3 fixed polynomials and either 3 or 4 (NE_G) random polynomials. Heterogeneous residual variances were estimated in 5 age groups (3-6 mo, 6 – 9 mo, 9 – 12 mo, 12 – 15 mo, and > 15 mo).

For all 3 variables the predicted values per individual were integrated over two periods, i.e. start of experimental feeding period to onset of puberty and from puberty to successful insemination. These cumulated variables were then entered into Model 2 in order to estimate treatment group effects.

Longitudinal Depiction of pre- and post-conception Heifer BW and BCS

Pre-conception heifer BW and BCS, 2,041 and 722 records (80 animals), respectively, minus outliers with residuals > 3 (17 and 8, respectively) were analyzed with **Model 4**:

$$Y_{ijkl} = \beta_0 \cdot LEG_0(T_i) + \ldots + \beta n \cdot LEGn(T_i) + \beta_{0i} \cdot LEG_0(T_i) + YS_j + f_k + \beta_I \cdot I + e_{ijkl}$$

where Y_{ijkl} is 1 observation of either BW or BCS in each period; $\beta_0, \ldots, \beta_n$ are fixed regression coefficients of 0^{th},…., n^{th} order *LEG* within T_i, the fixed effect of the *i*-th treatment group (*i* = 1, 2, 3, 4; HELP, HEHP, LELP, and LEHP); β_{0i} is the random regression coefficient of an animal within a treatment class $\sim \boldsymbol{N(0, \sigma^2_{animal})}$, and *e* is described in Model 1, YS_j and f_k in Model 3, and $\beta_I \cdot I$ in Model 2. Preconception variables had fixed polynomial order of 2. Heterogenous variances were estimated in 5 age groups as described in Model 3. In the pregnancy period, there were 1,354 BW and 819 BCS records (74 animals) and 6 and 15 outliers were removed. The polynomial orders were 4 and 3 for BW and BCS, respectively, and f_k was removed from the model (**Model 5**). Least-squares means were used to depict the trajectories over the two period.

Longitudinal Depiction of NorFor Output Variables from 3 mo to successful Insemination

All variables, i.e. DMI, NE_G, **NDFI** (in kg/d and defined as (NDF x DMI)/1000), PBV, CP_intake and AAT had 4,243 records (80 animals), from which in between 68 and 81 outliers were removed, and the remaining data analyzed with Model 4. All variables had a fixed polynomial complexity of 3^{rd} order except NE_G for which a second order polynomial

was used. Heterogeneous residual variances were estimated as with Model 3. Least-squares means were used to depict trajectories.

Longitudinal Depiction of first Lactation Test-Day Milk Yield, BW, BCS and NorFor output Variables

Test-day milk yield, BW, BCS (with 12,134, 9,146, and 2,192 records after outlier removal, respectively), DMI (kg DM), PBV_DM (protein balance in rumen, g/kg DM), NE_L_DM (NE_L/DMI, MJ/kg DM), AAT_NE_L (amino acids absorbed in the small intestine in g/MJ), NE_L_BAL (energy balance for cows, NE_L/ total net energy requirement, in %) and NE_L_ECM (NE_L/ ECM, MJ/kg ECM) (with 14,929 to 14,875 records), were all analyzed with Model 4. The order of the fixed effect polynomials were 3 for MY, BCS, AAT_NE_L, NE_L_DM, and NE_L_ECM, whereas a 4^{th} order polynomial was used for the 4 remaining variables. The YS comprised 6 classes (01.06.2012 – 31.12.2012, 01.01.2013 – 30.04.2013, 01.05.2013 –31.08.2013, 01.09.2013 – 31.12.2013, 01.01.2014 – 30.04.2014, 01.05.2014 – 31.05.2015), whereas *f* had 34 classes, i.e. combinations from fields of origin of the fed roughage. Five heterogeneous residual variances were defined (1 – 14 DIM, 15 – 56 DIM, 57 – 98 DIM, 99 – 140 DIM, 141 – 305 DIM). Again, least-squares means were used to depict trajectories.

Predicted 305-d ECM Yield in first, second, and third Lactation

To calculate 305-d ECM yield 643, 361 and 246 records in first, second and third lactation, respectively (after outlier removal), were extracted from the Norwegian Herd Recording System. Removing *f* from the model, the data were analyzed with Model 4 to obtain daily ECM yield per animal. The number of fixed polynomials was 4 and the number of random

polynomials was 3 in the first 2 lactations and 2 in the third. *YS* and heterogeneous variances were defined as when analyzing daily milk yield above. ECM yield was then cumulated over 305-d of lactation and treatment group effects were estimated with Model 2 after removing $\beta_I \cdot I$ from the model.

RESULTS

Figure 1 depicts heifer growth and BCS in the 4 treatment groups between 3 mo of age and successful insemination (Figures 1a and 1c) and throughout pregnancy (Figures 1b and 1d), whereas Figure 2 depicts measured DMI, NDFI, and CP_intake (Figure 2a, c and e) and calculated intake of NE_G, PBV and AAT (Figure 2b, d and f). Figure 1 also illustrates the age differences established between HE- and LE-groups at successful insemination (Figure 1a).

At the start of the experimental feeding period all heifers were the same age and BW, whereas BCS was not applicable (Table 4). At the onset of puberty, the heifers had reached the physiologically determined BW between 280 and 300 kg as planned (Table 4). HE heifers were significantly younger than LE heifers ($P < 0.0002$; Table 4), but LEHP animals were also significantly younger than LELP heifers ($P = 0.0062$). At the same time, BCS of the HE heifers was significantly higher than that of their LE counterparts ($P < 0.0001$; Table 4), but the BCS of HE animals as well as LE heifers were unaffected by protein level. At successful insemination, the age difference between HE- and LE heifers had increased further according to plan ($P < 0.0001$; Table 5). In addition, HELP heifers were significantly older than HEHP heifers ($P = 0.036$; Table 5), whereas there was no age difference between the LE groups. At successful insemination average BW was near the target weight of 400 kg for all 4 groups, but HELP heifers tended to be heavier than their HEHP contemporaries (*P*

= 0.052; Table 5) and were significantly heavier than LELP heifers (P = 0.019). BCS of HE heifers was still higher than that of LE heifers ($P < 0.0001$; Table 5). This is also illustrated in Figure 1c, where the dip of the polynomial towards the tail of the curve representing BCS of the HEHP group is likely due to few observations.

From successful insemination to calving, all animals were fed the same diet *ad libitum* and feed intake was not monitored. During the first 50 d of the gestation period the LE heifers apparently reduced their BW (Figure 1b), followed by a period of continuous increase in BW. Moreover, the differences in BCS established between HE and LE animals at successful insemination disappeared through the gestation period; HE-groups reduced their score whereas LE-groups remained unchanged as all groups converged towards a BCS of around 3.75 at calving (Figure 1d).

At calving, HE animals were some 4 months younger than LE heifers, around 22 and 26 mo of age, respectively ($P < 0.0001$; Table 5). Whereas LE-groups were near the target BW of 560 kg, HE-groups were some 30 kg lighter, significant ($P < 0.05$) for 3 of the 4 contrasts. The HEHP animals had the lowest BCS at calving, significant ($P < 0.018$) relative to the LEHP group.

Table 6 gives cumulative DMI, NE_G and CP_intake from 3 mo of age to onset of puberty, and from onset of puberty to successful insemination to separate intake in the prepubertal phase from that of the postpubertal period. Consequently, differences between groups were tested separately for the 2 periods. From start of the experimental feeding to onset of puberty LE heifers had a significantly higher cumulative DMI than HE heifers ($P < 0.031$; Table 6). The LELP group also had a higher cumulative DMI than LEHP ($P = 0.027$; Table 6), whereas HE-groups did not differ from one another. Between puberty and successful insemination, the differences were much smaller and significant only between HEHP and

LEHP heifers, the latter being the highest ($P = 0.003$; Table 6). With cumulated NE_G intake, the picture was like that of cumulative DMI with the exception that cumulative NE_G intake of the HE-groups from start to onset of puberty were now not significantly different from that of the LEHP heifers ($P > 0.05$; Table 6). The pattern of cumulative NE_G intake in the period between puberty and successful insemination was like that of cumulative DMI (Table 6). Cumulative CP_ intake did not differ between groups in either of the periods ($P > 0.05$; Table 6).

Figure 3 illustrates differences between groups in test-day milk yield (Figure 3a), in BW (Figure 3b) and in BCS (Figure 3c) during the first lactation. The HEHP group produced less than the other groups from the onset and throughout the first lactation, whereas differences between the other 3 were negligible (Figure 3a). Figure 3b illustrates that heifers were still growing, and Figure 3c that they reduce their BCS somewhat during the first lactation; again, the waiving tails of the curves are likely due to few observations.

Figure 4 depicts DMI, PBV_DM, NE_L_DM, AAT_NE_L, NE_L_BAL, and NE_L_ECM. Differences between groups are small and again, the explanation of the waiving tails is as before.

Table 7 lists 305-d ECM yield in the first, second and third lactation of the 4 groups and summed up over the 3 lactations. In the first lactation, i.e. in the experimental period, there were no significant differences between groups ($P > 0.05$). When ECM yield was summed up over the 3 lactations the HELP-group produced numerically the most and more than LELP followed by HEHP and LEHP.

DISCUSSION

Using a simple, roughage-based feeding strategy where all animals were fed just 1 kg of concentrate and both energy and protein supply were regulated with roughage quality employing the TINE Optifôr Ungdyr client of NorFor (Volden, 2011), we made 4 groups of heifers that entered puberty at ~ 9, 9, 11 and 12 mo of age with BCS of respectively 3.8, 3.8, 3.6 and 3.6, but near identical weight because they followed different targeted growth curves. Consequently, we could time the insemination of the heifers so that the fast-growing HE - groups calved at a somewhat (~30 kg) lower BW, but similar BCS within the targeted maximum of 3.75 at 22 mo, and thus 4 mo younger than their slower growing LE contemporaries, without negative effects on ECM yield over 3 lactations, i.e. over the average lifetime of a NR cow who goes through 2.7 lactations until culling (Norwegian Dairy Herd Recording System, 2017).

Age at puberty is an important trait in dairy production systems (Archbold et al., 2012, Wathes et al., 2014). High energy intake has previously been shown to reduce the age at which heifers reach puberty (Radcliff et al., 1997; Meyer et al., 2006a). Accordingly, our HE heifers were significantly younger at puberty than LE heifers ($P < 0.0002$; Table 4), consistent with the results of Radcliff et al. (1997) and Meyer et al. (2006a). LEHP animals were also significantly younger than LELP heifers ($P = 0.0062$). BW at puberty was, however, unaffected by nutrient intake (Table 4), again in line with the results of Radcliff et al. (1997) and Meyer et al. (2006a). All groups followed their targeted growth curves (Figure 1) from 3 mo of age to onset of puberty at the physiologically determined (Joubert, 1963) 280 to 300 kg (TINE Rådgiving, 2017), i.e. at 40 – 45% of their expected average mature BW, currently 658 kg, and thus similar to Holstein (Heinrichs, 1993; Van Amburgh et al., 1998), but somewhat below the National Research Council (2001) expectations of about 55% of the mean mature BW of the herd for dual-purpose and dairy heifers (Figure

1a, Table 4). Similarly, they had achieved their target BW of 400 kg, or around 60% of mature weight, at successful insemination (Figure 1b, Table 5). Current recommendations for the NR breed given by the Norwegian dairy cooperative (TINE Rådgiving, 2017) are to achieve around 60% of mature BW (390 – 415 kg) at breeding, and this also compares well with current recommendations for Holsteins heifers which should be bred between 55 and 60% of their mature BW (Troccon 1993; Soberon and Van Amburgh, 2011). BCS of the LE-groups stayed within the targeted maximum score of 3.75, whereas the HE-animals were somewhat over-conditioned already before the onset of puberty (Figure 1c). At puberty BCS of the HE heifers was significantly higher than that of their LE counterparts ($P < 0.0001$; Table 4). This is just due to the HE heifers having free access to roughage. That accelerated heifer rearing can lead to excessive fat deposition has long been known (Swanson 1960; Stelwagen and Grieve, 1990).

From 3 mo until successful insemination curves depicting DMI, NE_G, and CP_intake (Figure 2) all followed a consistent pattern with steeper curves for the HE-groups. Intake of NDF was virtually the same in both HE- and LE-groups until around 8 mo of age, indicating that all animals ate to satiation until 8 mo, whereafter an effect of the restricted straw-diluted roughage supply to LE-heifers became evident (NDFI; Figure 2c). Intake of CP (CP_intake; Figure 2e) shows that both HE-groups were in fact also HP-groups, whereas both LE-groups were LP-groups; the HP- and LP-groups could only be identified as marginal protein-level differences within the HE- and LE-groups. This means that what we compare in the present material are mainly effects of the 2 energy levels. Nevertheless, LEHP animals reached puberty some 30 days earlier than LELP heifers (P=0.006; Table 4), and at a somewhat lower weight (P = 0.38). AAT and PBV values (Figures 2d and f) give further insight into the effects of the protein supply in that both LE-groups, and in particular the LELP-group, had PBV-values well on the negative side. This might suggest that the LE-groups

experienced a situation where the available amount of degradable protein limited the synthesis of microbial protein, which to some extent may explain why the LELP heifers experienced the lowest growth rate of the 4 groups. In the Norfor system, the calculation of AAT assumes, however, an adequate amount of PBV in the ration. A too low PBV would lead to AAT being overestimated (Madsen, 1985; Madsen et al 1995), and thus explain why calculation of the AAT-balance (results not shown) could not confirm the protein shortage that is evident from the PBV calculations. It has previously been suggested (Drackley, 2008) that the diet should have a higher protein content when aiming to exploit the high lean tissue growth potential of young heifers and promote lean tissue growth without fattening. With our NR heifers this did not seem to be the case; a protein supply higher than NorFor recommendations did not promote lean growth from 3 mo of age through onset of puberty to successful insemination in any of the energy groups (Figure 1c, Tables 4 and 5), -and besides, our results suggested that protein supply was limiting in the LELP group. This would mean that protein supply above requirement does not pay off, whereas the opposite might constitute a problem.

When portraying nutrient intake as cumulative values (Table 6), and not as intake per d, we account for the fact that the number of d needed for the animals to reach a given stage of physiological development at nearly the same BW differed among the groups; otherwise it would be difficult to make fair comparisons between HE- and LE-groups. Looking at cumulative DMI and NE_G values, we found the lowest values in the HE-groups. This means that HE animals consistently used less feed and energy than LE animals to reach a given stage of physiological development at nearly the same BW. This is likely because LE-animals would require more nutrients for maintenance over their longer rearing period. On the other hand, when we consider cumulative CP_intake there were no significant

differences between groups ($P > 0.05$; Table 6), because HE animals consumed a considerably higher amount of CP per d for a shorter period than the LE-groups.

From confirmed pregnancy to calving the 4 groups were managed together with the rest of the university herd and were offered the same roughage *ad libitum*, but no concentrate; BW and BCS were recorded, but not feed intake. During pregnancy, HE-animals displayed growth rates towards the lower end of the planned 450 – 550 g/d, whereas the LE animals revealed growth rates higher than 550 g/d. For the HE-groups, this meant transfer from a diet, which until confirmed pregnancy had resulted in an ADG of between 900 and 1,000 g/d to one that halved this growth rate. The HE heifers responded by reducing their body condition score to end up with a score near equal to that of the LE-groups at the end of the gestation period (Figure 1d). The mechanism behind would be that the feed restriction lead to increased secretion of Growth hormone (GH) from the pituitary, but also a decreased number of GH receptors. The resulting high circulating levels of GH would increase mobilization of fatty acids to meet energy requirements (Hornick et al, 2000). Moreover, the animals were still growing, but the composition of growth in this situation would favor lean tissue growth whereas fat would barely be deposited (Hornick et al, 2000). During the part of the experimental feeding period until confirmed pregnancy the LE-groups had, on the other hand, experienced a diet where the straw content likely led to a physical regulation of roughage intake (Dulphy and Demarquilly, 1994). From confirmed pregnancy, they were offered roughage *ad libitum*, but now without added straw. Figure 1b suggests that they responded by switching to metabolic regulation of their feed intake (Dulphy and Demarquilly, 1994), meaning that they could reduce their roughage intake but still feel satiated. What appears as a reduction in growth rate in the first part of the pregnancy followed by compensatory growth in the remaining period (Figure 1b) is therefore likely just

an underlying linear growth rate during the whole pregnancy, this because their BCS remained unchanged throughout the gestation period (Figure 1d).

What distinguished primiparous HE animals from LE animals other than that they calved 4 months earlier, was that they gave birth at a somewhat lower body weight than the LE animals (Figure 1b and 3b, Table 5). HEHP heifers also calved nearly 3 weeks younger than HELP animals, in line with the findings of Radcliff et al. (2000). While HE heifers had achieved a mean post-calving BW of some 82% of the mean mature BW of the herd, the corresponding post-calving BW for the 4 mo older LE heifers were just above 85%, both comparable with growth bench marks of 82 – 85% of mature weight for Holstein heifer rearing (Fox et al., 1999; National Research Council, 2001). HE-heifers further calved with a close to similar BCS, around 3.7, to that of the LE animals (Figure 1d, Table 5), and near the nationally recommended maximum score of 3.75 for NR heifers (TINE Rådgiving, 2017). A higher BCS among the HE heifers was evident throughout the gestation period (Figure 1d), although the differences at the end of the gestation period were small, which compares well with the findings of Radcliff et al. (2000). The seeming inconsistency relative to the somewhat lower BCS displayed by the HE animals post calving (Figure 3c) is most likely due to the correction for YS in Model 4, taking effect on the least-squares means in the gestation period.

First-lactation curves of HELP animals closely followed that of their LE congeners, whereas that of HEHP animals followed a slightly flatter trajectory (Figure 3a). Dry matter intake increased towards peak lactation and followed the lactation curve, as would be expected Figure 4a); the low DMI at the start of the lactation is most likely a continuation of the dip that would have been initiated in late pregnancy (Ingvartsen and Andersen, 2000). The net energy value of the total ration (NE_L_DM, MJ/kgDM; Figure 4c) was similar for all groups, but of course somewhat higher for the HEHP group that produced the least and thus had the

lowest DMI (Figure 4a). All groups appeared to be in energy balance, with NE_L_BAL (Figure 4e) being close to 100 % throughout the lactation. All groups increased their BW during the first lactation to some 600 kg or just above 90% of mature weight (Figure 3b) according to expectations, and again comparable with target weights for Holstein (Fox et al., 1999). The LE groups lost condition for 60 – 70 days after calving because of upregulation of lipolysis in adipose tissue (Roche et al., 2009), and a less distinct decrease was evident in HELP animals Figure 3c); BCS of the HEHP group, however, seemed to follow the lactation curve rather than presenting a mirror of it (Roche et al., 2009). The protein balance in rumen (Figure 4b) was above the 10 g/kg DM recommended in NorFor in all groups, and the amino acids absorbed in the small intestine to NE_L ratio (AAT_NE_L, g/MJ, Figure 4 d) was in line with the recommended 15 g/MJ of AAT_NE_L (Volden, 2011).

In first lactation, there were no significant differences in ECM yield between groups (Table 7). Interestingly, cumulative ECM yield over 3 lactations was numerically highest in the HELP group (Table 7), meaning that accelerated growth during the rearing period leading to an AFC of 22 mo also did not impair milk production over 3 lactations; this is consistent with the results of Wathes et al. (2008), who showed that heifers calving at 22-23 mo of age had the highest lifetime yield over 5 yrs. As shown in our previous paper (Storli et al., 2017) for the highest individual growth rates a heifer's age becomes a limiting factor, explaining the curvilinear relationship between postpubertal ADG and first-lactation yield. The curvilinear relationship supports the suggestion that a heifer's physiological age is a major player in mammary development (Meyer at al., 2006b), and besides, suggests that an optimum ADG for first-lactation yield would be the one at which physiological age, BW and stage of mammary development are harmonized. The curvilinear relationship further emphasizes the importance of the post-pubertal period and also provides an explanation of why some studies have reported a positive association between postpubertal ADG and first-

lactation yield (Macdonald et al., 2005; Archbold et al., 2012; Krpálková et al., 2014) whereas others found no such effect (Hoffman et al., 1996). When the fastest growing HEHP animals experienced a postpubertal ADG of 1,000 g/d, we hypothesize that the reason why this group had the lowest first lactation yield of the 4 groups was that their growth rate exceeded the optimum for first-lactation yield in the postpubertal period (Storli et al. 2017). The HELP group, which grew more slowly, but still fast at 948 g/d post-pubertally may thus have been closer to their ADG optimum for subsequent yield. Why the LELP group produced on a par with the HELP animals even though they had experienced ADG just as far from their optimum could be because they were heavier than their HE peers at calving, and therefore nearer their mature weight. Interestingly, both HP groups produced numerically less in first lactation than their LP peers within energy level, and the tendency was also evident in second lactation (Table 7). Since level of dietary protein should not affect mammary development (Dobos et al., 2000) this could to some extent be due to HP animals calving 2 to 3 weeks younger (Radcliff et al., 2000) (Table 5). But we may also hypothesize that protein supply in excess of requirements in early life could affect future production through a change of rumen microbial composition and function (Malmuthuge and Guan, 2017).

The present study gives no insight into reproduction and disease traits that are crucial to milk production in Norway, where longevity hardly varies due to a high meat price for cows. Such information from a lifetime perspective can, however, be obtained from a re-analysis of the field dataset from Storli et al. (2017) when all animals either have finalized or are close to finalizing their productive life.

CONCLUSIONS

We have shown that it is possible to rear NR heifers for a rapid weight gain from 3 mo of age to successful insemination combined with a moderate ADG throughout pregnancy without negative effect on subsequent performance over 3 lactations. Thus, we recommend reducing AFC from the current national average of around 26 mo of age by 4 mo to 22 mo of age. This rearing practice would improve energy efficiency. Furthermore, there are indications that protein supply above requirement does not pay off, whereas the opposite might constitute a problem. Results further suggest that the TINE Optifôr Ungdyr client of NorFor (Volden, 2011) is a highly efficient tool to achieve a predefined, desired growth rate.

