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The Effect of Negative Energy Balance on Gene Expression in cattle

Key external stakeholders:
Dairy and beef farmers, Irish Cattle Breeding Federation (ICBF), nutrition, physiology and genetic scientists.

Practical implications for stakeholders:
This study

- The study provided new insights into the effects of short-term negative energy balance (NEB) on gene expression in the hypothalamic-pituitary-ovarian axis and on immune function in cattle.
- Some cattle are very sensitive to short term periods of NEB and rapidly become anoestrus.
- Systemic concentrations of insulin-like growth factor-1 (IGF-1) appear to be centrally involved in mediating the reproductive effects of NEB, and would appear to be a good predictor of the reproductive response to NEB.
- NEB alters the expression of genes involved in mediating an immune response.
- NEB does not cause a stress response in heifers if adequate concentrations of IGF-1 and insulin are present. Heifers that became anoestrus appear to experience a certain degree of stress and altered immune function but the response is less marked than that observed for other husbandry practices.
- Periods of severe negative energy balance must be avoided otherwise reproductive and immune competencies will be compromised.

Main results:

- Almost a third of heifers became anoestrous following a short period of NEB. Heifers with low systemic concentrations of IGF-1 were more likely to become anoestrus.
- In hypothalamic tissue over one thousand genes were shown to be differentially expressed (DE) in heifers that became anoestrous compared with heifers that remained cyclic. .
- Pathway analysis showed that these genes were associated with pathways involving neuroactive ligand-receptor, homeostasis and immune response.
- These data suggest that heifers became anoestrus following increased expression of molecules within the hypothalamus that provide GnRH neurons with information that body reserves are not adequate to continue oestrous cyclicity. The catabolic environment induced by negative energy balance may ultimately cause anoestrus by reducing oestradiol synthesis, FSH-responsiveness and IGF signaling in granulosa, and LH-responsiveness in theca cells of dominant follicles.
- After 18 days of alterations in eosinophil and monocyte numbers and altered expression of *IL8*, *IL2* and *TNFα* could be attributed to NEB.
- This study also suggests that negative energy balance does not cause a stress response in heifers if adequate concentrations of IGF-1 and insulin are present. Heifers that became anovulatory appear to experience a certain degree of stress and altered immune function but the response is less marked than that observed for other husbandry practices.

Opportunity / Benefit:

Provides an opportunity to identify heifers sensitive or tolerant to the effects of NEB at a young age. This could be invaluable in selecting cows with greater reproductive efficiency. Because of the central role of reproductive performance in determine productive and economic efficiency in Irish seasonal calving milk and beef herds this would be of immediate practical relevance to beef and dairy farmers

Collaborating Institutions:

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1. Project background:

Nutrition plays a fundamental role in the modulation of reproduction in cattle. It is likely that this is mediated through effects on the functioning of the hypothalamic-pituitary-ovarian axis. However, the underlying biological mechanisms are poorly understood. Previous work from our laboratory has clearly shown that following short term severe dietary restriction, some heifers will become anoestrus within approximately two weeks, whereas others will continue normal oestrous cyclicity. Studies of both short term and long term feed restriction in beef heifers have provided evidence that NEB, induced by dietary restriction, causes decreased growth rate and maximum diameter of dominant follicles (DF) and often results in anovulation due to reduced LH pulse frequency. During periods of metabolic stress, animals typically channel energy toward survival and away from processes such as reproduction. The reproductive axis, therefore, has the capacity to respond to changing systemic concentrations of metabolic cues. GnRH neurons, present in the hypothalamus, represent the final output pathway of the neural network that integrates a multitude of internal and environmental cues to regulate the secretion of LH and FSH from the anterior pituitary gland. However, little is known of the molecular regulation of the secretion of GnRH due mainly to the difficulty and expense of obtaining hypothalamic tissues in cattle.

Numerous molecules are believed to communicate with the GnRH neuron and are the primary focus of this study. Therefore, real-time RT-qPCR assays were designed to examine the effect of acute severe NEB on the expression of a number of genes with known direct or indirect effects on the secretion of GnRH in the hypothalamus. Given the critical importance of hypothalamic-pituitary signalling to the occurrence of ovulation, the expression of candidate genes in the anterior pituitary were also investigated.
The objective of this study was to investigate possible reasons for a differential reproductive response to short term diet restriction, specifically investigating systemic metabolites and metabolic hormone concentrations and gene expression in the hypothalamus and anterior pituitary glands of heifers.

The molecular basis for an apparent differential response among cattle to dietary restriction and energy balance has not been established. Thus the objective of this study was to compare differences in transcriptional profiles in hypothalamic tissue between two groups of animals in energy deficit and a third control group on a higher level of feed all with divergent reproductive performance using RNAseq technology and pathway analysis.

Our hypothesis was that gene expression profiles within these cells, along with the endocrine changes in response to brain activation, may provide biomarkers of NEB. In order to gain new insights into the effects of nutrition induced NEB on the stress-immune system our objectives were to characterise the effects of dietary restriction on haematological responses and leukocyte gene expression and 2), to investigate if heifers less tolerant of NEB experience a greater degree of stress.