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Table 1. Average (SD) nutrient composition and estimated feeding value of roughages and concentrates weighted by number of feed d during the experimental feeding period and pregnancy (g/kg DM if not stated otherwise). Animals were not fed any concentrate during pregnancy

	From 3 mo of age to successful insemination				Pregnancy to calving
	Roughage		Concentrate		Roughage
Variables[1]	HE (SD)	LE (SD)[2]	LP	HP	All groups
DM, g/kg	323 (40.8)	403 (48.9)	856	855	390 (35.5)
Ash	68 (8.0)	62 (6.5)	88	98	53 (11.9)
CP	140 (13.7)	107 (8.9)	155	233	113 (2.4)
Soluble CP, g/kg CP	567 (37.8)	518 (36.8)	.	.	519 (79.8)
Crude fat	34 (2.9)	28 (1.6)	44	53	35 (2.7)
Starch	.	.	372	263	.
NDF	542 (18.5)	631 (25.8)	176	178	569 (34.3)
iNDF, g/kg NDF	162 (40.2)	197 (23.4)	.	.	207 (50.1)
Sugar	38 (30.5)	29 (23.7)	.	.	27 (53.5)
AAT_{N8}	64	62	83	98	62
PBV_{N8}	42	15	43	103	21
NE_{LN8}, MJ/kg DM	7.0	6.5	7.6	7.7	6.6

[1]Neutral detergent fiber (NDF), indigestible NDF (iNDF), amino acids absorbed in the small intestine (AAT_{N8},), standard feed value for protein balance in rumen (PBV_{N8}), and net energy lactation (NE_{LN8}), the 3 latter calculated values (TINE Optifôr) at 8 kg DMI; to calculate these variables, a weighted (by feed d) average for the 3 roughages (HE, LE, pregnancy to calving) and 2 concentrates (LP, HP) were entered into TINE Optifôr. CP was calculated as Kjeldahl Nitrogen x 6.25.

[2]Straw included

Table 2. Description of data recorded during the heifer-rearing period and during the 3 lactations, with number of records

Variables	Frequency	n
3 mo to successful insemination		
BW, kg	Bi-weekly	2,041
BCS, 1-5 scale[1]	Monthly[2]	722
Roughage intake, kg/d	Weekly mean[3]	3,479
Concentrate intake, kg/d	Weekly mean[3]	3,479
Silage sample	Weekly, pooled	11[4]
Straw sample	Weekly, pooled	5
Concentrate sample	Weekly, pooled	2
Progesterone, ng/mL	Weekly[5]	1,198
Successful insemination to first calving		
BW, kg	Monthly	1,354
BCS, 1-5 scale[1]	Monthly	819
First lactation		
BW, kg	Twice daily, automatic	9,304
BCS, 1-5 scale[1]	Weekly	2,239
Silage intake, kg/d	Automatic	12,900
Concentrate intake, kg/d	Automatic	12,697
Silage sample	Weekly, pooled	34[4]
Concentrate sample	Weekly, pooled	4
Test-day milk yield, kg	Daily, automatic	12,312
Fat content, %	Monthly	787
Protein content, %	Monthly	792
Lactose content, %	Monthly	792
First, 2nd and 3rd lactation		
Test-day milk yield, kg ECM, 1st lactation	Monthly	654
Test-day milk yield, kg ECM, 2nd lactation	Monthly	365
Test-day milk yield, kg ECM, 3rd lactation	Monthly	246

[1] 1-5 scale with increments of 0.25 (Gillund et al., 1999)

[2] From 6 mo of age

[3] Based on 1-4 registrations per wk

[4] Equals number of fields of origin (heifers) or combinations from fields of origin (cows)

[5] Measured weekly from 220 kg to established cyclicity

Table 3. Average (SD) of nutrient composition, fermentation quality and estimated feeding value of silage and concentrates weighted by number of feed d in milk (g/kg DM if not stated otherwise)

Variables[1]	Silage (SD)	Concentrate (SD)
DM g/kg	343 (53.6)	854 (9.4)
Ash	73 (12.4)	78 (3.2)
OMD, %	76 (2.4)	.
CP	145 (13.0)	204 (22.8)[2]
Soluble CP, g/kg CP	619 (48.7)	.
Crude fat	.	55 (4.7)
Starch	.	312 (40.6)
NDF	483 (32.0)	172 (11.0)
iNDF, g/kg NDF	155 (29.0)	.
Sugar	91 (19.9)	.
Ammonia nitrogen, g N/kg N	70 (17.1)	.
Lactic acid	45 (9.3)	.
Acetic acid	8.1 (2.8)	.
Propionic acid	2.3 (1.9)	.
Butyric acid	0.0 (0.1)	.
Formic acid	6.3 (2.2)	.
Alcohol	7.7 (1.9)	.
pH	4.5 (0.2)	.
AAT_{N20},	85	139
PBV_{N20},	14	8
NE_{L20}, MJ/kg DM	6.4	7.3

[1] Apparent total digestibility of organic matter (OMD), neutral detergent fiber (NDF), indigestible NDF (iNDF). To calculate standard feed values for amino acids absorbed in the small intestine (AAT_{N20}), protein balance in rumen (PBV_{N20}), and net energy lactation (NE_{L20}) at 20 kg DMI, weighted (by feed d) averages of silage and concentrates were entered into Tine Optifôr

[2] Kjeldahl nitrogen × 6.25

Table 4. Least-squares means (diagonal) and contrasts (off-diagonal) of age (mo), BW (kg) and BCS (1-5 scale) for experimental groups (high-energy low-protein, HELP, high-energy high-protein, HEHP, low-energy low-protein, LELP and low-energy high-protein, LEHP) at start of experimental feeding and at onset of puberty. Level of significance in parentheses

		Start experimental feeding				Onset of puberty			
		HELP	HEHP	LELP	LEHP	HELP	HEHP	LELP	LEHP
Age	HELP	**3.0**	-0.02 (0.543)	0.00 (0.972)	0.00 (0.994)	**9.1**	0.22 (0.584)	-2.76 (<0.0001)	-1.59 (0.0002)
	HEHP		**3.0**	0.03 (0.520)	0.02 (0.538)		**8.8**	-2.98 (<0.0001)	-1.82 (<0.0001)
	LELP			**3.0**	-0.00 (0.978)			**11.8**	1.16 (0.0062)
	LEHP				**3.0**				**10.7**
BW	HELP	**114**	1.51 (0.648)	-0.21 (0.950)	-0.28 (0.932)	**282**	5.36 (0.616)	-8.05 (0.458)	1.50 (0.888)
	HEHP		**113**	-0.71 (0.603)	-1.79 (0.588)		**276**	-13.41 (0.217)	-3.86 (0.717)
	LELP			**114**	-0.07 (0.983)			**290**	9.55 (0.379)
	LEHP				**114**				**280**
BCS	HELP	.	.	.	.	**3.83**	0.04 (0.246)	0.23 (<0.0001)	0.26 (<0.0001)
	HEHP		.	.	.		**3.78**	0.19 (<0.0001)	0.21 (<0.0001)
	LELP			.	.			**3.59**	0.02 (0.539)
	LEHP				.				**3.57**

Table 5. Least-squares means (diagonal) and contrasts (off-diagonal) of age (mo), BW (kg) and BCS (1-5 scale) for experimental groups (high-energy low-protein, HELP, high-energy high-protein, HEHP, low-energy low-protein, LELP and low-energy high-protein, LEHP) at successful insemination and at calving. Level of significance in parentheses

		Successful insemination				Calving			
		HELP	HEHP	LELP	LEHP	HELP	HEHP	LELP	LEHP
Age	HELP	**13.6**	0.85 (0.036)	-3.44 (<0.0001)	-3.25 (<0.0001)	**22.5**	0.62 (0.131)	-3.61 (<0.0001)	-3.23 (<0.0001)
	HEHP		**12.8**	-4.29 (<0.0001)	-4.11 (<0.0001)		**21.9**	-4.23 (<0.0001)	-3.85 (<0.0001)
	LELP			**17.1**	0.19 (0.635)			**26.1**	0.38 (0.364)
	LEHP				**16.9**				**25.8**
BW	HELP	**411**	14.62 (0.052)	17.67 (0.019)	5.18 (0.481)	**537**	4.86 (0.756)	-33.88 (0.037)	-26.85 (0.106)
	HEHP		**396**	3.05 (0.682)	-9.44 (0.206)		**532**	-38.73 (0.014)	-31.71 (0.050)
	LELP			**393**	-12.49 (0.092)			**571**	7.02 (0.663)
	LEHP				**406**				**564**
BCS	HELP	**3.98**	0.054 (0.056)	0.25 (<0.0001)	0.25 (<0.0001)	**3.70**	0.05 (0.109)	-0.01 (0.822)	-0.03 (0.425)
	HEHP		**3.93**	0.20 (<0.0001)	0.19 (<0.0001)		**3.65**	0.06 (0.064)	-0.08 (0.018)
	LELP			**3.73**	-0.01 (0.85)			**3.71**	-0.02 (0.555)
	LEHP				**3.73**				**3.73**

Table 6. Least-squares means (diagonal) and contrasts (off-diagonal) of cumulated DMI (kg), cumulated NE_G (MJ), and cumulated CP_intake (kg) for experimental groups (high-energy low-protein, HELP, high-energy high-protein, HEHP, low-energy low-protein, LELP and low-energy high-protein, LEHP) from start of experimental feeding to onset of puberty and from puberty to successful insemination. Level of significance in parentheses

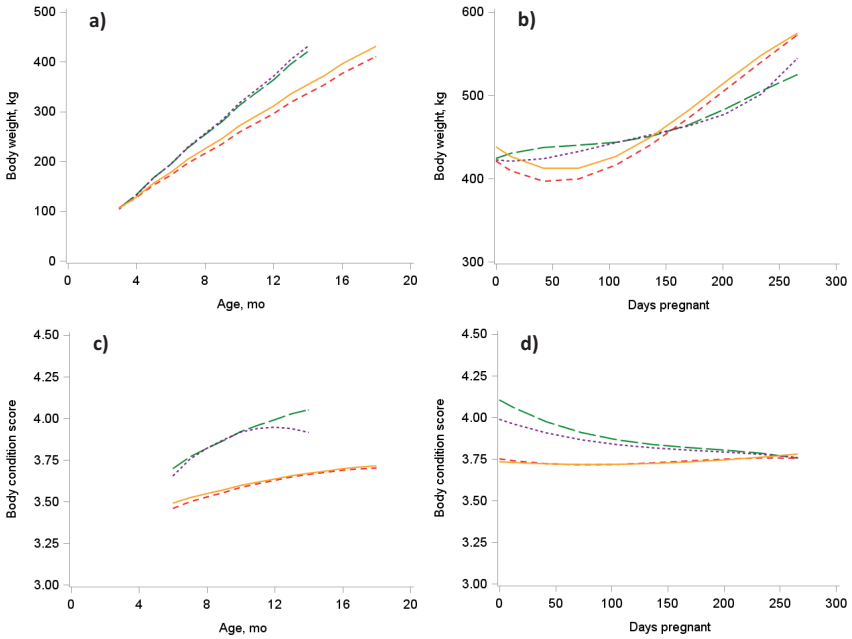
		Start experimental feeding to onset of puberty				Puberty to successful insemination			
Variables		HELP	HEHP	LELP	LEHP	HELP	HEHP	LELP	LEHP
DMI	HELP	**144**	7.3 (0.574)	-57.7 (<0.0001)	-28.3 (0.031)	**163**	22.6 (0.205)	-10.4 (0.561)	-31.9 (0.071)
	HEHP		**136**	-65.0 (<0.0001)	-35.6 (0.007)		**140**	-32.9 (0.070)	-54.5 (0.003)
	LELP			**201**	29.5 (0.027)			**173**	-21.5 (0.227)
	LEHP				**172**				**195**
NE_G	HELP	**849**	40.0 (0.567)	-253.4 (0.0006)	-91.6 (0.191)	**953**	134.6 (0.186)	18.4 (0.856)	-114.8 (0.252)
	HEHP		**809**	-293.3 (<0.0001)	-131.6 (0.062)		**818**	-116.2 (0.259)	-249.3 (0.016)
	LELP			**1103**	161.8 (0.025)			**934**	-133.1 (0.191)
	LEHP				**941**				**1068**
CP_intake	HELP	**20.9**	-0.8 (0.650)	-1.9 (0.286)	-1.0. (0.571)	**21.9**	1.7 (0.427)	3.4 (0.125)	-0.8 (0.720)
	HEHP		**21.7**	-1.1 (0.532)	-0.2 (0.911)		**20.1**	1.6 (0.457)	-2.5 (0.252)
	LELP			**22.8**	0.9 (0.607)			**18.5**	-4.2 (0.060)
	LEHP				**21.9**				**22.7**

Table 7. Estimated 305-d kg ECM yield in first, second and third lactation of high-energy low-protein (HELP), high-energy high-protein (HEHP), low-energy low-protein (LELP), and low-energy high-protein (LEHP) groups, with number of animals within each group (n)

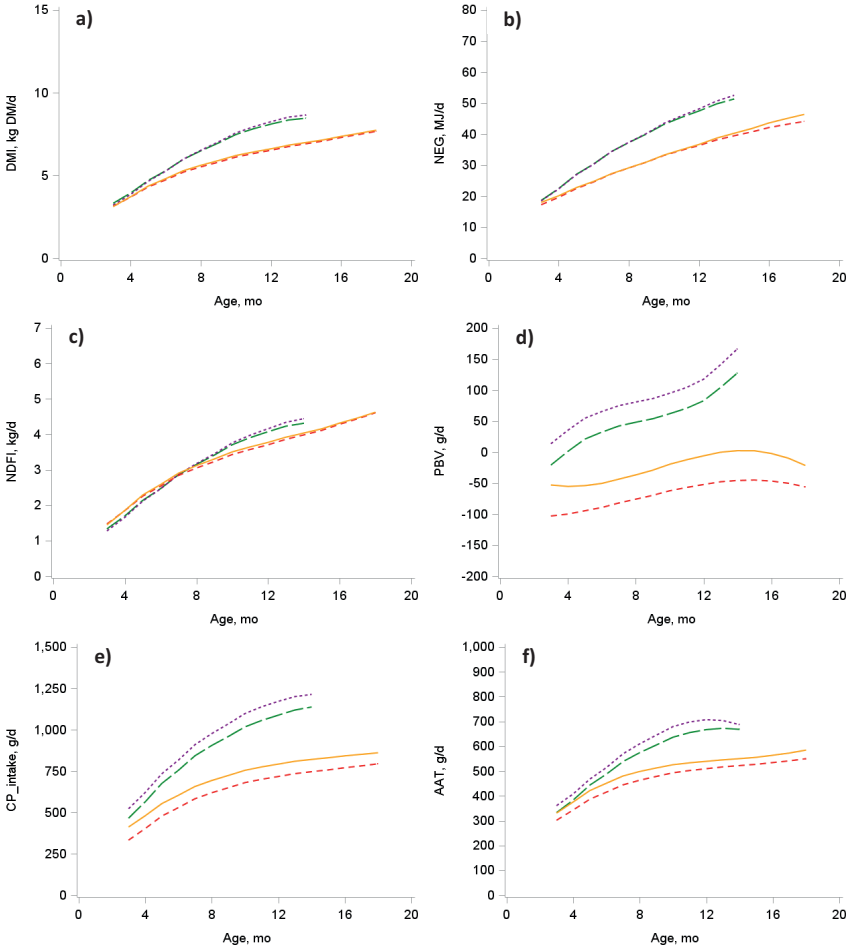
	Treatment							
Lactation	n	HELP	n	HEHP	n	LELP	n	LEHP
1st	18	6,994[a1]	19	6,629[a]	18	7,164[a]	17	7,060[a]
2nd	12	8,300[a]	15	8,018[a]	8	8,709[a]	10	8,196[a]
3rd	8	9,135[a]	11	9,083[a]	5	8,355[ab]	6	7,365[b]
Sum		24,429		23,730		24,228		22,621

[1)] Significant ($P < 0.05$) differences within lactation when letters are different

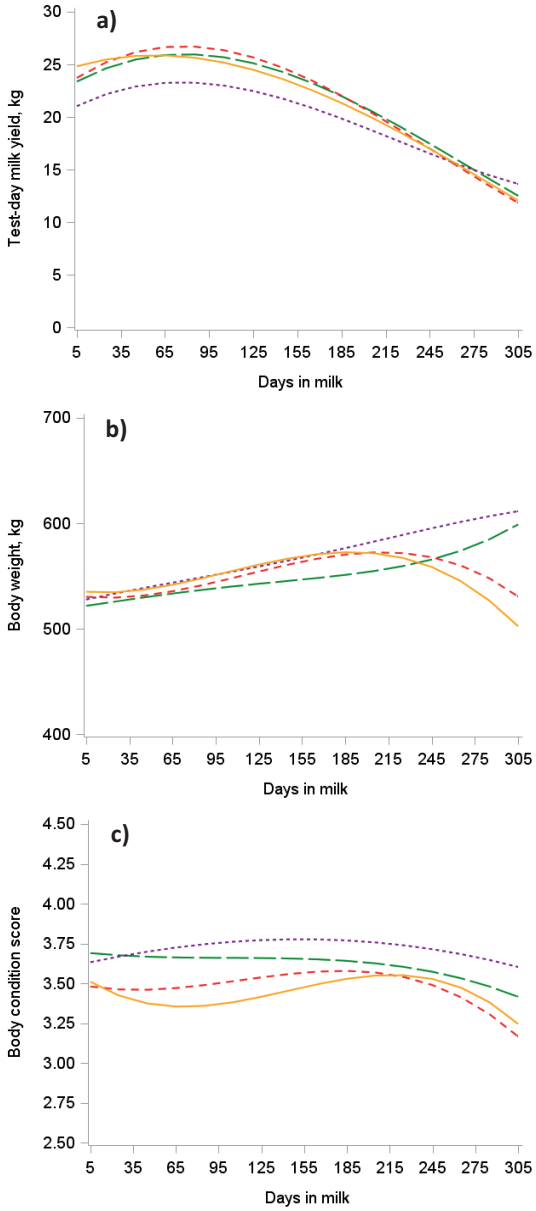
Salte, Figure 1.



Salte, Figure 2.



Salte, Figure 3



Salte, Figure 4.

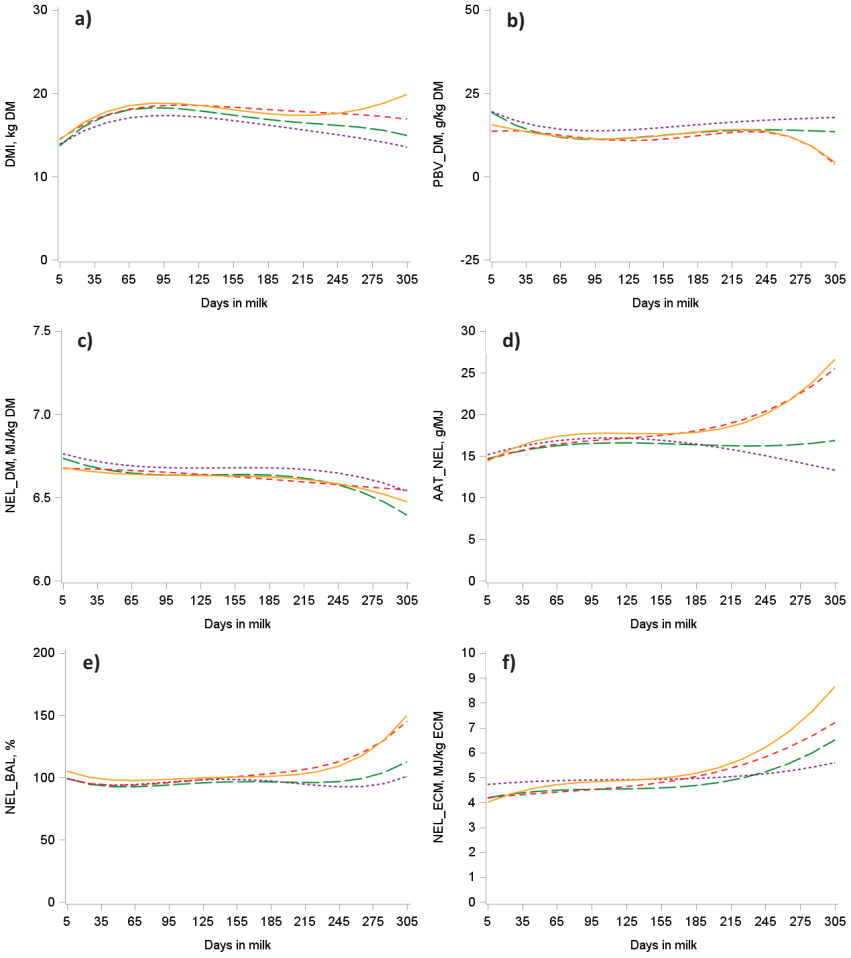


Figure 1. Estimated least-squares means of body weight (panel a and b) and body condition score (1 -5 scale, panel c and d) of high-energy low-protein (long-dashed line), high-energy high-protein (dotted line), low-energy low-protein (short-dashed line) and low-energy high-protein (solid line) groups between 3 mo of age and successful insemination and through gestation.

Figure 2. Estimated least-squares means of a) DMI, b) NE_G, c) intake of NDF in total ration (NDFI, (defined as (NDF x DMI)/1000, kg/d), d) PBV (protein balance in rumen, g/d), e) CP_intake (g/d), f) AAT (amino acids absorbed in the small intestine, g/d) in the high-energy low-protein (long-dashed line), high-energy high-protein (dotted line), low-energy low-protein (short-dashed line) and low-energy high-protein (solid line) groups from 3 mo of age to successful insemination

Figure 3. Estimated least-squares means of first lactation a) test-day milk yield, b) body weight, and c) body condition score (1-5 scale) in the high-energy low-protein (long-dashed line), high-energy high-protein (dotted line), low-energy low-protein (short-dashed line) and low-energy high-protein (solid line) groups from 5 to 305 DIM.

Figure 4. Estimated least-squares means of first lactation a) DMI, b) PBV_DM (protein balance in rumen, g/kg DM) c) NE_L_DM (NE_L/DMI), d) AAT_NE_L (amino acids absorbed in the small intestine in g per NE_L), e) NE_L_BAL (energy balance for cows; NE_L/ total net energy requirement, in %), and f) NE_L_ECM (NE_L/ ECM) in the high-energy low-protein

(long-dashed line), high-energy-high-protein (dotted line), low-energy low-protein (short-dashed line) and low-energy high-protein (solid line) groups from 5 to 305 DIM.

Paper II

PLOS ONE

RESEARCH ARTICLE

RNA-seq analysis of bovine adipose tissue in heifers fed diets differing in energy and protein content

Hilde K. L. Wærp[1], Sinéad M. Waters[2]*, Matthew S. McCabe[2], Paul Cormican[2], Ragnar Salte[1]

1 Department of Animal and Aquacultural Sciences, Norwegian University of Life Sciences, Ås, Norway, **2** Animal and Bioscience Research Department, Animal and Grassland Research and Innovation Centre, Teagasc, Dunsany, Co. Meath, Ireland

* Sinead.Waters@teagasc.ie

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Abstract

Adipose tissue is no longer considered a mere energy reserve, but a metabolically and hormonally active organ strongly associated with the regulation of whole-body metabolism. Knowledge of adipose metabolic regulatory function is of great importance in cattle management, as it affects the efficiency and manner with which an animal converts feedstuff to milk, meat and fat. However, the molecular mechanisms regulating metabolism in bovine adipose tissue are still not fully elucidated. The emergence of next-generation sequencing technologies has facilitated the analysis of metabolic function and regulation at the global gene expression level. The aim of this study was to investigate the effect of diets differing in protein and energy density level on gene expression in adipose tissue of growing replacement dairy heifers using next-generation RNA sequencing (RNAseq). Norwegian Red heifers were fed either a high- or low-protein concentrate (HP/LP) and a high- or low-energy roughage (HE/LE) diet from 3 months of age until confirmed pregnancy to give four treatments (*viz*, HPHE, HPLE, LPHE, LPLE) with different growth profiles. Subcutaneous adipose tissue sampled at 12 months of age was analyzed for gene expression differences using RNA-seq. The largest difference in gene expression was found between LPHE and LPLE heifers, for which 1092 genes were significantly differentially expressed, representing an up-regulation of mitochondrial function, lipid, carbohydrate and amino acid metabolism as well as changes in the antioxidant system in adipose tissue of LPHE heifers. Differences between HPHE and HPLE heifers were much smaller, and dominated by genes representing NAD biosynthesis, as was the significantly differentially expressed genes (DEG) common to both HE-LE contrasts. Differences between HP and LP groups within each energy treatment were minimal. This study emphasizes the importance of transcriptional regulation of adipose tissue energy metabolism, and identifies candidate genes for further studies on early-stage obesity and glucose load in dairy cattle.

and analysis, decision to publish, or preparation of the manuscript.

Competing interests: We therefore have the following interests: This study is partly funded by TINE SA Norwegian dairies, Felleskjøpet agricultural cooperative and Animalia AS. There are no patents, products in development or marketed products to declare. This does not alter our adherence to all the PLOS ONE policies on sharing data and materials, as detailed online in the guide for authors.

Introduction

The global human population is steadily increasing, and with it so are the demands for efficient animal production systems [1, 2]. Within the dairy industry, efficient meat and milk production is dependent on animals with a well-functioning metabolism and an efficient and purposeful nutrient allocation [3–5]. For growing cattle this implies a rapid growth rate without excess fattening, as the number of days spent to achieve productive size is proportional to total rearing costs [6], while excessive fattening has a negative impact on both feed efficiency, subsequent health and carcass value [5, 7]. High growth rates can be achieved by both breeding and feeding strategies. Growth is also affected by management and environmental conditions, although to a lesser degree [8, 9]. As the capacity for rapid genetic progress at a farm level is limited and management and environment yield smaller effects, nutritional management is the main strategy applied in practice. However, nutritionally induced high growth rates are associated with increased fatness [10]. There is a plethora of heifer studies reporting the effects of feeding regimes during the rearing period on growth rate and characteristics, as well as on subsequent milk yield in dairy heifers [11, 12]. However, the molecular mechanisms by which these effects are exerted are still not fully elucidated.

An important role of adipose tissue in this regard has emerged, as adipose is no longer considered merely a passive energy storage tissue, but is a transcriptionally active endocrine organ with an important role in whole-body metabolism and energy balance [13, 14]. When compared to monogastrics this is especially true for ruminants, in which adipose tissue is the main site for both lipid synthesis, storage and mobilization [15]. Increased knowledge of how transcriptomic regulation in adipose tissue is affected by nutrition in dairy heifers may contribute to the development of both feeding and breeding strategies to produce more efficient animals. Several studies on adipose tissue from cattle fed differently have been performed in the recent years, elucidating the connection between plane of nutrition and the expression of genes associated with lipogenesis, adipogenesis and lipolysis [16–18]. However, most gene expression studies on adipose tissue in cattle to date have been performed using microarrays and targeted gene analysis [14–16]. Recent increased availability and reduced costs of next generation sequencing techniques have led to more gene expression studies being performed by RNA-sequencing (RNAseq) technology. Compared to microarrays, RNA-seq is more sensitive and has a much wider dynamic range. It analyzes the whole transcriptome in an unbiased fashion, and is therefore not limited by predetermined target genes, array setups or a need for existing genome annotations [19].

Data in the published literature provides evidence of an effect of nutrition on adipose expression of genes, but until now such information have only been available through microarrays and targeted gene expression analyses performed on adult cows [16–18]. Hence, the objective of this study was to investigate the effects of diets differing in protein and energy density on the full adipose transcriptome of growing dairy heifers, and to identify gene networks related to critical metabolic pathways which can be targeted in further studies to enhance replacement heifer rearing strategies and production efficiency in the Norwegian Red cow.

Materials and methods

Ethics statement

All experimental procedures involving animals were approved by the Norwegian Animal Research Authority (FOTS ID 2955, Reference number: 2010/203231). Biopsies were harvested under epidural anesthesia, and all animals were given 3 mg/kg BW of Ketoprofen

intramuscularly (Comforion vet., Orion Pharma Animal Health) to prevent pain or inflammation at the biopsy site.

Animal model

This study was part of a large experiment designed to examine the effects of diets with differing energy and protein contents on growth characteristics, fat deposition and subsequent milk yield in Norwegian Red heifers, and from this to determine the optimal growth curve in the pre- and post-pubertal periods for a replacement dairy heifer. Eighty heifers from the dairy herd at the Norwegian University of Life Sciences (years 2010 and 2011) were assigned either to a high (HE) or low (LE) energy group, fed according to a BW gain of 800–1000 or 600–750 g/day from three months of age to confirmed pregnancy, respectively. Each energy group was further subdivided into two protein groups, i.e. low (LP) or high (HP) to give four dietary treatments with 20 animals in each group, *viz* low-protein high-energy (LPHE), high-protein high-energy (HPHE), low-protein low-energy (LPLE) and high-protein low-energy (HPLE). The university herd consists of two genetic groups, one group with high milk-yield (HMY) and one group with low occurrence of clinical mastitis (LCM) [20]. As this genetic difference could affect several variables of interest to our experiment, it was taken into account when allocating heifers to dietary treatments to generate groups which were balanced according to genetic group. Apart from this consideration, heifers were randomly allocated to treatment group. Body weights [21] were measured every second week, and body condition scores (BCS) and height at withers (WH) recorded every month for all experimental animals. The Norwegian 1–5 scale BCS system is based on Edmonson et al. [22] but calibrated for Norwegian Red [23].

Heifer feeding and management. All heifer calves were fed the same diet from birth to three mo of age. From three months of age until confirmed pregnancy, heifers were housed in a tie-stall barn and fed one of the four experimental diets. Fresh feed was offered twice daily and individual feed intake was recorded four days a week. HE groups were fed grass silage ad libitum whereas LE groups were fed the same silage mixed with 10 to 40% wheat straw (on a dry matter basis) given as restricted rations. All heifers were daily fed 1 kg of either of two custom-made concentrates, differing in protein content. Energy density of the diets was adjusted with the roughage quality. Diet protein content was adjusted with both roughage quality and type of concentrate. Table 1 gives the average nutrient content of roughage and custom made concentrates. Table 2 gives average daily intake of dry matter, energy, and protein for the different treatment groups.

Adipose tissue biopsy sampling

Adipose tissue biopsies were collected at 12 months of age. The heifers were given a low epidural anesthesia of 3–5 ml of a local anesthetic (Lidokel-Adrenalin vet, Kela Laboratoria NV) prior to sampling. Subcutaneous adipose tissue biopsies were harvested lateral to the tail base. Each biopsy consisted of 0.5–1.5 g of adipose tissue. Biopsies were immediately snap frozen in liquid nitrogen, and subsequently stored at -80˚C.

RNA extraction and sequencing

Each adipose tissue biopsy was crushed on dry ice, and 100 mg was used for RNA extraction. RNA extraction was carried out using the Qiagen RNeasy lipid tissue mini kit (Qiagen). All samples were homogenized in Trizol using a PRO 200 handheld homogenizer (PRO Scientific Inc., Oxford, CT USA). Except for the homogenization step, the RNA extraction methodology was performed according to the manufacturer's protocol. The quantity and quality of RNA

Table 1. Average nutrient content of high- and low-energy (HE and LE) roughages and high and low protein (HP and LP) concentrates, all weighted by number of feed days during the experimental feeding period from 3 mo of age to confirmed pregnancy (g/kg of dry matter if not stated otherwise).

	Start experimental feeding to confirmed pregnancy			
	Roughage		Concentrate	
Variables[1]	HE (SD)	LE (SD)	LP	HP
Dry matter, g/kg	323 (40.8)	403 (48.9)	856	855
Ash	68.0 (8.0)	61.5 (6.5)	87.8	98.1
Crude protein	140 (13.7)	107 (8.9)	155	233
Crude fat	34.0 (2.9)	27.9 (1.6)	44	53
Starch	.	.	372	263
NDF	542 (18.5)	630.7 (25.8)	176	178
Sugar	38.3 (30.5)	28.8 (23.7)	.	.
AAT_{N8}	64	62	83	98
PBV_{N8}	42	15	43	103
NEL_{N8}, MJ/kg DM	7.0	6.5	7.6	7.7

[1] Neutral detergent fiber (NDF). Amino acids absorbed in the small intestine (AAT_{N8}), protein balance in rumen (PBV_{N8}), and net energy lactation (NEL_{N8}), are all calculated values (TINE Optifôr, (Volden, 2011)) at 8 kg DMI; to calculate these variables a weighted (by feed days) average for the 2 roughages (HE, LE) and concentrates (LP, HP) were entered into TINE Optifôr as fictive feeds; note that standard values have no SD. Crude protein was analysed as Kjeldahl Nitrogen x 6.25.

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was determined using a Nanodrop spectrophotometer ND-1000 (Nanodrop Technologies, Wilmington, DE, USA) and the RNA 6000 Nano Lab Chip kit (Agilent Technologies Ireland Ltd., Dublin, Ireland), respectively. The best-quality RNA samples to give a balanced sample set with respect to genetic group and dietary treatment were selected for further RNA sequencing (n = 6 for LPHE, HPHE, LPLE and HPLE, respectively). RIN values for these samples varied between 7.8 and 9.2. To avoid DNA contamination, all RNA samples were subjected to a DNAse treatment after extraction (Turbo DNA-free, Ambion, Life Technologies) and a subsequent clean-up (RNA Clean and Concentrator -25, Zymo Research Corp.).

Library preparation was performed using the Illumina TruSeq RNA sample prep kit v2, according to the manufacturer's instructions (Illumina, San Diego, CA, USA). Sequencing was carried out on an Illumina HiSeq 2000 workstation, with 100 bp paired end reads, and the 48 samples distributed randomly over 12 lanes (Clinical Genomics, Toronto).

Statistics and bioinformatics

The fastq files containing the raw sequence reads were examined using the software FASTQC (v 0.11.2.). Adapters were trimmed from all sample fastq files using Cutadapt (v 1.3). Reads were simultaneously quality trimmed by removing reads shorter than 20 bp, or with Phred scores below 25. Following adapter and quality trimming, all files were checked again in FASTQC. Paired-end read files were aligned to the UMD3.1 bovine genome assembly [24], using Tophat 2.0.12 [25]. The number of reads per gene in each sample was counted using HTSeq-count (v 0.6.1) [26]. Statistical analysis of read counts were carried out in EdgeR (v 3.1.2), a Bioconductor software package run in the statistical software environment R (v 3.1.2) [27]. Differential gene expression was tested in EdgeR using tagwise dispersions. The statistical tests were corrected for multiple testing using the Benjamini-Hochberg method as implemented in EdgeR. A generalized linear model which included dietary treatment and genetic group was fitted to the data using the glmFit function [28]. The comparisons were performed

Table 2. Calculated average daily intake in the ration of dry matter (DMI, kg DM/d), net energy growth (NEG, MJ/d), crude protein (CP_intake, g/d), and amino acids absorbed in the small intestine (AAT, g/d) in the 4 treatment groups (high-energy low-protein (LPHE), high-energy high-protein (HPHE), low-energy low-protein (LPLE), low-energy high-protein (HPLE) during the entire experimental feeding period.

Stage[1]	Treatment	Variables			
		DMI	NEG	CP_intake	AAT
3 mo of age to onset of puberty	LPHE	5.3	31.3	771	485
	HPHE	5.2	30.7	825	510
	LPLE	5.1	28.0	580	432
	HPLE	5.0	27.3	637	456
Puberty to confirmed pregnancy	LPHE	7.9	46.4	1,067	665
	HPHE	7.8	45.6	1,118	693
	LPLE	7.4	39.5	785	558
	HPLE	7.2	39.4	840	578

[1]Norwegian Red heifers reach puberty at about 280 kg regardless of age. HE animals entered puberty at 9 months of age, whereas LE animals entered puberty at 11.5 months of age. Pregnancy start was targeted at 400 kg BW for all heifers, and therefore confirmed pregnancy occurred later for LE heifers than for the faster-growing HE heifers (17 and 13 months of age, respectively).

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as pairwise comparisons and the differences between contrasting treatments, i.e. LPHE-LPLE, HPHE-HPLE, LPHE-HPHE and LPLE-HPLE, were tested. Significantly differentially expressed genes (DEG) were called at a FDR (false discovery rate adjusted p-value as given in EdgeR) of 0.1, and retained for further analysis. Subsequently, the resulting lists of DEG from the low and high-energy HP/LP contrasts and the low and high-protein HE/LE contrasts were compared to identify any DEG common to both comparisons within an energy or protein level. This suggests this gene was affected by energy or protein level regardless of the level of the other dietary variable. The DEG were subsequently analyzed using Ingenuity® Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity) to identify affected biological pathways and cellular functions represented by the DEG. An affected pathway is defined as a pathway containing more DEG than expected by chance, given the number of genes belonging to the pathway. Significance level for the pathway analyses was set at $p < 0.05$. Pathways or functions specifically pertaining to irrelevant diseases, species or tissues were omitted.

Results and discussion

Animal performance

At 12 months, BW of heifers were significantly different ($p < 0.05$) between all dietary treatments, but less so between different protein treatments within an energy treatment than across energy treatments. Body condition scores were different between different energy treatments ($p < 0.05$), but not between protein treatments within an energy treatment. Height at withers were significantly different ($p < 0.05$) between all treatment groups, but the largest difference was found between different energy levels. Least-squares means of BW, BCS and WH at 12 months of age are shown in Table 3.

Differential gene expression

Sequencing yielded on average 57.2 million raw reads per sample. Alignment analysis resulted in an average overall read mapping rate of 95.1% and a concordant pair alignment rate of 89.6%. Genes with a minimum CPM (counts per million) of two in at least three samples were

Table 3. LSMEANS of body weight(BW), body condition score (BCS) and withers height (WH) at 12 months for different treatment groups (SE).

	BW, kg	BCS, points	WH, cm
LPHE	363 (0.9)	3.99 (0.016)	114.3 (0.2)
HPHE	371 (1.1)	3.95 (0.017)	115.3 (0.2)
LPLE	295 (0.8)	3.63 (0.014)	111.7 (0.2)
HPLE	311 (0.8)	3.63 (0.013)	112.4 (0.2)

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considered to be expressed and were included in the differential expression analysis. Out of 26,740 gene transcripts in the UMD3.1 bovine genome assembly, 12,476 gene transcripts fulfilled this criterion. A multidimensional scaling plot visualizing the level of similarity between dietary treatments is presented in Fig 1. The greatest difference in adipose gene expression profiles was found between LPHE and LPLE heifers. This contrast yielded 1092 DEG, 40 times more than any other comparison. The number of DEG identified by contrasting treatments, energy or protein levels are shown in Table 4. Contrasts between the HP and LP groups within each energy level yielded only two DEG for the HE contrast, and one DEG for the LE contrast. Details on these DEG are presented in Table 5. The full lists of DEG between the LPHE-LPLE and the HPHE-HPLE contrasts are shown in S1 and S2 Tables, respectively. The RNAseq raw data are deposited and accessible through NCBI's Gene Expression Omnibus with the GEO Series accession number GSE79347 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE79347).

Pathway analysis

Differentially expressed genes were grouped in IPA according to their biological functions. The functional groups most highly represented by DEG found for LPHE-LPLE contrasts were nucleic acid metabolism, amino acid metabolism and small molecule biochemistry. Small molecule biochemistry was also the functional group most highly represented by the HPHE-HPLE DEG. Figs 2 and 3 display further cellular function differences found for DEG from the two LPHE-LPLE and HPHE-HPLE contrasts, respectively. Pathway analysis of the DEG between LPHE and LPLE heifers resulted in 99 significantly affected pathways ($p < 0.05$). The main pathways affected by the difference in LPHE and LPLE feeding regimes pertained to mitochondrial functions (mitochondrial dysfunction, oxidative phosphorylation and TCA cycle), amino acids (valine, isoleucine, leucine, tryptophan, arginine, proline and cysteine metabolism and degradation) and carbohydrate metabolism (pyruvate, propanoate, butanoate and ketone body metabolism, glycolysis and gluconeogenesis), antioxidant defense systems and fatty acid biosynthesis and degradation. The most highly affected pathways for this contrast are shown in Fig 4. Pathway analysis of the 24 DEG from the HPHE-HPLE comparison yielded the IPA pathways shown in Fig 5. Significantly affected pathways for this contrast were mainly related to glycosaminoglycan and NAD biosynthesis. NAD biosynthesis was also the main pathway represented by the 17 DEG common to both HE-LE contrasts. DEG from the two HP-LP contrasts were not subjected to pathway analysis due to low numbers.

Gene expression differences between energy treatments

The massive difference in DEG numbers identified for the examined contrasts suggests a profound interaction between energy and protein level in the diet on adipose tissue metabolism, and that this interaction effect is in fact larger than the effects of protein or energy independently. The differences in gene expression between LPHE and LPLE heifers illustrates the

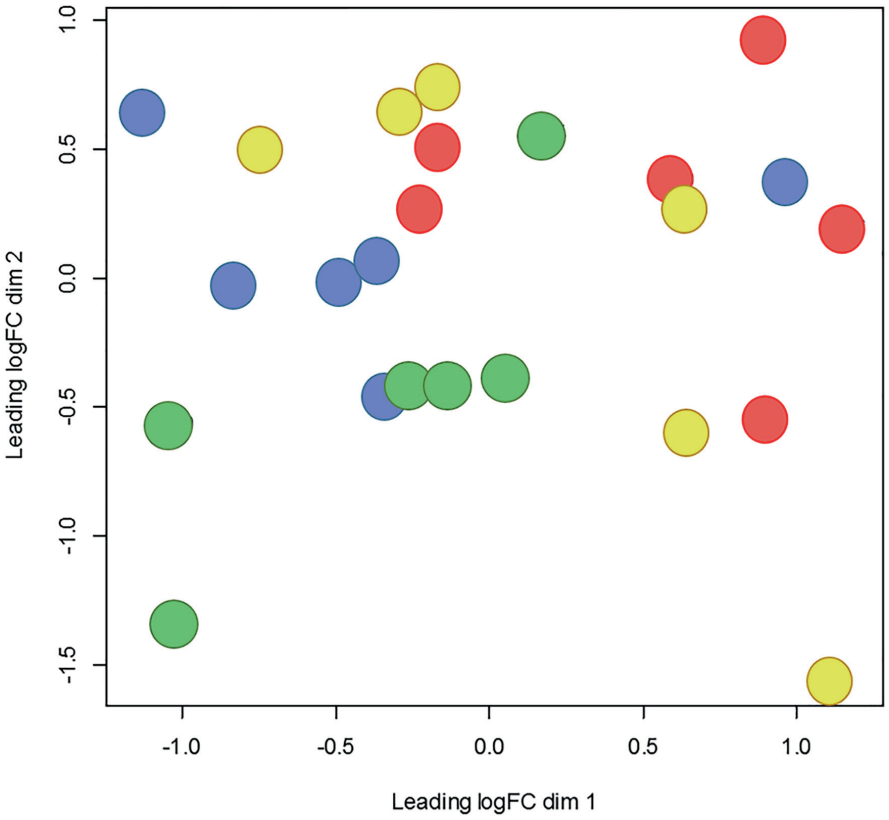

Fig 1. MDS plot of individual samples plotted 2-dimensionally by log fold change (logFC). Green = Low-protein, high-energy (LPHE), Blue = High-protein, high-energy (HPHE), Red = Low-protein, low-energy (LPLE), Yellow = High-protein, low-energy (HPLE).

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importance of transcriptional regulation in metabolism, as several of the main affected pathways such as oxidative phosphorylation, TCA cycle, amino acid and carbohydrate metabolism clearly reflect changes in energy metabolism which are expected to differ in animals fed high- or low-energy diets. The DEG between HPHE and HPLE heifers suggest the main differences

Table 4. Number of up- and down-regulated differentially expressed genes between compared treatment groups.

Comparison	DEG total	Upregulated	Downregulated
LPHE-LPLE	1092	712	380
HPHE-HPLE	24	13	11
HPHE-LPHE	2	1	1
HPLE-LPLE	1	0	1
(LPHE-LPLE)–(HPHE-HPLE)	16	12	4
(LPHE-HPHE)—(LPLE-HPLE)	0	-	-

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Table 5. DEG from HP-LP comparisons.

Gene symbol	Ensembl Gene ID	Description	logFC[a]	FDR[b]
HPLE-LPLE				
CRYM	ENSBTAG00000009842	crystallin, mu	-3.217	0.097394
HPHE-LPHE				
ACTA1	ENSBTAG00000046332	Actin, alpha 1, skeletal muscle	7.751	0.020466
DSP	ENSBTAG00000015106	Desmoplakin	-3.720	0.020466

[a] logFC = log2 fold change

[b] FDR = False discovery rate.

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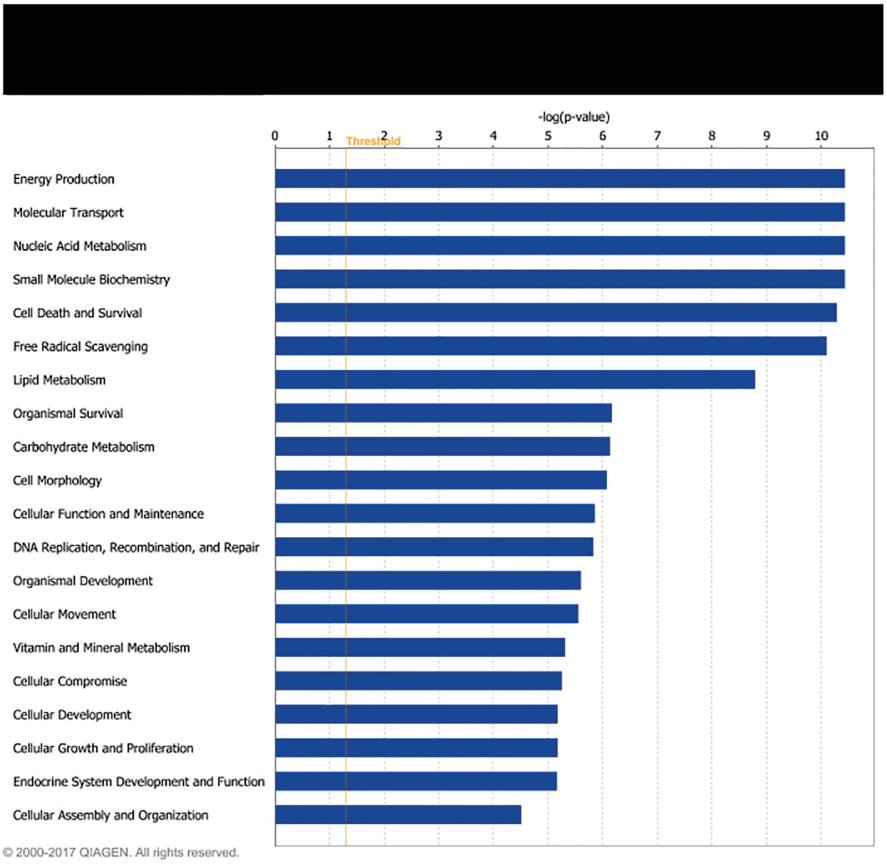

Fig 2. Cellular functions of differentially expressed genes between low-protein fed heifers on high- or low-energy diets. The bars indicate the likelihood [-log (p-value)] that the specific cellular function was affected by dietary energy level compared with other functions represented by the list of differentially expressed genes. Threshold–log p-value (orange line) is set to 1.3, which equals a p-value of 0.05.

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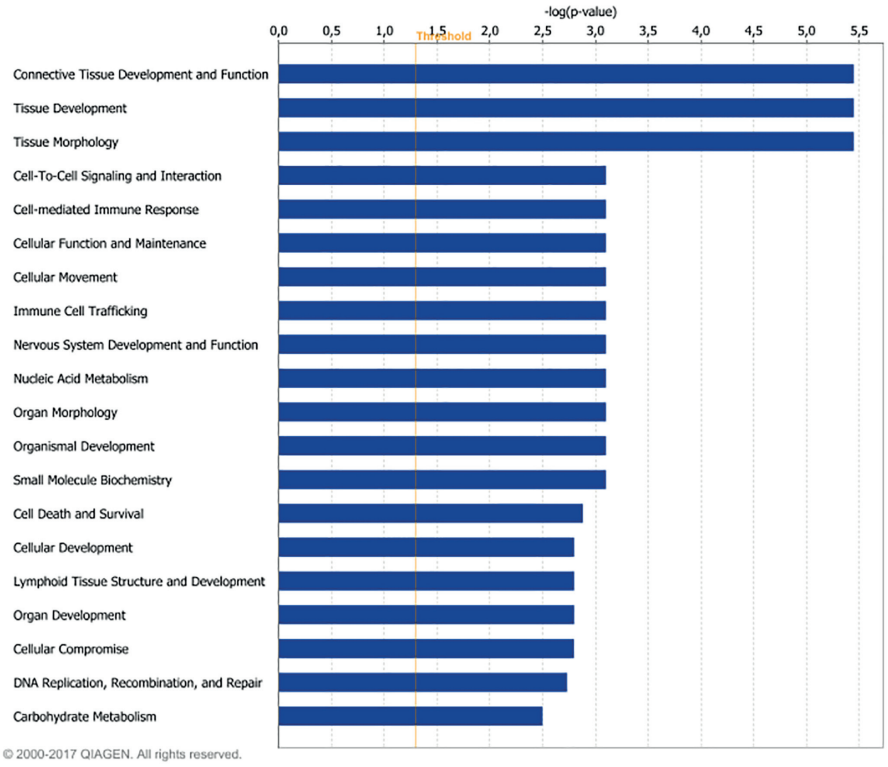

Fig 3. Cellular functions of differentially expressed genes between high-protein fed heifers on high-or low-energy diets. The bars indicate the likelihood [-log (p-value)] that the specific cellular function was affected by dietary energy level compared with other functions represented by the list of differentially expressed genes. Threshold–log p-value (orange line) is set to 1.3, which equals a p-value of 0.05.

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between these two groups was related to connective tissue structure (upregulated glycosaminoglycan synthesis by xylosyltransferase 1, *XYLT1*), and NAD synthesis by quinolinate phosphoribosyltransferase (*QPRT*). The low number of DEG in this contrast also affects the number of DEG common to both HE-LE contrasts, and leaves NAD synthesis as the pathway mainly affected by energy level regardless of protein level in the diet. This is an interesting finding, although not surprising, as nicotinamide adenine dinucleotide (NAD) is a coenzyme well known to take part in a plethora of cellular oxidation-reduction reactions, which constitute the basis of cellular energetics [29]. Based on the results of other gene expression studies, we expected certain genes to be differentially expressed in both energy contrasts. These genes included *FASN* [18, 30], *SCD* [18, 30], *ELOVL6* [30], *ACACA* [18, 30] and *OB* [31]. We would like to emphasize that even though these "classical" and expected DEG from the LPHE-LPLE contrast, did not obtain an FDR < 0.1 in the HPHE-HPLE contrast, 991 of the 1092 DEG from the LP comparison displayed the same directional change in the HP contrast. Thus, differences were mainly the same, only smaller, and given a bigger sample set, the results for the two contrasts would probably be more similar. For comparative purposes, we also performed

Fig 4. Main pathways differentially expressed between low-protein/ high-energy (LPHE) and low-protein/ low-energy (LPLE) fed heifers. Red bars indicate percent upregulated, and green bars indicate percent downregulated genes in the pathway in LPHE versus LPLE heifers. Number to the right of bars display total number of genes pertaining to each pathway. Orange squares indicate the negative logarithm of p-value of observation (-log p-value = 1.3 equals p-value = 0.05).

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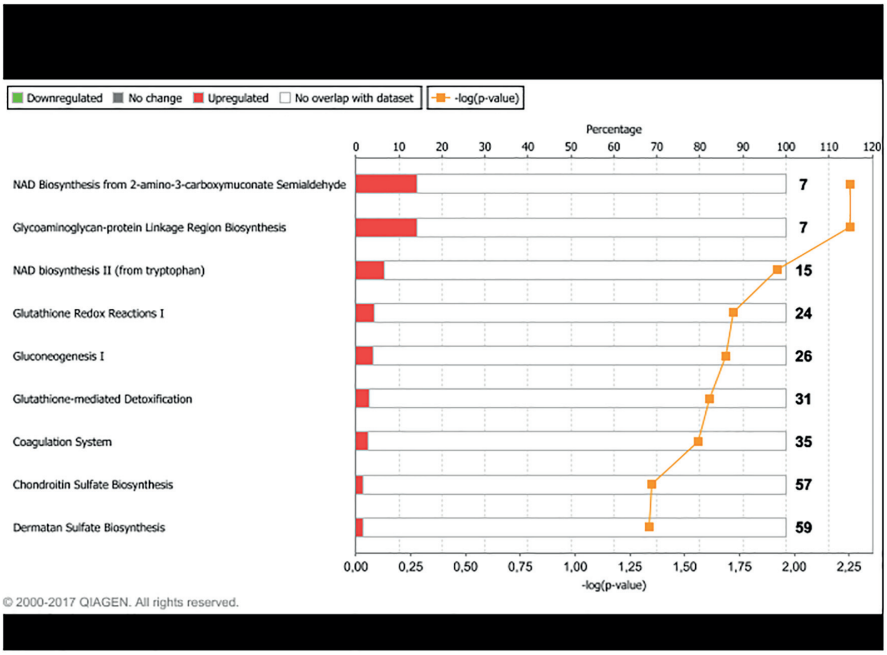

Fig 5. Main pathways differentially expressed between high-protein/ high-energy (HPHE) and high-protein/ low-energy (HPLE) fed heifers. Red bars indicate percent upregulated, and green bars indicate percent downregulated genes in the pathway in HPHE versus HPLE heifers. Number to the right of bars display total number of genes pertaining to each pathway. Orange squares indicate the negative logarithm of p-value of observation (-log p-value = 1.3 equals p-value = 0.05).

https://doi.org/10.1371/journal.pone.0201284.g005

an overall comparison of all HE animals against all LE animals within the same fitted model as described above. With an FDR cutoff value of 0.05 (because of the increased power of this test), the resulting DEG list was strikingly similar to the DEG list for the LPHE-LPLE contrast. In the following 4 sub-sections the main differences in adipose gene expression between LPHE and LPLE heifers are discussed.

Mitochondrial function. The tricarboxylic acid (TCA) cycle and the electron transport chain performing oxidative phosphorylation take place in the mitochondria. The TCA cycle is central to many anabolic and catabolic pathways in a mammalian cell as its main substrate, acetyl-CoA is produced from the initial breakdown of carbohydrates, protein and lipids. It also provides substrates for the synthesis of fatty acids, sterols, amino acids and glucose. The cycle consists of eight steps. Genes associated with all eight steps were up-regulated in LPHE heifers which indicates increased mitochondrial citrate production in LPHE heifers. Citrate is the main fatty acid precursor originating from oxidative metabolism. Citrate destined for fatty acid synthesis is transported out of the mitochondria by a citrate transport protein encoded by *SLC25A1*, which was up-regulated in LPHE heifers. In the cytosol, citrate is converted to acetyl-CoA by ATP citrate lyase (*ACLY*). In ruminants, acetate (from ruminal fermentation) has been shown to be the main carbon source for fatty acid synthesis [32]. Citrate lyase has traditionally been regarded as virtually inactive in ruminant adipose tissue, because of the low levels

of glucose available for metabolism in peripheral tissues [32]. However, *ACLY* was up-regulated in LPHE compared to LPLE heifers. Recent findings, including this study show that the *ACLY* gene displays expression differences in the adipose tissue of cattle with different energy states [18, 30]. This suggests that citrate lyase does indeed play a part in ruminant metabolism and metabolic regulation, and may indicate an increased incorporation of glucose into fatty acids in animals with a positive energy balance. Genes associated with all of the 5 complexes in the electron transport chain were up-regulated in the LPHE heifers, indicating an increase in electron transport chain activity and energy (ATP) production. This makes sense as the role of adipose tissue as a metabolic excess energy endpoint requires it to deal with all forms of energy-yielding nutrients, transforming them in to lipids or to the energy required to produce these lipids. The genes associated with changes in mitochondrial function between LPHE and LPLE heifers, are presented in Fig 6.

Fatty acid biosynthesis and degradation. Differentially expressed genes associated with fatty acid synthesis and degradation are shown in Table 6. The up-regulation of genes associated with fatty acid synthesis (*ACACA*, *SCD*, *FASN* and *ELOVL6)*, fatty acid activation (*ACSL4*, *SLC27A3*, *SLC27A6*), and triacylglycerol (**TAG**) synthesis (*AGPAT2*, *DGAT2*, *ELOVL6*, *LPCAT4*, *PLPP3*) in the LPHE heifers is a reflection of their increased energy intake, leading to a higher production of TAG from citrate and other precursors than in the LPLE heifers. The activity of delta-9-desaturase is important in ruminant adipose tissue compared to monogastrics. This is because of the extensive microbial biohydrogenation of dietary unsaturated fatty acids occurring in the rumen, leaving almost exclusively saturated fatty acids (**SFA**) to be transported from viscera to the peripheral tissues in ruminants [33]. However, the high melting point of SFA makes them unsuitable as the sole source of fatty acids incorporated into cell membranes and adipose tissue. The high delta-9-desaturase activity of ruminant adipose tissue alleviates this effect, and makes oleic acid the most abundant FA in ruminant tissues [34].

Amino acid metabolism. Differentially expressed genes pertaining to amino acid metabolism are shown in Table 7. Genes associated with the metabolism and degradation of several amino acids (isoleucine, leucine, phenylalanine, valine, cysteine, lysine, alanine, tryptophan and aspartate) were up-regulated in the LPHE heifers. This is likely a general response, which would be present also in e.g. muscle because HE animals grow much faster than the LE heifers and thus have both an increasingly higher daily crude protein intake as well as an increased metabolism and degradation of amino acids.

Carbohydrate metabolism. Differentially expressed genes pertaining to carbohydrate metabolism are shown in Table 8. Carbohydrates are energy-yielding nutrients, and several genes associated with carbohydrate degradation such as glycolytic reactions and mitochondrial transport and breakdown of pyruvate were up-regulated in the LPHE heifers. The fact that glycolysis-associated genes were DE between the two energy levels, suggests that the LPHE heifers had sufficient glucose available to direct some of it towards adipose tissue energy storage. This is not considered a common fate for glucose in ruminants which under most physiological conditions are dependent on *de novo* gluconeogenesis in the liver, as most of the glucose in the diet will be degraded by rumen microbes [32]. However, more recent studies have shown that hepatic *de novo* gluconeogenesis is highly prioritized in cows, and proceeds at a relatively high rate even in the absence of lactational demands or in the presence of available dietary glucose [35]. Thus, glucose directed towards adipose energy storage in HE heifers may originate from hepatic synthesis. The pentose-phosphate pathway is mainly associated with anabolic metabolism. It's main functions are to provide ribose-5-phosphate for nucleic acid biosynthesis and reducing equivalents (NADPH) for the biosynthesis of fatty acids and steroids, as well as for the reduction of reactive oxygen species (ROS) [36]. The up-regulation of genes coding for pentose-phosphate pathway enzymes in HE heifers is in concordance with the up-regulation

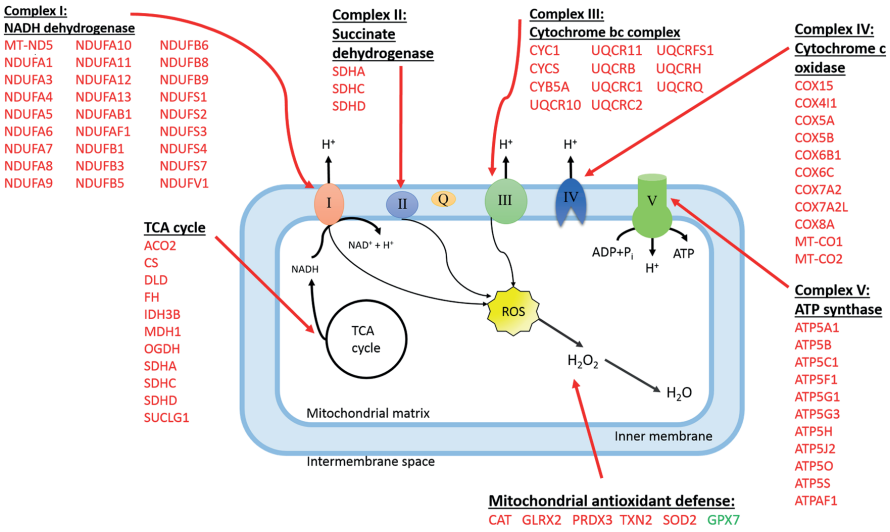

Fig 6. Genes associated with mitochondrial function, differentially expressed between low-protein fed heifers on high- (LPHE) or low- (LPLE) energy diets. Red colour indicate upregulated, and green colour indicate downregulated genes in LPHE versus LPLE heifers.

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of genes associated with fatty acid synthesis and glutathione redox reactions (Tables 6 and 9, respectively). The up-regulation of genes associated with propionate, butyrate and ketone body metabolism and transport in LPHE heifers indicate an increased flux/transport of propionate and 3-hydroxybutyrate from blood into adipocytes. This is possible because the LPHE heifers had an increasingly higher energy intake per day than the LPLE heifers, and as a result microbial fermentation in ruminants causes the energy in feed to enter the animal's bloodstream mainly as volatile fatty acids (VFA), not as glucose or fatty acids [37]. The difference in energy intake between HPHE and HPLE heifers was comparable to the LP energy contrast, however there was no significant up-regulation of genes pertaining to VFA transport between these two groups. A possible explanation is that the increased fraction of protein-rich feedstuffs in the experimental concentrate given to the HP groups, was substituted by barley, which is rich in starch, in the low-protein concentrate fed to the LP groups. This led to a difference in daily starch intake of approximately 130 grams between protein treatments. Starch is rapidly degraded into VFA (mainly propionate) in the rumen. The increased amount in the LP diets combined with the energy surplus in the HE roughage may have resulted in a sufficiently increased VFA flux from the rumen to the blood to evoke an up-regulation of 11 genes in LPHE heifers, enabling their adipose tissue to deal with the energy excess in the form of VFA and direct this energy towards fat deposition.

Antioxidant functions. Differentially expressed genes pertaining to cellular antioxidant functions are shown in Table 9. Virtually any metabolic reaction involving oxygen may yield reactive oxygen species (ROS) as byproducts. Consequently, both mitochondria, endoplasmic reticulum (ER) and peroxisomes are major sites of ROS production in the cell [38]. In controlled quantities, these substances have a function in cell signaling and regulation [38]. However, accumulation of ROS will lead to a state of oxidative stress, causing uncontrolled

Table 6. Fatty acid synthesis and degradation-associated genes differentially expressed between low-protein, high-energy heifers and low-protein, low-energy heifers.

Symbol	Ensembl	Entrez Gene Name	LogFC	FDR
ACAA2	ENSBTAG00000002863	acetyl-CoA acyltransferase 2	0,746	1,37E-02
ACAT1	ENSBTAG00000012885	acetyl-CoA acetyltransferase 1	0,866	1,94E-03
ACSL4	ENSBTAG00000018986	acyl-CoA synthetase long-chain family member 4	0,834	6,48E-02
AGPAT2	ENSBTAG00000025161	1-acylglycerol-3-phosphate O-acyltransferase 2	0,950	1,17E-02
ALDH6A1	ENSBTAG00000018469	aldehyde dehydrogenase 6 family, member A1	0,631	5,74E-02
CYB5A	ENSBTAG00000012012	cytochrome b5 type A (microsomal)	0,770	4,45E-02
DGAT2	ENSBTAG00000001154	diacylglycerol O-acyltransferase 2	0,628	6,36E-02
DHCR24	ENSBTAG00000004688	24-dehydrocholesterol reductase	0,544	4,32E-02
DLAT	ENSBTAG00000010709	dihydrolipoamide S-acetyltransferase	0,860	3,71E-03
DLD	ENSBTAG00000001908	dihydrolipoamide dehydrogenase	0,734	8,62E-03
ECHS1	ENSBTAG00000017710	enoyl CoA hydratase, short chain, 1, mitochondrial	0,943	1,95E-03
EHHADH	ENSBTAG00000019625	enoyl-CoA, hydratase/3-hydroxyacyl CoA dehydrogenase	0,730	4,70E-02
ELOVL6	ENSBTAG00000010564	ELOVL fatty acid elongase 6	1,586	2,44E-07
FADS2	ENSBTAG00000015505	fatty acid desaturase 2	-1,129	1,06E-03
FASN	ENSBTAG00000015980	fatty acid synthase	0,759	1,59E-02
HSD17B10	ENSBTAG00000017779	hydroxysteroid (17-beta) dehydrogenase 10	0,734	2,39E-03
IDI1	ENSBTAG00000004075	isopentenyl-diphosphate delta isomerase 1	1,316	2,52E-04
IVD	ENSBTAG00000004409	isovaleryl-CoA dehydrogenase	0,574	3,25E-02
LPCAT4	ENSBTAG00000020040	lysophosphatidylcholine acyltransferase 4	-0,597	8,63E-02
LSS	ENSBTAG00000018936	lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase)	1,044	1,58E-03
MVD	ENSBTAG00000012059	mevalonate (diphospho) decarboxylase	0,785	5,88E-02
NSDHL	ENSBTAG00000009231	NAD(P) dependent steroid dehydrogenase-like	0,791	8,64E-04
PDHA1	ENSBTAG00000019852	pyruvate dehydrogenase (lipoamide) alpha 1	0,658	6,69E-03
PDHB	ENSBTAG00000021724	pyruvate dehydrogenase (lipoamide) beta	0,434	6,49E-02
PLPP3	ENSBTAG00000011640	phospholipid phosphatase 3	-0,820	1,84E-02
SCD	ENSBTAG00000045728	stearoyl-CoA desaturase (delta-9-desaturase)	1,102	3,90E-03
SCP2	ENSBTAG00000003746	sterol carrier protein 2	0,689	1,16E-02
SLC27A3	ENSBTAG00000021862	solute carrier family 27 (fatty acid transporter), member 3	-1,150	4,79E-03
SLC27A6	ENSBTAG00000004860	solute carrier family 27 (fatty acid transporter), member 6	0,935	8,21E-02
THEM4	ENSBTAG00000004772	thioesterase superfamily member 4	0,671	5,91E-02

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oxidation of DNA and proteins in the cell and cellular dysfunction [39]. Therefore, it is imperative that cells have well-functioning systems to regulate and control the level of ROS at all times. The glutathione system plays an important role in the cellular defense against oxidative damage and harmful substances. Glutathione is synthetized by glutamate cysteine ligase (GCL, encoded by *GCLC*) and glutathione synthetase (GSS, encoded by *GSS*) from glycine and cysteine. It functions as an electron donor for reactive oxygen species (ROS), itself being dimerized to glutathione disulfide (GSSG) in the reaction. Glutathione disulfide may be recycled back to 2 GSH molecules by glutathione reductase, using NADPH as an H+ donor. In concordance with studies performed on mouse models, there was an up-regulation of *GCLC* and *GSS* and a down-regulation of the glutathione peroxidase gene *GPX7* in LPHE heifers [40, 41]. When examined in isolation, a down-regulation of *GPX7* indicates a decreased level of redox reactions producing GSSG and is associated with increases in oxidative stress [40], inflammatory status and insulin resistance [41]. However, as shown in Fig 6, a whole range of other antioxidant system genes were upregulated in LPHE heifers. Among these were several genes coding for glutathione-S-transferases (*GSTT1*, *GSTT3*, *GSTM1*, *MGST1* and *MGST3*). These enzymes

Table 7. Amino acid metabolism-associated genes differentially expressed between low-protein, high-energy heifers and low-protein, low-energy heifers.

Gene symbol	Ensembl gene ID	Description	logFC	FDR
AADAT	ENSBTAG00000010326	aminoadipate aminotransferase	0,988	4,93E-04
ABAT	ENSBTAG00000004038	4-aminobutyrate aminotransferase	0,769	2,14E-02
ACADSB	ENSBTAG00000018041	acyl-CoA dehydrogenase, short/branched chain	0,749	7,26E-02
ACAT1	ENSBTAG00000012885	acetyl-CoA acetyltransferase 1	0,866	1,94E-03
ALDH6A1	ENSBTAG00000018469	aldehyde dehydrogenase 6 family, member A1	0,631	5,74E-02
ALDH7A1	ENSBTAG00000009646	aldehyde dehydrogenase 7 family, member A1	0,503	2,24E-02
ASNS	ENSBTAG00000003222	Asparagine synthetase	0,416	6.64E-02
BCAT2	ENSBTAG00000009172	branched chain amino-acid transaminase 2, mitochondrial	0,595	4,89E-02
BCKDHA	ENSBTAG00000016037	branched chain keto acid dehydrogenase E1, alpha polypeptide	0,669	1,44E-02
BCKDHB	ENSBTAG00000012096	branched chain keto acid dehydrogenase E1, beta polypeptide	0,737	1,47E-03
CDO1	ENSBTAG00000017442	cysteine dioxygenase type 1	-1,363	2,25E-02
DLD	ENSBTAG00000001908	dihydrolipoamide dehydrogenase	0,734	8,62E-03
ECHS1	ENSBTAG00000017710	enoyl CoA hydratase, short chain, 1, mitochondrial	0,943	1,95E-03
EHHADH	ENSBTAG00000019625	enoyl-CoA, hydratase/3-hydroxyacyl CoA dehydrogenase	0,730	4,70E-02
GOT1	ENSBTAG00000011960	glutamic-oxaloacetic transaminase 1, soluble	0,547	3,19E-02
GOT2	ENSBTAG00000007172	glutamic-oxaloacetic transaminase 2, mitochondrial	0,747	6,27E-04
HIBADH	ENSBTAG00000001036	3-hydroxyisobutyrate dehydrogenase	0,427	7,16E-02
HIBCH	ENSBTAG00000007787	3-hydroxyisobutyryl-CoA hydrolase	0,492	6,46E-02
HMGCL	ENSBTAG00000021832	3-hydroxymethyl-3-methylglutaryl-CoA lyase	0,405	8,54E-02
HPD	ENSBTAG00000004175	4-hydroxyphenylpyruvate dioxygenase	1,532	3,56E-02
HSD17B10	ENSBTAG00000017779	hydroxysteroid (17-beta) dehydrogenase 10	0,734	2,39E-03
IDO1	ENSBTAG00000020602	indoleamine 2,3-dioxygenase 1	2,388	1,21E-02
IVD	ENSBTAG00000004409	isovaleryl-CoA dehydrogenase	0,574	3,25E-02
KYNU	ENSBTAG00000032277	Kynureninase	1,343	4,90E-02
MDH1	ENSBTAG00000019295	malate dehydrogenase 1, NAD (soluble)	0,818	1,36E-03
NFS1	ENSBTAG00000006962	NFS1 cysteine desulfurase	0,410	8.94E-02
PIPOX	ENSBTAG00000007946	pipecolic acid oxidase	1,219	2,16E-02
SLC27A3	ENSBTAG00000021862	solute carrier family 27 (fatty acid transporter), member 3	-1,150	4,79E-03

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Table 8. Carbohydrate metabolism-associated genes differentially expressed between low-protein, high-energy- and low-protein, low-energy fed heifers.

Gene symbol	Ensembl Gene ID	Description	LogFC	FDR
ALDOA	ENSBTAG00000012927	aldolase A, fructose-bisphosphate	0,666	4,11E-03
DLD	ENSBTAG00000001908	dihydrolipoamide dehydrogenase	0,734	8,62E-03
ENO1	ENSBTAG00000013411	enolase 1, (alpha)	0,550	3,01E-02
G6PD	ENSBTAG00000019512	glucose-6-phosphate dehydrogenase	1,219	2,02E-05
GPI	ENSBTAG00000006396	glucose-6-phosphate isomerase	0,886	9,57E-04
MCEE	ENSBTAG00000035247	methylmalonyl CoA epimerase	0,939	8,90E-04
MUT	ENSBTAG00000014272	methylmalonyl CoA mutase	0,763	1,24E-02
PCCB	ENSBTAG00000015221	propionyl CoA carboxylase, beta polypeptide	0,741	8,80E-03
PGAM1	ENSBTAG00000012697	phosphoglycerate mutase 1 (brain)	0,604	9,47E-03
PGD	ENSBTAG00000013527	phosphogluconate dehydrogenase	1,038	2,52E-04
PGK1	ENSBTAG00000000894	phosphoglycerate kinase 1	0,623	9,08E-03
TALDO1	ENSBTAG00000010336	transaldolase 1	0,805	1,01E-03
TKT	ENSBTAG00000003758	Transketolase	0,770	5,11E-03
TPI1	ENSBTAG00000019782	triosephosphate isomerase 1	0,654	2,08E-02

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Table 9. Antioxidant system-associated genes differentially expressed between low-protein, high-energy heifers and low-protein, low-energy heifers.

Gene symbol	Ensembl Gene ID	Description	LogFC	FDR
ACTA1	ENSBTAG00000046332	actin, alpha 1, skeletal muscle	-6,524	2,11E-03
ACTG2	ENSBTAG00000015441	actin, gamma 2, smooth muscle, enteric	-2,960	7,45E-03
CAT	ENSBTAG00000020980	Catalase	0,620	1,02E-02
GCLC	ENSBTAG00000015571	glutamate-cysteine ligase, catalytic subunit	0,778	1,94E-03
GPX7	ENSBTAG00000018281	glutathione peroxidase 7	-0,675	3,37E-02
GSS	ENSBTAG00000003504	glutathione synthetase	1,145	8,21E-04
GSTM1	ENSBTAG00000037673	glutathione S-transferase mu 1	1,020	2,27E-04
Gstt1	ENSBTAG00000040298	glutathione S-transferase, theta 1	1,308	1,81E-06
Gstt3	ENSBTAG00000008587	glutathione S-transferase, theta 3	1,151	8,65E-03
MAF	ENSBTAG00000044192	v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog	-0,647	7,34E-02
MAP2K1	ENSBTAG00000033983	mitogen-activated protein kinase kinase 1	0,489	3,33E-02
MGST1	ENSBTAG00000008541	microsomal glutathione S-transferase 1	0,792	2,23E-02
MGST3	ENSBTAG00000010265	microsomal glutathione S-transferase 3	0,639	2,16E-02
MRAS	ENSBTAG00000001497	muscle RAS oncogene homolog	0,692	8,01E-03
NQO1	ENSBTAG00000020632	NAD(P)H dehydrogenase, quinone 1	1,302	2,25E-05
PRDX1	ENSBTAG00000003642	peroxiredoxin 1	0,777	3,77E-04
PRDX6	ENSBTAG00000004855	peroxiredoxin 6	0,542	1,47E-02
PRKCZ	ENSBTAG00000014119	protein kinase C, zeta	-1,589	1,44E-02
SCARB1	ENSBTAG00000014269	scavenger receptor class B, member 1	0,601	1,44E-02
SOD1	ENSBTAG00000018854	superoxide dismutase 1, soluble	0,490	8,54E-02
SOD2	ENSBTAG00000006523	superoxide dismutase 2, mitochondrial	0,673	2,39E-02
SQSTM1	ENSBTAG00000015591	sequestosome 1	0,724	2,34E-02
UBE2K	ENSBTAG00000020175	ubiquitin-conjugating enzyme E2K	0,452	9,97E-02
USP14	ENSBTAG00000019214	ubiquitin specific peptidase 14 (tRNA-guanine transglycosylase)	0,652	5,12E-02

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represent another part of the glutathione system. They are multifunctional, and may act as peroxidases as well as catalyzing the conjugation of glutathione with unwanted substrates such as epoxides and lipid hydroperoxides, making them eligible for transport out of the cell [42]. The shift towards a conjugate-forming oxidative defense rather than one relying solely on redox reactions involving GSH/GSSG also indicates an increased cellular export of glutathione in HE heifers, as these conjugates are excreted and catabolized extracellularly [43]. An increased export of glutathione conjugates out of the cell may explain the need for increased production of GSH and the up-regulation of *GCLC* and *GSS*. An increased cellular excretion of harmful substances could also be expected from the upregulation of *EPHX2*. This gene codes for soluble epoxide hydrolase (SEH), a peroxisomal enzyme playing a role in cellular detoxification by converting lipid epoxides to trans-hydro-diols, which are eligible for conjugation and subsequent excretion [44].

The genes *SOD2* and *CAT* were also up-regulated in HE heifers. Superoxide dismutase (SOD) converts superoxides to H_2O_2, which may subsequently be reduced by either GPX or catalase. If regarded in isolation, the up-regulation of these two genes probably would represent a deterioration of the antioxidant defense in the HE heifers, as catalase exclusively binds H_2O_2, and at a low affinity, while *GPX* has the ability to reduce several other organic peroxides as well. For this reason, catalase has been regarded as a second line antioxidant defense, taking effect when peroxide levels become supraphysiological [45].

The combination of DEG representing mitochondrial energy metabolism pathways such as the TCA cycle and oxidative phosphorylation as well as the antioxidant system, is what makes

the mitochondrial dysfunction pathway so highly ranked in the LPHE-LPLE contrast. During the last decade, increasing evidence has highlighted the associations between nutritional overload, mitochondrial changes in redox and energy state, insulin resistance and impaired adipocyte function [39–41, 45]. Although our HE heifers were clinically healthy, it is possible that these heifers, at 12 months of age and a BCS of almost 4 were on the verge of a harmful degree of fatness, and thus displayed a gene expression profile with similarities to that of morbidly obese individuals of other species. If this is the case, we may have revealed candidate genes for the early detection of obesity. Among these are genes coding for glutathione-S-transferases (*GSTT1*, *GSTT3*, *GSTM1*, *MGST1* and *MGST3*), *SOD2* and *CAT*.

Expression differences between protein treatments

The differences between the protein treatments within each energy group were minimal. Only *CRYM*, coding for μ-crystallin, was DE for the HPLE-LPLE contrast, and was down-regulated in the HPLE heifers. The expression of *CRYM* is negatively associated with serum glucose levels, and positively with insulin sensitivity [46]. Therefore, it is interesting that this gene was DE in heifers fed different protein levels, but not different energy levels. Depending on passage rate and heat treatment, the ruminal digestibility of ground barley (present in varying amounts in our experimental concentrates) varies from 80–90% [47, 48], and the main product is propionate. Thus, only 10–20% of ingested starch may pass undegraded from the rumen to the intestine and can be absorbed into the blood as glucose. Given the higher starch intake of LPLE heifers, one would expect a higher blood glucose and a decreased *CRYM* expression in these animals, but it is possible that the association of both *CRYM* expression and blood glucose levels with insulin sensitivity may actually cause the opposite effect if insulin sensitivity is sufficiently high. If a difference in daily starch intake has indeed resulted in the observed up-regulation of *CRYM*, this indicates that certain parts of the metabolic regulation in heifers are sensitive to small changes in protein and carbohydrates available through feed, even on isocaloric diets, and that *CRYM* may be a sensitive indicator of glucose load in cattle. For the HPHE-LPHE contrast only *DSP* and *ACTA1* were DE, for which any notable association to dietary protein or starch are as of yet unknown. Thus, we have not found any genes to be differentially expressed as an effect of different feed protein levels regardless of energy level. This emphasizes further the dominant effect of total ration composition above the single effects of dietary energy or protein on the adipose tissue gene expression of growing heifers.

Conclusions

Dietary energy content has a massive impact on the bovine adipose tissue transcriptome regulating several aspects of energy metabolism. The size of the effect is modulated by dietary protein content. The different protein levels applied in this study had only minor effects on adipose gene expression *per se*. Compared to heifers with a BCS of 3.63, heifers fed to a BCS of 3.95 at 12 mo of age displayed several transcriptional responses which have been associated with increased oxidative stress in other species. This study is a first step towards the identification of biomarkers for early-stage obesity (*GSTT1*, *GSTT3*, *GSTM1*, *MGST1*, *MGST3*, *SOD2*, *CAT*) and glucose load (*CRYM)* in cattle. However, certain biomarker identification will require further validation studies such as functional assays, lipidomics and proteomics. The current study underpins the importance of adipose transcriptional regulation of metabolism in growing cattle and highlights how total ration characteristics have a larger impact on adipose tissue transcriptomes than the single effects of protein and energy.

Supporting information

S1 Table. All differentially expressed genes from comparison of low-protein, high-energy- and low-protein, low-energy fed heifers. Pivot table of all differentially expressed genes found by comparing low-protein,high-energy (LPHE) and low-protein, low-energy (LPLE) fed heifers. Red rows show upregulated and green rows show downregulated genes in the adipose tissue of LPHE versus LPLE heifers.
(XLSX)

S2 Table. All differentially expressed genes from comparison of low-protein, high-energy- and high-protein, low-energy fed heifers. Pivot table of all differentially expressed genes found by comparing high-protein,high-energy (HPHE) and high-protein, low-energy (HPLE) fed heifers. Red rows show upregulated and green rows show downregulated genes in the adipose tissue of HPHE versus HPLE heifers.
(XLSX)

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Author Contributions

Conceptualization: Ragnar Salte.

Data curation: Hilde K. L. Wærp, Sinéad M. Waters, Matthew S. McCabe, Ragnar Salte.

Formal analysis: Hilde K. L. Wærp, Sinéad M. Waters, Matthew S. McCabe, Paul Cormican.

Funding acquisition: Ragnar Salte.

Investigation: Hilde K. L. Wærp, Sinéad M. Waters, Matthew S. McCabe, Paul Cormican, Ragnar Salte.

Methodology: Hilde K. L. Wærp, Sinéad M. Waters, Matthew S. McCabe, Paul Cormican.

Project administration: Sinéad M. Waters, Ragnar Salte.

Resources: Ragnar Salte.

Software: Paul Cormican.

Supervision: Sinéad M. Waters, Matthew S. McCabe, Paul Cormican, Ragnar Salte.

Visualization: Sinéad M. Waters, Paul Cormican, Ragnar Salte.

Writing – original draft: Hilde K. L. Wærp, Sinéad M. Waters.

Writing – review & editing: Sinéad M. Waters, Matthew S. McCabe, Paul Cormican, Ragnar Salte.

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Paper III

Long-term effects of prior diets, dietary transition and pregnancy on adipose gene expression in dairy heifers

Hilde Lyby Wærp[1*], Sinéad M.Waters[2], Matthew S. McCabe[2], Paul Cormican[2], Ragnar Salte[1]

1. Department of Animal and Aquacultural Sciences, Norwegian University of Life Sciences, Ås, Norway.
2. Animal and Bioscience Research Department, Animal and Grassland Research and Innovation Centre, Teagasc, Dunsany, County Meath, Ireland.

* Corresponding author

E-mail: hilde.lyby@nmbu.no

Abstract

Adipose tissue is highly involved in whole-body metabolism and is the main site for lipid synthesis, storage and mobilization in ruminants. Therefore, knowledge about adipose tissue responses to different diets is important, especially in growing heifers as the feeding regimes of replacement heifers affect their future success as dairy cows. However, at gene expression level such knowledge is limited. As part of a larger feed trial, adipose tissue biopsies from 24 Norwegian Red heifers were collected at 12 months of age (12MO) and at month seven of gestation (PREG) and analyzed by next-generation mRNA sequencing. Between these two sampling points, all heifers had gone through a successful conception and a feed change from four dietary treatments of high or low energy (HE/LE) and protein (HP/LP) content (treatments LPHE, HPHE, LPLE and HPLE) to a low-energy, low-protein pregnancy feed given to all animals. Main objectives of this study were to examine post-treatment effects between the treatment groups at month seven of gestation; 6 months after the termination of experimental feeding, and to determine the long-term gene expression changes occurring in the adipose tissue between 12MO and PREG. Post-treatment group comparisons showed evidence of long-term effects of dietary treatment on adipose gene expression. Differences between protein treatments were smaller than between energy treatments. Adipose gene expression changes from 12MO to PREG were much larger for the HE than the LE treatments and seemed to mostly be explained by the characteristics of the diet change. 97 genes displayed a unidirectional expression change for all groups from 12MO to PREG, and are considered to be caused by pregnancy or increased age. This study provides candidate genes and key regulators for further studies on pregnancy preservation (TGFB1*, CFD*) and metabolic regulation and efficiency (PI3K, RICTOR, MAP4K4,) in dairy cattle.

Introduction

-The heifer is the cow of the future. Through the years, this recognition has brought about a plethora of heifer rearing studies investigating the relationships between heifer rearing strategies and animal performance including growth (1), mammary development (2-5), endocrinology (6), metabolic profile (7) and subsequent milk yield (7, 8).

The importance of a well-functioning metabolic regulation and an appropriate nutrient allocation in a successful dairy cow management is commonly known, and have been subject of intense research down to the level of hepatic (9-15), mammary (16) and adipose gene expression (17-23) in adult cows.

However, if one acknowledges the fact that a cow is not just a function of its genome and its environment at a given moment, but rather a function of its genome and all its previous environments, it is clear that if we are to optimize dairy management, we also need information about metabolic regulatory mechanisms at earlier life stages. In this regard, adipose tissue plays a key role as it is not only the end point for feed excess energy and an energy reserve for periods of higher energy consumption than input (24), but is also a transcriptionally and endocrinologically active organ strongly involved in the regulation of whole-body metabolism (25, 26). There are several published gene expression studies on adipose tissue in growing cattle (23, 27-31) , but so far no reports of post-treatment dietary effects or effects of pregnancy on the total adipose transcriptome. As part of a larger feed trial, adipose tissue biopsies were collected at 12 months of age (12MO) and at month seven of gestation (PREG, 20 (HE) and 24 (LE) months of age) from 24 Norwegian Red heifers and analyzed by next-generation RNA sequencing (RNA-seq). Between these two sampling points, all heifers had gone through a successful conception and a feed change from four dietary treatments differing in energy and protein content, to a low-energy, low-protein

pregnancy feed given to all animals. During pregnancy, we observed different growth responses to the pregnancy feed between the dietary treatment groups. Such post-treatment differences have been described by others, and have been found to account for a larger fraction of milk yield variability than the experimental feeding per se (32). It is therefore of great interest to investigate the metabolic regulation underlying these post-treatment effects. Thus, the main objective of this study was to examine post-treatment gene expression differences between the experimental feeding groups at PREG; six months after change to a consistent feeding regime. A secondary objective of this study was to describe the gene expression changes occurring in the heifers between 12MO and PREG. Finally, differentially expressed genes associated with pregnancy, diet change or post-treatment dietary effects identified in this study could potentially yield candidate genes for further studies on breeding and feeding for improved feed efficiency, production and reproduction in Norwegian Red cattle.

Materials and methods

Ethics statement

All experimental procedures involving animals were approved by the Norwegian Animal Research Authority (FOTS id 2955, reference no. 2010/203231). Biopsies were harvested under local anesthesia, and all animals were given 3 mg/ kg BW of Ketoprofen intramuscularly (Comforion vet., Orion Pharma Animal Health) to prevent pain or inflammation at the biopsy site.

Animal model

Eighty Norwegian Red heifers from the university herd (born in 2010 and 2011) were randomly assigned either to a high (HE) or low (LE) energy group, destined for a BW gain of 850 – 1000 or 600 – 750 g/day from three months of age to confirmed pregnancy, respectively. Each of the energy groups were split into two protein groups, low (LP) or high (HP), to give four dietary treatment groups with 20 animals in each group, *viz* Low-protein high-energy (LPHE), high-protein high-energy (HPHE), low-protein low-energy (LPLE) and high-protein low-energy (HPLE). Target body weight at conception was 400 kg for all heifers. Thus, heifers on HE treatments were inseminated at an earlier age than LE heifers, because of their faster growth. Age at successful insemination was 13.6 months for LPHE, 12.8 months for HPHE, 17.1 months for LPLE and 16.9 months for LPHE heifers, and PREG samples were taken 7 months after these age points. After confirmed pregnancy (at day 28-42 of gestation), all heifers were group-fed the same diet *ad libitum.* The pregnancy diet was designed for a moderate growth of about 550 g/ d until calving to reach a target BW of 560 kg and a maximum BCS of 3.75 at calving. Nutrient content in the experimental diets at 12 months of age and in the pregnancy diet at month seven of gestation is shown in Table 1.

Table 1. Average nutrient content of experimental diets at 12 months and common diet offered during pregnancy.

	12MO				PREG
	LPHE	HPHE	LPLE	HPLE	
Net energy (MJ/kg DM)	6.43	6.43	5.98	5.98	5.66
Crude protein (g/kg DM)	142	149	113	122	113
Neutral detergent fiber (g/kg DM)	508	510	579	581	569
Starch (g/kg DM)	40	26	49	32	0

Experimental diets were Low-protein, High-energy (LPHE), High-protein, High-energy (HPHE), Low-protein, Low-energy (LPLE) and High-protein, Low-energy (HPLE). PREG= Pregnancy diet given to all animals. Total ration content for 12MO diets as calculated from average nutrient content of roughages and concentrates used and least square means of dry matter intake of roughage and concentrate at 12 months for each treatment. These data were put into Optifôr, a digital feed planning tool based on NorFôr, the nordic feed evaluation system for cattle (33). Total ration content for the PREG diet was calculated as the average nutrient content of the roughages offered during pregnancy (single feed ration).

Adipose biopsy sampling and sample preparation

Adipose tissue biopsies were harvested at 12 months of age (12MO) and at month seven of gestation (PREG). These time points were chosen to assess the long-term between-treatment differences and within-treatment changes when the heifers were in a steady state, without the interference of a recent feed change or an imminent calving.

Biopsy sampling procedure, RNA extraction and library preparation were performed as described by Wærp et al. (34). Briefly, 0.5-1.5 g of adipose tissue was harvested from the tail base of the heifers under epidural anesthesia. Biopsies were snap frozen in liquid nitrogen, and subsequently stored at -80°C. 48 Adipose tissue samples from 24 heifers were selected for RNA sequencing (n=6 for each group). RNA extraction was carried out using the Qiagen RNeasy lipid tissue mini kit (Qiagen). The quality and RIN values of the extracted samples were assessed using an Agilent bioanalyzer. RIN values varied between 7.8 and 9.2. To avoid DNA contamination, all RNA samples were subject to a DNAse treatment after extraction (Turbo DNA-free, Ambion, Life technologies) and a subsequent cleanup (RNA Clean & Concentrator -25, Zymo Research Corp).

Library preparation was performed using the Illumina TruSeq RNA sample prep kit v2, according to the manufacturer's manual.

Sequencing was performed in an Illumina HiSeq 2000 workstation, with 100 bp paired end reads, and four samples per lane (Clinical Genomics, Toronto).

Statistics and Bioinformatics

The fastq files containing the raw sequence reads were examined using FASTQC (v 0.11.2.) Adapters were trimmed from all sample fastq files using Cutadapt (v 1.3). Reads were simultaneously quality trimmed by removing reads shorter than 20 bp, or with Phred scores below 25. Following adapter and quality trimming, all files were checked again in FASTQC. Paired-end read files were aligned to the UMD3.1 bovine genome assembly (35), using Tophat (v 2.0.12) (36). The number of reads per gene in each sample was counted using HTSeq-count (v 0.6.1) (37). Statistical analysis of read counts were carried out in EdgeR (v 3.1.2), a Bioconductor software package run in the statistical software

environment R (v 3.1.2) (38). All differential gene expression analyses were performed in EdgeR, using tagwise dispersions. The statistical tests were corrected for multiple testing using the Benjamini-Hochberg method as implemented in EdgeR (39, 40). To compare gene expression differences between treatments, a generalized linear model which included dietary treatment and genetic group was fitted to the data using the glmFit function (39). As EdgeR cannot estimate multiple levels of variation, the 12MO and PREG sample sets were divided into subsets and analyzed for treatment differences in separate runs, to avoid bias caused by unaccounted within-individual variation (due to the paired nature of the total data set).

The comparisons were performed as pairwise comparisons between contrasting treatments, i.e. LPHE-LPLE, HPHE-HPLE, LPHE-HPHE and LPLE-HPLE. Significantly differentially expressed genes (DEG) were called at a FDR (false discovery rate adjusted p-value as given in EdgeR) of 0.05. A full description of the DEG representing treatment differences at 12 months of age (12MO samples), is presented in a separate paper by our group (34). To correct for any effect of individual animals, the difference in gene expression between 12MO and PREG was analyzed as a pairwise comparison within each treatment group. Subsequently, the resulting DEG lists were compared to find the DEG common to all treatments. Gene expression changes from 12MO to PREG which were common to all treatments was assumed to have occurred independently of previous dietary treatment, and to rather be caused by pregnancy or increased age. The lists of differentially expressed genes from each comparison were subsequently analyzed through the use of the core analysis function in QIAGEN's Ingenuity® Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity) to find over-represented biological pathways and upstream regulators represented by the DEG. Significance level for the pathway analyses was set at $p < 0.05$. Finally, we run an IPA comparison analysis of the 12MO-PREG DEG

lists within each treatment was to compare the gene expression changes between 12MO and PREG and the upstream regulators controlling them for each dietary treatment. Pathways or functions specifically pertaining to irrelevant diseases, species or tissues were omitted. The RNAseq raw data are deposited and accessible through NCBI's Gene Expression Omnibus with the GEO Series accession number GSE79347.

Results and discussion

Animal performance

Body weight and BCS at 12 months of age, conception, 7 months of pregnancy and calving are shown in Table 2. Both HE and LE heifers followed their planned growth trajectories throughout the experimental feeding period, with an average daily gain (ADG) of 930 and 660 g/d, respectively. However, the response to the pregnancy diet differed between treatment groups: LE heifers displayed a faster growth than expected with an ADG of 600 g/d., while the HE heifers only achieved an ADG of 470 g/d during pregnancy. This led to the HE heifers calving in at a BW of approximately 535 kg at 22 months of age, while the LE heifers calved in at 26 months at a BW of approximately 565 kg.

Table 2. Least square means of body weight (BW) and body condition score (BCS) at 12 months of age, conception, 7 months of pregnancy and calving.

	Treatment	Age (mo)	BW (kg)	BCS (scale 1-5)
12 months	LPHE	12	363 (0.9)[a]	3.99 (0.016)[a]
	HPHE	12	371 (1.1)[b]	3.95 (0.017)[a]
	LPLE	12	295 (0.8)[c]	3.63 (0.014)[b]
	HPLE	12	311 (0.8)[d]	3.63 (0.013)[b]
Conception	LPHE	13.6	411 (5.2)[a]	3.98 (0.02)
	HPHE	12.8	396 (5.3)[ab]	3.93 (0.02)
	LPLE	17.1	393 (5.2)[b]	3.73 (0.02)
	HPLE	16.9	406 (5.2)[ab]	3.73 (0.02)
7 months pregnant	LPHE	20.6	483 (3.8)[a]	3.80 (0.010)[a]
	HPHE	19.8	477 (3.7)[a]	3.79 (0.010)[a]
	LPLE	24.1	506 (3.9)[b]	3.75 (0.011)[b]
	HPLE	23.9	516 (3.9)[c]	3.74 (0.011)[b]
Calving	LPHE	22.5	537 (11.4)[a]	3.70 (0.02)
	HPHE	21.9	532 (10.7)[a]	3.65 (0.02)
	LPLE	26.1	571 (11.0)[b]	3.71 (0.02)
	HPLE	25.8	564 (11.7)[a]	3.73 (0.02)

LPHE= Low-protein, High-energy; HPHE= High-protein, High-energy; LPLE= Low-protein, Low-energy and HPLE= High-protein, Low-energy. N=80 (all animals in the feeding experiment). Numbers with different superscripts differ. Standard errors are presented in parentheses.

Read alignment and overall results

Sequencing yielded on average 57.2 million raw reads per sample. Alignment gave an average overall read mapping rate of 95.1 % and a concordant pair alignment rate of 89.6 %. Genes with a minimum CPM (counts per million) of two in at least six (n=6) samples were considered to be expressed and were included in the differential expression analysis. Out of 26,740 gene transcripts in the UMD3.1 bovine genome assembly, 13,396 gene transcripts fulfilled this criterion. The DEG lists resulting from the examined contrasts varied greatly in size. The number of DEG found for each contrast is presented in Table 3. At PREG, the LPHE-LPLE and HPHE-HPLE comparisons resulted in 79 and 57 DEG, respectively. The main affected pathways for these comparisons are shown in Figs 1 and 2. The HPLE-LPLE comparison yielded only five DEG, while we found no DEG when comparing HPHE and LPHE. All post-treatment comparison DEG are listed in Supplementary Table S1, and complete lists of significantly affected pathways for the LPHE-LPLE and HPHE-HPLE comparisons are given in Table S2. The HP-LP comparisons yielded no significantly affected pathways.

The HE treated heifers underwent a larger change in ration characteristics after conception than heifers on LE treatments and this seems to be reflected in their degree of alteration in gene expression between 12MO and PREG, as these groups displayed more than 7 times as many DEG as any of the LE groups. The 12MO-to-PREG change in gene expression was massive for both HE treatments; The LPHE heifers displayed 3987 DEG, and the HPHE heifers displayed 3997 DEG with an FDR < 0.05. The 12MO-to-PREG gene expression change for the two LE treatments was less pronounced but still considerable, with 534 and 334 DEG (FDR < 0.05) for the LPLE and HPLE groups, respectively. The within-treatment 12MO-to-PREG gene expression changes were larger than the differences found between the dietary treatment groups during 12MO (34), and much larger than any

differences found between treatments at PREG. The full lists of DEG between 12MO and PREG are shown in Supplementary Tables S3-S6 for the LPHE, HPHE, LPLE and HPLE treatments, respectively.

As illustrated in Fig 3, the 12MO-to-PREG DEG for each group displayed a complex pattern of overlapping: The two HE groups, which had the greatest change in total gene expression had about three quarters of their DEG in common, with each group also displaying a large number of unique DEG. The two LE groups displayed a much smaller overall gene expression change, but comparable fractions of shared and unique DEG. IPA pathway analysis yielded 506, 493, 314 and 215 significantly affected pathways for LPHE, HPHE, LPLE and HPLE heifers from 12MO to PREG, respectively. Complete lists of significantly affected pathways for each treatment group are presented in Supplementary Tables S7-S10. The main over-represented pathways for each treatment group from 12MO to PREG are shown in Figs 4-7.

Table 3. Number of differentially expressed genes (DEG) for all examined contrasts.

DEG numbers for all contrasts analyzed in EdgeR. LPHE= Low-protein, High-energy; HPHE= High-protein, High-energy; LPLE= Low-protein, Low-energy and HPLE= High-protein, Low-energy (HPLE). Upregulated means refers to upregulated in the first element of the contrast relative to the second. Genes with an FDR < 0.05 are defined as DE.

	DEG	Upregulated	Downregulated	Uncharacterized
Within-treatment changes from 12MO to PREG:				
12MO-PREG LPHE	3988	2873	1115	226
12MO-PREG HPHE	3997	2711	1286	244
12MO-PREG LPLE	534	471	63	31
12MO-PREG HPLE	334	160	174	19
Between-treatment differences at 12MO (34):				
LPHE-LPLE	693	493	200	111
HPHE-HPLE	18	11	7	7
HPHE-LPHE	2	1	1	0
HPLE-LPLE	0	0	0	0
Between-treatment differences at PREG:				
LPHE-LPLE	79	21	58	12
HPHE-HPLE	57	19	38	13
HPHE-LPHE	0	0	0	0
HPLE-LPLE	5	0	5	1

Fig 1. Main pathways affected by differently expressed genes between Low-Protein, High-Energy and High-Protein, High-Energy treated heifers at month seven of pregnancy.

Main affected pathways between heifers fed Low-Protein, High-Energy (LPHE) or Low-Protein, Low-Energy (LPLE) diets until pregnancy when comparing them at month 7 of gestation. Red bars indicate percent upregulated, and green bars indicate percent downregulated genes pertaining to the pathway in LPHE versus LPLE heifers. Number to the right of bars display total number of genes pertaining to each pathway. Orange squares indicate the negative logarithm of p-value of observation (-log p-value = 1.3 equals p-value = 0.05).

Fig 2. Main pathways affected by differently expressed genes between High-Protein, High-Energy- and High-Protein, Low- Energy heifers at month seven of pregnancy.

Main affected pathways between heifers fed High-Protein, High-Energy (HPHE) or High-Protein, Low-Energy (HPLE) diets until pregnancy when comparing them at month 7 of gestation. Red bars indicate percent upregulated, and green bars indicate percent downregulated genes pertaining to the pathway in HPHE versus HPLE heifers. Number to the right of bars display total number of genes pertaining to each pathway. Orange squares indicate the negative logarithm of p-value of observation (-log p-value = 1.3 equals p-value = 0.05).

Fig 3. **Unique and shared differentially expressed genes (DEG) from the experimental feeding period (12MO) to pregnancy (PREG).** The diagram shows the number of genes unique to or shared between experimental treatment groups with a change in expression during development from experimental feeding at 12 months of age, to established similar feeding regimes at month 7 of gestation for each dietary treatment. LPHE= Low-protein, High-energy; HPHE= High-protein, High-energy; LPLE= Low-protein, Low-energy and HPLE= High-protein, Low-energy (HPLE). Numbers in parentheses are the total number of differentially expressed genes found for each treatment. (Venn diagram modified from VennDiagram and Venneuler R packages).

Fig 4. Main affected pathways from experimental feeding period (12MO) to pregnancy (PREG) for Low-protein, High-energy fed heifers (LPHE). Main affected pathways for LPHE heifers during development from experimental feeding at 12 months of age, to established similar feeding regimes at month 7 of gestation. Red bars indicate percent upregulated, and green bars indicate percent downregulated genes pertaining to the pathway during PREG versus 12MO. Number to the right of bars display total number of genes pertaining to each pathway. Orange squares indicate the negative logarithm of p-value of observation (-log p-value = 1.3 equals p-value = 0.05).

Fig 5. Main affected pathways from experimental feeding period (12MO) to pregnancy (PREG) for High-protein, High-energy fed heifers (HPHE). Main affected pathways for HPHE heifers during development from experimental feeding at 12 months of age, to established similar feeding regimes at month 7 of gestation. Red bars indicate percent upregulated, and green bars indicate percent downregulated genes pertaining to the pathway during PREG versus 12MO. Number to the right of bars display total number of genes pertaining to each pathway. Orange squares indicate the negative logarithm of p-value of observation (-log p-value = 1.3 equals p-value = 0.05).

Fig 6. Main affected pathways from experimental feeding period (12MO) to pregnancy (PREG) for Low-protein, Low-energy fed heifers (LPLE). Main affected pathways for LPLE heifers during development from experimental feeding at 12 months of age, to established similar feeding regimes at month 7 of gestation. Red bars indicate percent upregulated, and green bars indicate percent downregulated genes pertaining to the pathway during PREG versus 12MO. Number to the right of bars display total number of genes pertaining to each pathway. Orange squares indicate the negative logarithm of p-value of observation (-log p-value = 1.3 equals p-value = 0.05).

Fig 7. **Main affected pathways from experimental feeding period (12MO) to pregnancy (PREG) for High-protein, Low-energy fed heifers (HPLE).** Main affected pathways for HPLE heifers during development from experimental feeding at 12 months of age, to established similar feeding regimes at month 7 of gestation. Red bars indicate percent upregulated, and green bars indicate percent downregulated genes pertaining to the pathway during PREG versus 12MO. Number to the right of bars display total number of genes pertaining to each pathway. Orange squares indicate the negative logarithm of p-value of observation (-log p-value = 1.3 equals p-value = 0.05).

Post-treatment differences between dietary treatments

Although offered the same feed ad libitum during pregnancy and entering pregnancy at a similar BW, the HE and LE heifers were not the same age (they were inseminated at about 13 and 17 months of age), they did not respond to the pregnancy diet in the same way and consequently seemed to be in two different physiological states during pregnancy. The LE-treated heifers maintained the same growth in response to the new ad libitum pregnancy feeding compared to the restricted rations they were offered earlier, and as BCS also remained constant the growth seemed to consist of proportional amounts of lean and fat tissue (Table 2). Compared to the experimental feeding period, the HE-treated heifers decreased their growth rate and BCS during pregnancy. However, the HE heifers maintained a steady growth of almost 500 grams per day throughout pregnancy, meaning they were in a positive energy balance. The concomitant decrease in BCS may indicate one of two things: Either, the HE heifers were able to maintain a lean mass gain from protein while mobilizing a net flux of lipids from the adipose tissue for energy consumption, or they deposited both protein and fat, but with an increased ratio of lean mass relative to adipose tissue, while "growing into" their already well-established fat depots. The LPHE-LPLE and HPHE-HPLE comparisons resulted in 79 and 57 DEG, respectively. Cholesterol biosynthesis, thyroid receptor complex (TR/RXR) activation, liver X receptor (LXR/RXR) activation and sirtuin signaling pathways were among the pathways which were significantly affected in both comparisons, and they were all mostly downregulated in the HE- compared to the LE-heifers. Sirtuins are a class of proteins involved in a wide range of cellular processes, including aging, transcription, apoptosis, inflammation, energy efficiency and adaptation to low calorie intake (41-43). The TR/RXR and LXR/RXR activation pathways are involved in the regulation of both lipid, carbohydrate and steroid metabolism (44, 45), and the latter (cholesterol metabolism) was significantly affected and

downregulated in HE heifers in both comparisons. As the HE heifers decreased their BCS through pregnancy, a decreased need for cholesterol in adipose cell membranes and downregulation of its production is logical.

The TR/RXR and LXR/RXR activation and sirtuin signaling pathways were represented mainly by genes which are also highly involved in energy metabolism pathways, such as *ACACA, FASN, ACLY, ACSS2, G6PD* and *SCD,* and no genes coding directly for sirtuins, thyroid or liver X receptors or RXR complexes were DE. Therefore we cannot ascertain whether the downregulation of these genes in HE heifers is mainly associated with cell signaling or with metabolic pathways.

A gene coding for an uncoupling protein, *UCP2,* is also associated with TR/RXR, LXR/RXR and Sirtuin signaling pathways and was upregulated in HE heifers in both comparisons. Uncoupling proteins are embedded in the inner mitochondrial wall of many cell types, but is the best known to be present in brown adipose tissue, where UCP1 is a prerequisite for the essential heat production function of this tissue (46). The uncoupling proteins function as proton carriers mediating a regulated proton leakage through the inner mitochondrial membrane, free-coupled from the reactions of the respiratory chain (47). Free-coupling proton flow from ATP production in the respiratory chain causes a futile cycle where energy spent on pumping protons against an electrical gradient is lost as the protons flow back out along the gradient without energy-rich molecules being produced. However, the roles of the other UCPs (UCP2-5) seem to be more complex than the well-known thermogenetic function of UCP1 (48). UCP2 has been found to act as a free radical scavenger, and it also plays a role in regulating insulin (48).

The *UCP2* gene is almost ubiquitously expressed throughout body tissues (48). It is regulated by TR and PPARγ and was recently shown to be regulated by diet in subcutaneous

white adipose tissue of cattle (49). However, our heifers had been offered the same diet for 6 mo before PREG biopsises were taken. Thus, the observed upregulation of *UCP2* in HE heifers is not a direct, contemporary effect of diet, and we do not know the reason for this post-treatment effect. It may represent a lasting change in the regulation of insulin secretion and glucose metabolism or an increased need for free radical scavenging because of a higher lipid turnover (BCS decrease) in the adipose tissue of pregnant HE heifers, but several other scenarios are also possible. Nonetheless, we find that the post-treatment upregulation of UCP2 in animals fed high-energy diets should be studied further.

The difference in gene expression between HPLE and LPLE heifers, although small, increased post-treatment; from zero at 12MO to five at PREG. This is surprising, however it is in agreement with the results of Van Amburgh et al, who found that post-treatment effects of prepubertal feeding regimes differing in energy and protein content had a larger impact on subsequent milk yield than the experimental feeding itself (32).

Three DEG maintained their direction of differential expression between treatment groups from 12MO to pregnancy, despite the feed change, the age difference and the different growth profiles obtained during pregnancy. Two of these genes were found to be DEG in the LPHE-LPLE comparison. The first gene was *RNS1*, coding for pancreatic ribonuclease. This was upregulated in LPHE relative to LPLE heifers at 12MO as well as PREG with an FDR<0.02, and fold changes of 2.5 and 5.7 at 12MO and PREG, respectively. The other gene found for the LPHE-LPLE contrasts was *ENSBTAG00000007075,* coding for a major histocompatibility complex class I (A-like precursor). This gene was also upregulated in LPHE heifers relative to LPLE heifers at both time points with an FDR<0.02. Log fold change was 3.4 at 12MO and 12.0 at PREG. The third gene displaying a persisting treatment effect at PREG was *ENSBTAG00000048049,* a novel gene with immunoglobulin-like sequences, with several orthologues in other species found to be an *IGHM* gene (50).

This was downregulated for HPHE with a log fold change of -2.9 and -6.1 in the HPHE-HPLE contrast at 12MO and PREG, respectively, and with an FDR <0.02 at both time points. As bovine pancreatic ribonuclease represents a very basic and general cellular function, it is hard to deduce exactly what function a persisting expression represents in the adipose tissue of heifers in our study. Furthermore, this gene was also one of the genes commonly upregulated across all treatment groups from 12MO to PREG. This indicates that several mechanisms has influenced the expression of this gene, and the apparently persistent treatment effect may in fact be the result of different underlying causes. The two immunologically related genes on the other hand displayed different, treatment-dependent changes from 12MO to PREG as well as the persistent DE between treatment groups at these time points. Together with the large expression differences (log fold change), this may indicate that the experimental diets have in fact elicited a lasting difference in the expression of *ENSBTAG00000007075* and *ENSBTAG00000048049*. If this is true, it may indicate a small window still open for some type of epigenetic programming acting through an interaction between nutrition and the immune system after three months of age, which is when the experimental treatments were initiated. To our knowledge, this has not been shown before, but further studies are required to either confirm or reject this hypothesis.

Within-treatment gene expression changes from 12MO to PREG

LPHE and HPHE treatments

For the LPHE and HPHE heifers, the gene expression changes occurring between 12MO and PREG were very similar. Apart from slight differences in the ranking of the significantly affected pathways and number of DEG, the main pathway results were nearly identical. Therefore, gene expression changes for these two treatments are presented and

discussed commonly in this section, under the term HE treatments (Any specific changes pertaining only to one group are specified).

Gene expression changes from 12MO to PREG for the HE treatments were dominated by a downregulation of pathways pertaining to energy metabolism and mitochondrial functions, namely oxidative phosphorylation, mitochondrial dysfunction, cholesterol biosynthesis and the TCA cycle. Fifteen genes representing all eight steps in the TCA cycle and 85 genes associated with either of the five complexes in the respiratory chain were significantly downregulated. This finding is in concordance with observations from the LPHE-LPLE contrast at 12MO: At this point, oxidative phosphorylation, mitochondrial dysfunction and the TCA cycle was clearly upregulated in the high-energy fed LPHE heifers compared to the restrictively fed LPLE heifers (34). The transition to the pregnancy diet implied a reduction in energy intake for the HE heifers. This possibly explains a downregulation of pathways connected to the production of energy-rich molecules available for lipid synthesis.

A small proportion of the DEG associated with mitochondrial dysfunction were upregulated instead of downregulated. None of these genes were directly associated with energy-yielding reactions, and therefore do not change the overall massive downregulation of genes associated with energy production in the adipose of HE heifers. However, among the few upregulated DEG pertaining to mitochondrial dysfunction were *NCSTN, APHA1, BACE1, APP, MAOB* and *CASP9.* These genes are all part of a mitochondrial pathway leading to production of amyloid-beta from the amyloid precursor protein (APP) and apoptosis (51). Such changes are associated with oxidative stress and lipid peroxidation (52), but studies on this pathway has so far been performed mainly with a focus on human diseases and little is known about its function in the adipose tissue of ruminants. However, apoptosis induced by caspases have also been associated with programmed cell death in lipoatrophy (53). These

DEG may therefore be an indication of apoptosis associated with the decreasing fatness of the HE heifers from 12MO to PREG (Table 2).

Cholesterol biosynthesis was a major affected pathway for the HE treatments. 17 out of 28 genes pertaining to this pathway were downregulated at PREG in HE treatments. Cholesterol is synthesized in adipose tissue, where it is stored as a free sterol in the phospholipid membrane of lipid droplets (54). Thus, it is logical to find a downregulation of cholesterol synthesizing pathways in adipose tissue when dietary energy is reduced.

Besides metabolic pathways, the list of highly affected pathways for the HE treatments was dominated by signaling pathways, and the one most significantly affected for the LPHE heifers was axonal guidance signaling (144 DEG). PI3K signaling in B lymphocytes (55 DEG) and leukocyte extravasation signaling (71 DEG) was highly ranked for both HE treatments.

The axonal guidance pathway was represented by 121 upregulated and 23 downregulated DEG for the LPHE heifers. Axonal guidance refers to the directional guidance of axons towards their target tissues or cells during tissue development or restructuring. White adipose tissue has both sympathetic and sensoric innervation, and the sympathetic system has been shown to regulate lipolysis (55). Axonal guidance is signaled via a large range of molecules, collectively named “guidance cues” which includes semaphorins, netrins, ephrins, SLIT ligands, morphogens (Wnt), growth factors, EPH receptors, ROBO receptors and cytokines (56, 57). Genes coding for all these types of molecules were upregulated in HE heifers at pregnancy. Even though there are no neuronal cell bodies present in adipose tissue, the mRNA reads for these gene products in our data may originate from neurons, as transport to and local translation of neuronal mRNA in axons, far away from the nuclei in which they were transcribed, constitutes an important regulation mechanism in axon

guidance (57). As the axonal guidance pathway was so clearly upregulated in LPHE heifers, and not in any of the other treatment groups, we assume it to be affected by their dietary treatment. If so, it is reasonable to assume that axonal guidance occurred to a greater degree in this group because these heifers experienced the greatest alteration in diet and the largest decrease in BCS causing these heifers to have the greatest degree of adipose tissue restructuring.

Phosphatidylinositol 3-kinases (PI3K) exert their effects on membrane signaling complexes within many pathways and systems (58, 59). In B lymphocytes, PI3K regulates both activation, proliferation and differentiation (58). More recently, PI3K signaling has also been shown to play an important role in energy balance regulation: Experimentally induced reduction of PI3K signaling increases energy expenditure and protects from obesity and obesity-related disorders (59, 60). Thus, the upregulation of PI3K signaling in adipose B lymphocytes of HE heifers after transition to low-energy feeding may be part of an energy-saving strategy. If so, manipulation of PI3K signalling may provide a means for increased feed efficiency in cattle, the opposite goal of ongoing human research, which focuses on alleviating obesity by increasing energy expenditure through PI3K functions.

Leukocyte extravasation and immune cell infiltration of adipose tissue has been the subject of intense research over the last decade, as obesity, metabolic syndrome and adipose tissue dysfunction in humans is associated with low-grade chronic inflammation and increased immune cell trafficking of adipose tissue (61). However, our finding is an upregulation of leukocyte extravasation signals in animals subjected to a reduction in feed energy, displaying no increase in adiposity. This is in line with recent research on mouse models, stating that adipocyte inflammation is not only associated with adipocyte-related diseases, but is also essential for physiological tissue remodeling (62). The type, degree, and duration

of leukocyte migration and infiltration is interdependent on the type of physiological or pathological change occurring in the adipose tissue (61, 62).

LPLE

The LPLE treatment expression changes from 12MO to PREG clearly differed from the other three dietary treatments: The LPLE experimental diet was the one most similar to the pregnancy diet, and this seems to be reflected in the gene expression of the LPLE heifers through the lack of affected metabolic pathways. The top list of affected pathways due to/or during diet change for this treatment group almost exclusively consisted of pathways associated with immunological and inflammatory functions (Fig 6). It is also worth mentioning that out of the eleven most affected pathways for this group, six were specifically associated with T lymphocytes (CD28 signaling in T helper cells, NFAT regulation of the immune response, PKCθ signaling in T-lymphocytes, iCOS-iCOSL signaling in T helper cells, Th1 and Th2 activation and Th2 pathway). The type of immune cells involved in adipose tissue infiltration is dependent on the current stage and physiology / pathology of adipose tissue (61). T cell infiltration of the adipose tissue has been described as a primary event in the development of obesity and obesity-related disorders such as adipose inflammation and insulin resistance in humans (63). However, the LPLE heifers were never obese during our trial. Therefore, it is possible that T cell infiltration in adipose is not necessarily associated with morbidity but is rather a response to a positive energy balance and increased energy flux towards the adipose.

HPLE

For the HPLE heifers, the diet change from their experimental feed treatment to the pregnancy feed meant a transition to a ration of lower protein density. From 12MO to PREG, we observed a downregulation of several pathways associated with amino acid

degradation in the HPLE heifers; As shown in Fig 7, 11 of the 20 most highly affected pathways were implicated in the degradation of valine (Val), leucine (Leu), methionine (Met), alanine (Ala), phenylalanine (Phe), glycine (Gly) and glutamate (Glu). Except for Met and Gly, showing a mixed pattern of up- and downregulated DEG, all DEG pertaining to these pathways were downregulated at PREG. No other treatments displayed a similar downregulation of amino acid degrading pathways at PREG, and the reason for HPLE heifers to do so remains unknown. However, we allow ourselves to contemplate upon some possible explanations: The capacity of adipose tissue to both synthesize and degrade AA in reaction to concentrations of insulin, glucose or specific AA as part of whole-body metabolism and regulation has been known for decades (64-67). The pregnancy diet and the experimental diet given to the HPLE heifers differed both in protein content and protein sources (because of the inclusion of concentrate in the experimental diet). Thus, both the total and relative amounts of different AA available to the animals could be expected to differ between the two diets, and the allocation and metabolic fate of specific AA likewise. However, most of the AA represented by significantly affected degradation pathways also have important functions other than acting merely as protein precursors and energy substrates: Methionine also acts as a methyl donor for transmethylation reactions in lipid biosynthesis and this function may also be the cause of Met degradation in the adipose tissue of HPLE heifers (68). Levels of circulating branched chain amino acids (BCAA; Val, Leu, Isoleu) are associated with metabolic regulation, obesity and insulin resistance (69, 70) , and are regulated by BCAA metabolism in adipose tissue (71). The other AAs represented by downregulated degradation pathways (Phe, Ala, Gly and Glu) also possess vital functions in addition to being energy substrates and protein precursors (72), and the background for the downregulation of their degradation pathways at PREG is almost certainly more complex than a simple up- and downregulation because of their availability as protein

precursors in peripheral tissues. However, as HPLE was the only group displaying this effect, it must be assumed to be dietary related.

The HPLE heifers received a diet of similar energy content but more protein than the LPLE heifers during 12MO, and the net energy intake for HPLE was very similar to that of the LPLE heifers (Table 1). Therefore, one would expect the change in energy intake from 12MO to PREG for the two LE groups to be similar, and small. Interestingly, the HPLE heifers still displayed a pathway analysis result with a closer resemblance to the results for the HE treatments, although at a smaller scale, with a downregulation of mitochondrial dysfunction and oxidative phosphorylation indicating a decrease in energy flux towards adipose tissue. Together with the downregulation of amino acid degradation pathways this indicates that the effect of total ration characteristics during 12MO has a larger effect than dietary energy and protein in isolation.

Changes from 12MO to PREG common to all groups

Among the genes found to be differentially expressed between 12MO and PREG samples for all four groups, 97 displayed a similar up- or down-regulation in all groups. These genes are listed in Supplementary Table S11. We interpret the differential expression of these genes to be treatment-independent, and that they were caused by pregnancy, aging or by characteristics of the pregnancy feed per se. Pathway analysis of the common DEG yielded 23 significantly over-represented pathways. The main pathways are shown in Fig 8, and among these were the complement system, leucotriene biosynthesis and eicosanoid signaling. The immune system is known to undergo several changes during pregnancy, in order to avoid rejection and expulsion of the fetus while maintaining an adequate immune competence to combat infections and other threats. (73-76). A characteristic part of these

changes seems to be an activation of the innate immune system, including the complement system and natural killer cells (74, 75, 77). Adipsin (also known as Complement factor D, coded by *CFD,* upregulated in PREG heifers), has been subject to extra interest as it seems to have an association with pre-eclampsia and abortion (78-80). Thus it seems complement activation may be part of both physiological and pathological immune changes related to pregnancy.

The upregulation of genes coding for arachidonate 5-lipoxygenase (*ALOX5*) and its activating protein (*ALOX5AP*) together with leukotriene C4 synthase (*LTC4*) indicates an increased leukotriene production, especially of LTC4, from arachidonic acid in adipose tissue during pregnancy. Expression changes of *ALOX5* and *ALOX5AP* through gestation and during labour in human chorionic membranes have been shown, suggesting a role for these genes both at parturition as well as earlier in pregnancy (81). Concentration of LTC4 has also been shown to increase in human amniotic fluid during labour (82). An increased capacity for leukotriene synthesis during pregnancy has recently been detected in human blood (73), but so far, no studies have reported such effects in adipose tissue. As the heifers in our study underwent normal pregnancies we assume the observed changes in their gene expression to be physiological. However, further studies should be performed to establish whether any of these genes are potential biomarkers for gestational health or preservation.

Fig 8. Main affected pathways common to all treatments during the period from 12MO to PREG. Main affected pathways associated with differentially expressed genes common to all heifers during development from experimental feeding at 12 months of age, to established similar feeding regimes at month 7 of gestation. Red bars indicate percent

upregulated, and green bars indicate percent downregulated genes pertaining to the pathway during PREG versus 12MO. Number to the right of bars display total number of genes pertaining to each pathway. Orange squares indicate the negative logarithm of p-value of observation (-log p-value = 1.3 equals p-value = 0.05).

Upstream regulators

Despite the differences in DEG, growth rates and BCS development during the period from 12MO to PREG the treatment groups displayed many similarities in their main upstream regulators as analyzed by IPA. A heatmap containing key regulators and their most highly affected DEG is shown in Fig 9. The comparison analysis proved transforming growth factor-β 1 (TGFB1) to be the highest ranked upstream regulator of identified DEG overall. The *TGFB1* gene itself was significantly upregulated in the HE groups, but not in the LE groups. In the HE groups, TGFB1 was directly or indirectly associated with > 450 of the identified DEG between 12MO and PREG. These results indicate a widespread involvement in several cell functions, but most DEG connected to TGFB1 could be associated with immune functions for all treatments. In adipose tissue, TGFB1 is mainly secreted by cells other than adipocytes (83). However, an increased secretion of TGFB1 has been associated with obesity in other species (83). This is contradictive to our study, where TGFB1 was upregulated only in the HE heifers, which underwent a decrease in energy intake and BCS during this period. This supports the notion that the main function of TGFB1 in our heifers were in the regulation of pregnancy-induced immune system changes, but that these changes to some extent were different because of different preceding dietary treatments. Gene

polymorphisms and plasma levels of TGFB1 have also shown associaton with the risk of preeclampsia and recurrent abortions in humans (84). As such, it would be interesting to investigate whether TGFB1 or any of its affected downstream molecules proves to be indicators of pregnancy preservation capabilities in cattle as it is in humans.

Rapamycin-insensitive companion of mammalian target of rapamycin, encoded by *RICTOR,* is part of a protein complex which regulates cell growth based on nutritional status and growth factor-derived signals. This signal protein seem to be highly involved in the changes occurring in the HE-treated heifers from 12MO to PREG, but much less so in the LE-treated heifers. An IPA core analysis run on the DEG associated with RICTOR in the HE heifers showed it to be mostly involved in downregulation of genes associated with mitochondrial energy production. These functions were clearly downregulated in the HE heifers compared to the LE heifers due to the larger reduction in dietary energy for these groups. This explains the difference in RICTOR involvement between the two energy treatments, and assigns RICTOR to a role in the regulation of energy production in adipose tissue.

Interferon Gamma (IFNG) is a cytokine regulating a wide range of immune and inflammatory functions. In our study, IFNG was most highly associated with function of T cells, the complement system, cell differentiation and maturation and interleukin signaling across treatments. It was least active in HPLE heifers and most in HPHE and LPLE. Compared to the other treatments, the overall impact of IFNG in the HPLE heifers was also slightly skewed with involvement in citrulline metabolism highly ranked together with a more narrow range of immune functions, such as the complement system and phagosome formation. The reason for this is not known.

Mitogen-activated protein kinase 4 (MAP4K4) displayed increased activity from 12MO to PREG for all treatments except LPLE. The activation also seemed lower for HPLE heifers

than for the HE treatments. As for RICTOR, this is explained by the DEG associated with MAP4K4 and to the changes occurring within each treatment group from 12MO to PREG: MAP4K4 was mostly associated with DEG pertaining to energy production, and these were downregulated in HE heifers and to a lesser degree in HPLE heifers (Figs 4, 5 and 7). The LPLE heifers, which underwent the smallest dietary change, did not display such changes (Fig 6). Thus, it seems the regulating function of MAP4K4 in our experimental heifers was mainly to decrease the production of energy rich compounds in adipose tissue when subjected to a decrease in dietary energy flux.

Tumor necrosis factor (TNF) was also ranked high among the upstream regulators for all groups. Tumor necrosis factors are a family of multifunctional cytokines with known effects on apoptosis, inflammation, lipid metabolism and cellular redox balance among others (85). In the context of our study, it is of interest that TNF is an activator of leukotriene production pathways, which were upregulated in all groups at PREG (Figs 4-7 and 9). TNF also acts upon TP53, and mediates some of its effects through this transcription factor (86), and TP53 was also ranked very high among upstream regulators for all treatment groups. *TNF* itself was not DE for any group, but all groups displayed between three and 19 DEG associated with activity regulation of TNF such as *TNFAIP3, TNFAIP6, C1QTNF6, TNFSF18, LITAF, TNIP2, TRAF5* and *TNFRSF1B.* This is a good example of the versatility and flexibility of gene regulation: It may act directly upon the gene coding for a main regulator (as in this case with *TGF1B*), or it may act upon several other genes coding for regulators of the regulator (as here with TNF).

Fig 9: Heatmap displaying top 20 upstream regulators active across all treatments from the experimental feeding period (12MO, 12 mo) to month 7 of pregnancy (PREG,

20 (HE) and 24 (LE) mo) and most highly differentially expressed genes (DEG) within each treatment for the top 4 upstream regulators.

LPHE= Low-protein, High-energy; HPHE= High-protein, High-energy; LPLE= Low-protein, Low-energy and HPLE= High-protein, Low-energy (HPLE). LogFC = log Fold Change.

a) Heatmap of upstream regulators with activation z-score. Orange squares indicate activation, blue squares indicate deactivation of the regulator functions. A $-2 \geq$ z-score ≤ 2 indicates that the activation /deactivation was statistically significant ($p < 0.05$).

b) Most highly up- or downregulated genes pertaining to upstream regulator Transforming Growth Factor 1 (TGFB1), as identified by IPA comparison analysis. Red/pink squares indicate upregulation, green squares indicate downregulation as measured by logFC.

c) Most highly up- or downregulated genes pertaining to the upstream regulator Rapamycin-insensitive companion of mammalian target of rapamycin (RICTOR), as identified by IPA comparison analysis. Red/pink squares indicate upregulation, green squares indicate downregulation as measured by logFC.

d) Most highly up- or downregulated genes pertaining to upstream regulator interferon gamma (IFNG) as identified by IPA comparison analysis. Red/pink squares indicate upregulation, green squares indicate downregulation as measured by logFC

e) Most highly up- or downregulated genes pertaining to the upstream regulator mitogen-acticated protein kinase 4 (MAP4K4), as identified by IPA comparison analysis. Red/pink squares indicate upregulation, green squares indicate downregulation as measured by logFC.

Summary

Gene expression differences between heifers fed different levels of energy and protein are evident even 6 months after the termination of different feeding regimes and transition to the same diet. Only a few of the gene differences observed while being fed different diets are still present post-treatment, indicating a possible immunologic or epigenetic programming, while most post-treatment differences seem to be reflections of different physiological responses to the new diet, rather than the dietary content itself. Still, diet also has a major direct impact on adipose gene expression, which is clear from the treatment-dependent and varied gene expression changes occurring within treatment groups from experimental feeding at 12 months of age until similar feeding at 7 months pregnant. Pregnancy is known to cause immunologic changes, and in the adipose tissue of our heifers, these changes seem to consist mainly of complement activation and increased leukotriene synthesis. Through our study, we have identified several candidate genes and key gene regulators which should be studied further with regards to pregnancy preservation (TGFB1*, CFD*) and metabolic regulation and efficiency (*UCP2,* PI3K, RICTOR, MAP4K4,) in dairy cattle.

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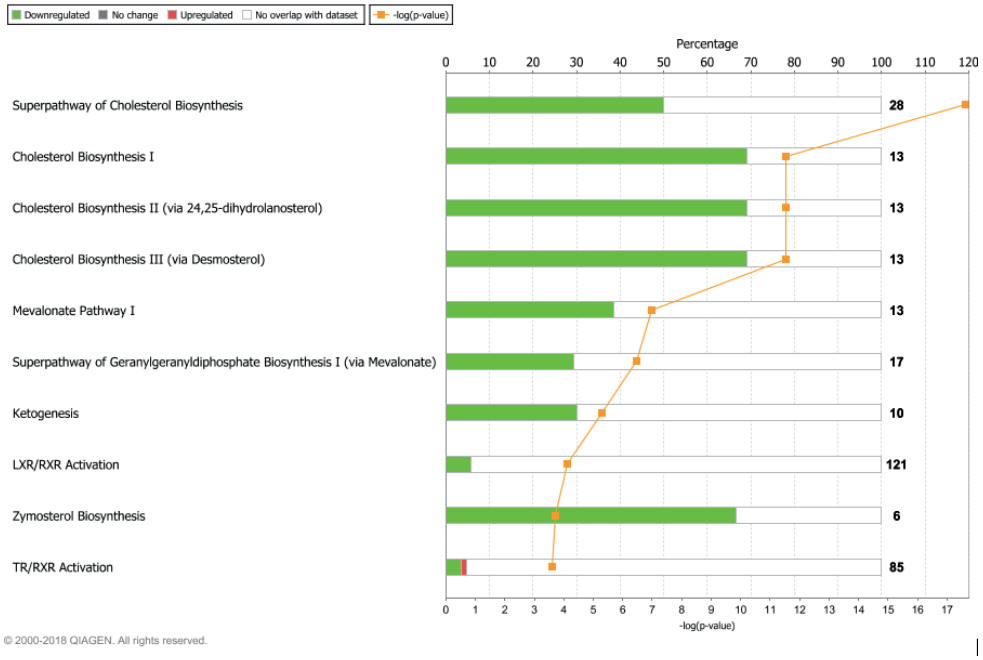
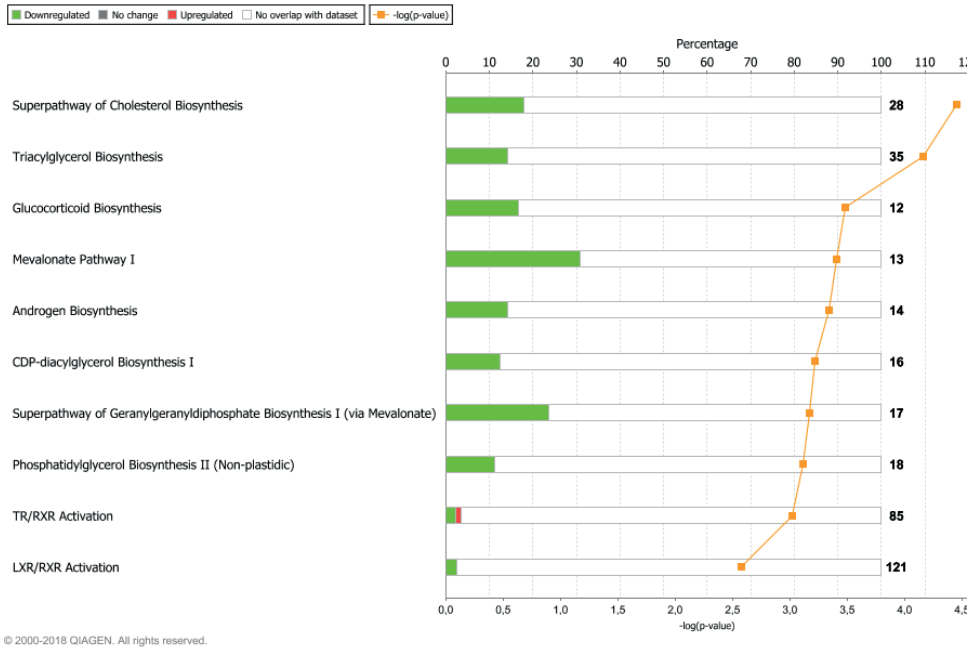


Figure 1.



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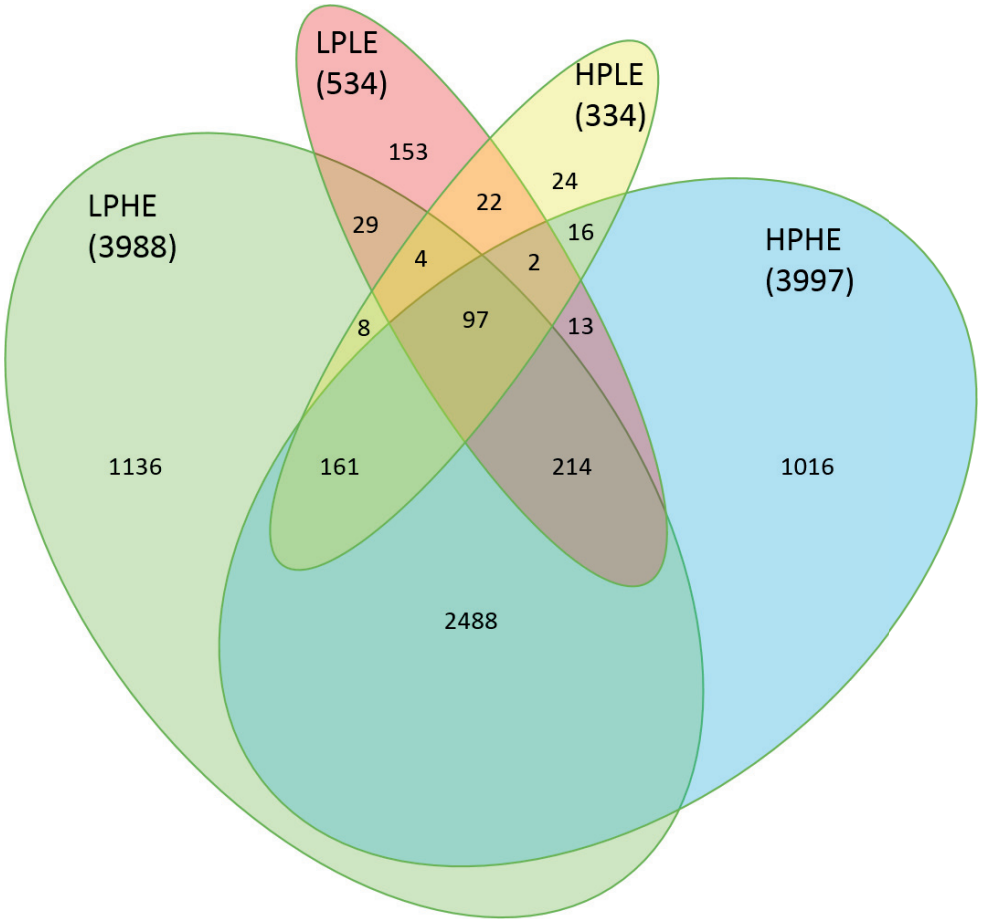
LPLE
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Figure 3.

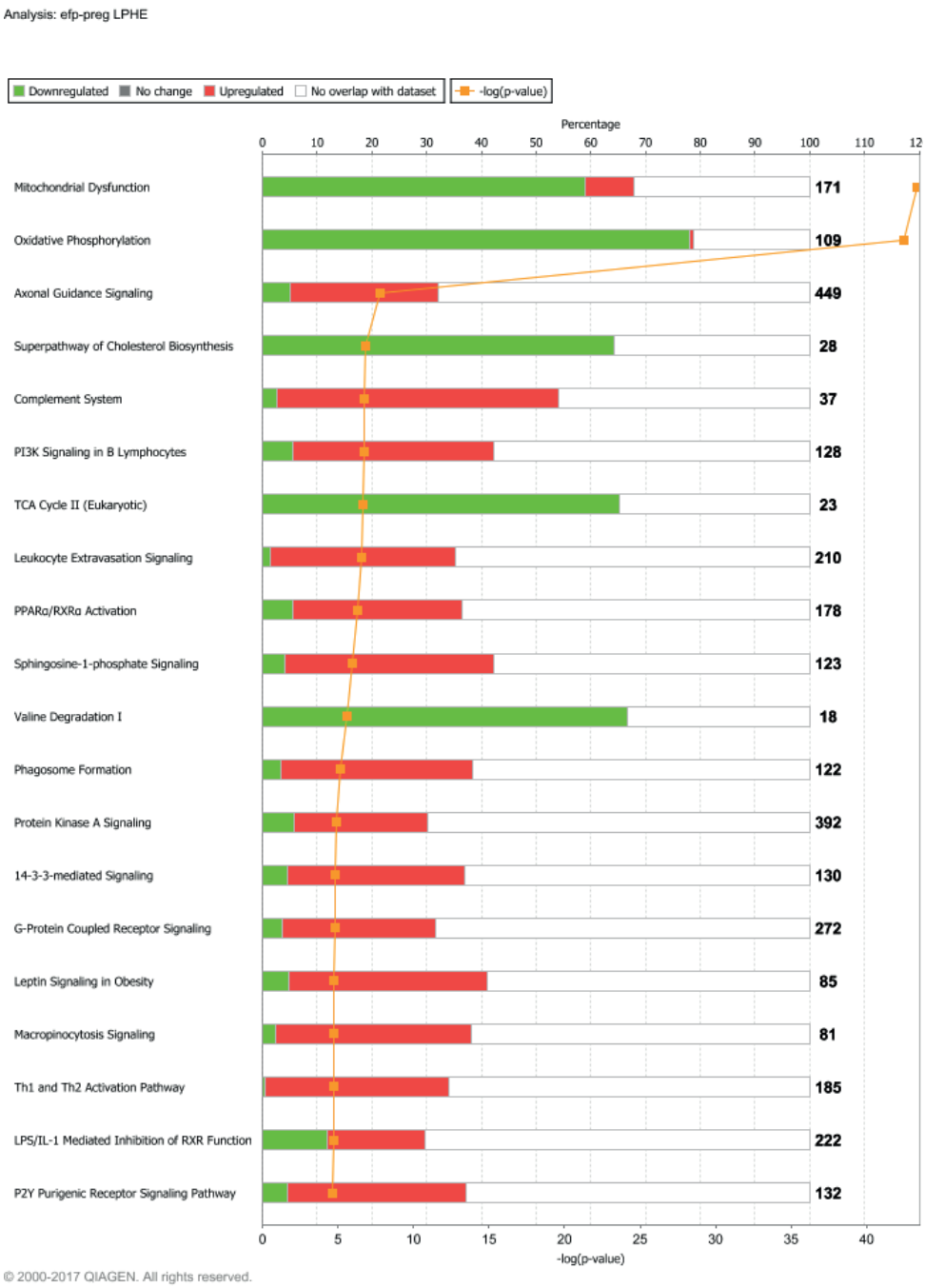
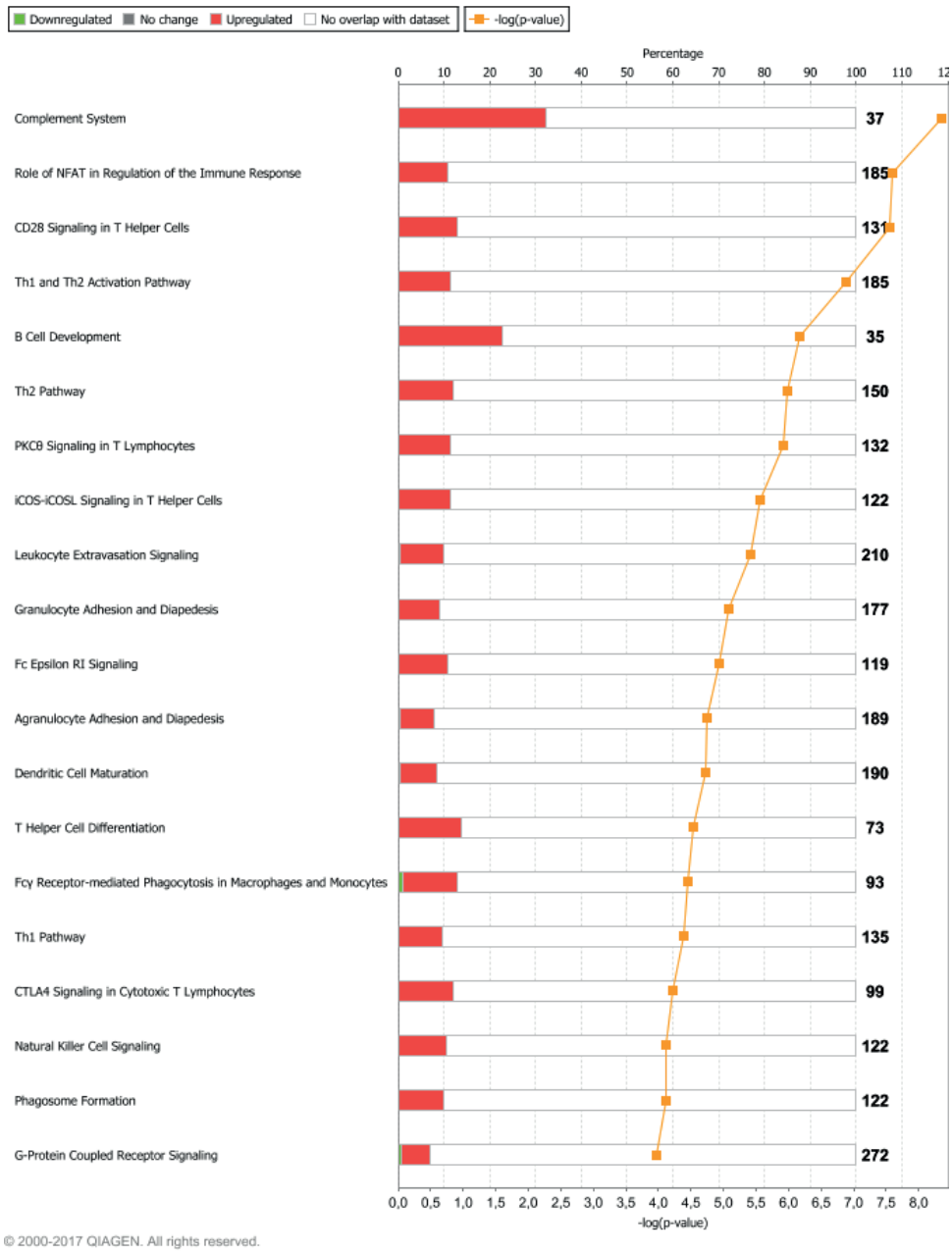


Figure 4.

Figure 5.


Analysis: efp-preg LPLE
Downregulated
No change
Upregulated
No overlap with dataset
-log(p-value)
Percentage
0
10
20
30
40
50
60
70
80
90
100
110
Complement System
37
Role of NFAT in Regulation of the Immune Response
185
CD28 Signaling in T Helper Cells
131
Th1 and Th2 Activation Pathway
185
B Cell Development
35
Th2 Pathway
150
PKCθ Signaling in T Lymphocytes
132
iCOS-iCOSL Signaling in T Helper Cells
122
Leukocyte Extravasation Signaling
210
Granulocyte Adhesion and Diapedesis
177
Fc Epsilon RI Signaling
119
Agranulocyte Adhesion and Diapedesis
189
Dendritic Cell Maturation
190
T Helper Cell Differentiation
73
Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes
93
Th1 Pathway
135
CTLA4 Signaling in Cytotoxic T Lymphocytes
99
Natural Killer Cell Signaling
122
Phagosome Formation
122
G-Protein Coupled Receptor Signaling
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-log(p-value)
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Figure 6.


Analysis: efp-preg HPLE
Downregulated
No change
Upregulated
No overlap with dataset
-log(p-value)
Percentage
0
10
20
30
40
50
60
70
80
90
100
110
120
Mitochondrial Dysfunction
171
Oxidative Phosphorylation
109
Methylmalonyl Pathway
4
2-oxobutanoate Degradation I
5
Valine Degradation I
18
β-alanine Degradation I
2
Estrogen Biosynthesis
39
Leucine Degradation I
9
Glycine Betaine Degradation
10
Triacylglycerol Biosynthesis
46
4-aminobutyrate Degradation I
3
Phenylalanine Degradation IV (Mammalian, via Side Chain)
14
Superpathway of Methionine Degradation
32
Branched-chain α-keto acid Dehydrogenase Complex
4
Melatonin Degradation II
4
LPS/IL-1 Mediated Inhibition of RXR Function
222
Complement System
37
Citrulline-Nitric Oxide Cycle
5
Glutamate Degradation III (via 4-aminobutyrate)
5
Superpathway of Melatonin Degradation
67
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-log(p-value)
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Figure 7.

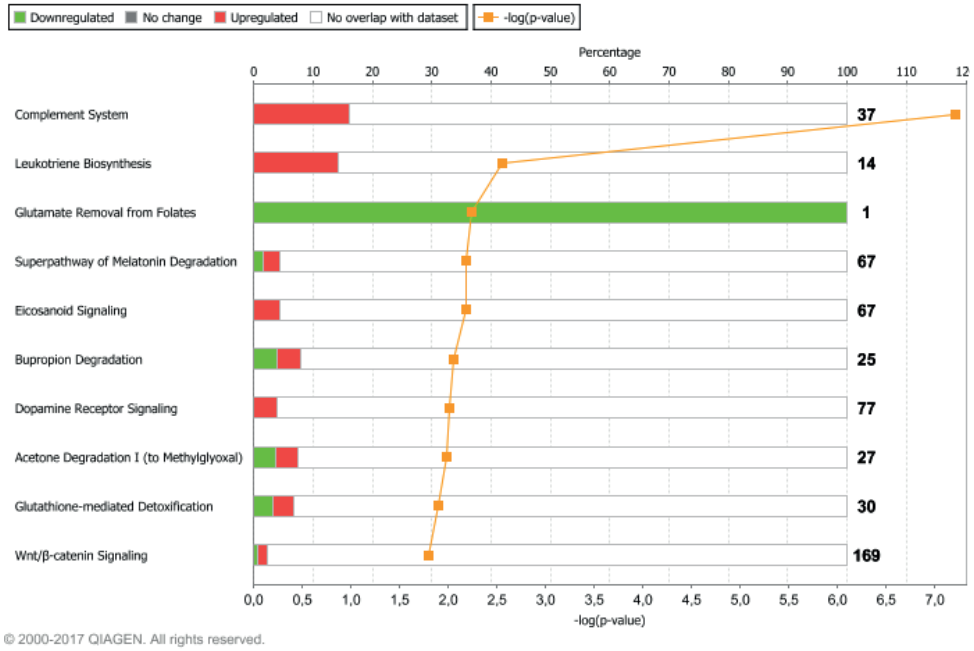

Figure 8.

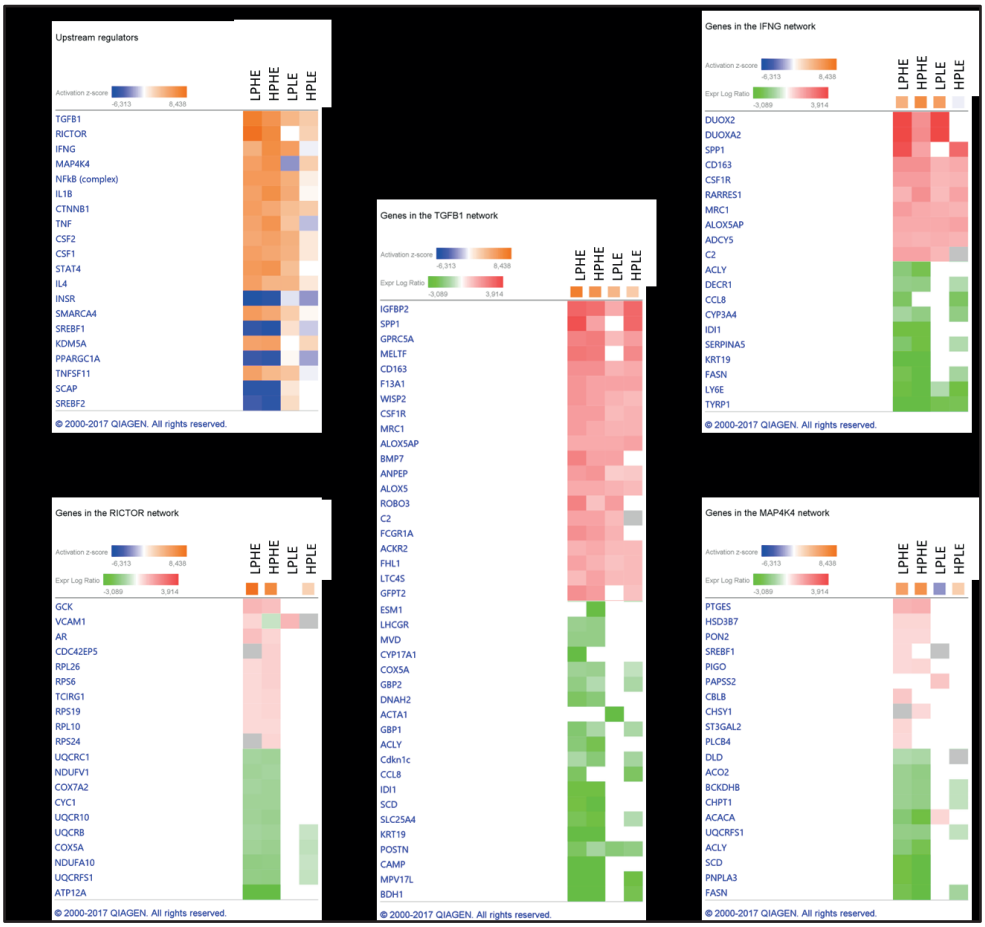

Figure 9.

Supporting information

S1 Table: Post-treatment differences between dietary treatments. All differentially expressed genes (DEG) from energy treatment comparisons at month 7 of pregnancy.

S2 Table: All pathways affected by differently expressed genes between treatment comparisons at month seven of pregnancy.

S3 Table: All DEG (FDR<0.05) between experimental feeding period (12MO) and month 7 of pregnancy (PREG) for heifers fed a high-protein, high-energy diet (HPHE) during the experimental feeding period..

S4 Table: All DEG (FDR<0.05) between experimental feeding period (12MO) and month 7 of pregnancy (PREG) for heifers fed a high-protein, high-energy diet (HPHE) during the experimental feeding period.

S5 Table: All DEG (FDR<0.05) between experimental feeding period (12MO) and month 7 of pregnancy (PREG) for heifers fed a low-protein, low-energy diet (LPLE) during the experimental feeding period.

S6 Table: All DEG (FDR<0.05) between experimental feeding period (12MO) and month 7 of pregnancy (PREG) for heifers fed a high-protein, high-energy diet (HPHE) during the experimental feeding period.

S7 Table: Over-represented pathways representing differentially expressed genes between experimental feeding period (12MO) and month 7 of pregnancy (PREG, 20 (HE) and 24 (LE) mo of age) for heifers on a low-protein, high-energy diet (LPHE) during the experimental feeding period.

S8 Table: Over-represented pathways affected by transition between experimental feeding period (12MO) and month 7 of pregnancy (PREG, 20 (HE) and 24 (LE) mo of age, low-protein, low-energy diet) for heifers on a high-protein, high-energy diet (HPHE) during the experimental feeding period.

S9 Table: Over-represented pathways affected by transition between experimental feeding period (12MO) and month 7 of pregnancy (PREG, 20 (HE) and 24 (LE) mo of age, low-protein, low-energy diet) for heifers on a low-protein, low-energy diet (LPLE) during the experimental feeding period.

S10 Table: Over-represented pathways affected by transition between experimental feeding period (12MO) and month 7 of pregnancy (PREG, 20 (HE) and 24 (LE) mo of age, low-protein, low-energy diet) for heifers on a high-protein, low-energy diet (HPLE) during the experimental feeding period.S11 Table: Genes differentially expressed between experimental feeding period (12MO) and month 7 of pregnancy (PREG, 20 (HE) and 24 (LE) mo of age) common to all treatment groups.

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Norwegian University
of Life Sciences

Postboks 5003
NO-1432 Ås, Norway
+47 67 23 00 00
www.nmbu.no

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