2. Questions addressed by the project:

- What is the molecular biological basis for the differential reproductive response to short term diet restriction?
- What are the systemic metabolite, metabolic hormone and gene expression changes in the hypothalamus and anterior pituitary glands of heifers to short term diet restriction?
- Does acute nutritional restriction influences the expression of genes regulating gonadotrophin and IGF response in ovarian follicles, thereby reducing their functional capacity for differentiation and ovulation?
- What are the effects of dietary restriction on haematological responses and leukocyte gene expression?
- Do heifers that are less tolerant to NEB experience a greater degree of physiological stress?

3. The experimental studies:

A total of 4 studies were carried out.

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Study 1. A total of 40 heifers were used to determine the effect of severe short term dietary restriction on systemic metabolites, metabolic hormones and provide tissues for subsequent gene expression of candidate genes in the hypothalamus and anterior pituitary of beef heifers. To facilitate the study, the oestrous cycles of all heifers were synchronised using an 8 day combined controlled internal drug-releasing device Ten days after CIDR withdrawal luteolysis was induced using PGF2α to allow ovulation of the DF of the subsequent oestrous cycle (2nd DF). During the oestrous synchronisation period heifers were individually offered a grass silage and concentrate diet (fed 1:1 on an energy content basis) supplying the energy for 1.2 times maintenance (1.2 Mn). One day before CIDR removal (day 0), heifers were allocated randomly to either a restricted diet supplying 0.4 Mn (n=28) or retained on 1.2 Mn as control (n=12). Ovarian follicular growth and incidence of ovulation were monitored by daily transrectal ultrasonography. Blood samples were collected twice daily (0900 and 2100 h) prior to feeding all heifers from the day of diet allocation (day 0) until the day of slaughter for reproductive (progesterone, FSH and oestradiol) and metaboloic (IGF-1, insulin and leptin) hormones and metabolite (glucose, urea, and β-hydroxybutyrate) analyses. Heifers were slaughtered on day 18 and hypothalamic, anterior pituitary and ovarian tissues collected, snap frozen and stored at -80^{o}C.
The relative expression of 18 genes in the hypothalamus, and 11 genes in anterior pituitary was measured by RT-qPCR, in a subset of 21 heifers (7 Control (C)), seven Restricted Ovulatory (RO) and seven Restricted Anovulatory (RA)).
Study 2. The effect of severe short term dietary restriction on gene expression in the bovine hypothalamus using next generation RNA sequencing technology was investigated.
Study 3. Haematological variables known to be reliable biomarkers of husbandry stressors and gene expression of seven leukocyte cytokine genes and five immunological biomarkers were investigated. Heifers were blood sampled on selected days during the period of dietary restriction and total leukocyte, neutrophil , lymphocyte, eosinophil and monocyte number, red blood cell number, haemoglobin, mean corpuscular haemoglobin concentration, haematocrit percentage and platelet number were measured. The relative expression of 12 genes were then measured on days 0,5 9, 13 and 18.
Study 4. Following slaughter, each pair of ovaries were removed, follicles were dissected from the stroma, follicular fluid was aspirated from both the dominant and the largest subordinate follicle and snap frozen for subsequent assay of oestradiol, progesterone and IGF-1. Theca and granulosa cells were isolated from the follicle wall. Follicle total cholesterol, β-hydroxy butyrate, and glucose were analysed using commercial biochemical assay kits. Total RNA was extracted from theca and granulosa cells using Trizol® reagent. Complementary DNA was synthesised from 1 µg of total RNA using random primers and the High Capacity cDNA reverse transcriptase kit. Quantitative real-time PCR (Q-RT-PCR) was carried out on the 7500 Fast Real-Time PCR System. All primers were designed using PrimerBLAST and manufactured by Eurofins MWG (Ebersberg, Germany). Primer sequences for genes within the IGF family were those previously validated for bovine endometrial gene expression analyses.

4. Main results:

Study 1

- Nine of 28 heifers became anoestrous due to diet restriction.
- Follicular growth rate and maximum diameter were reduced by dietary restriction,
- Systemic concentrations of IGF-1 were positively associated with the probability of ovulation on days -2, 0, 5 and 9, relative to diet allocation. The results indicate that IGF-1 concentrations define if a heifer is capable of ovulating during an energy deficit.
- The expression of 18 candidate genes in the hypothalamus and 11 candidate genes in the anterior pituitary of heifers was also measured. Of these (*GHSR)* was highest in the anterior pituitary of RA heifers.

Study 2

- A total of 15,295 genes were expressed in hypothalamic tissue.
- The largest number of differentially expressed genes was observed between RO and RA heifers, with 1094 genes shown to be differentially expressed (DE).
- Innatedb pathway analysis showed that these DE genes were associated with 6 canonical pathways ($P < 0.01$), of which neuroactive ligand-receptor interaction was the most significant.
- Pathways regulating homeostasis and immune response were significantly altered between restricted anovulatory and restricted ovulatory heifers.
- We conclude that heifers became anovulatory following increased expression of molecules within the hypothalamus that provide GnRH neurons with information that body reserves are not adequate to continue oestrous cyclicity.

Study 3

- After 18 days of differential feeding alterations in eosinophil and monocyte numbers and altered

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expression of *IL8*, *IL2* and *TNFα* could be attributed to diet restriction.

- More specifically, effects on these five variables occurred in animals that became anovulatory. It has previously been described that heifers that become anovulatory have lower circulating concentrations of IGF-1 and insulin. Therefore, we conclude that diet restriction does not cause a stress response in heifers if adequate concentrations of IGF-1 and insulin are present. Heifers that became anovulatory appear to experience a certain degree of stress and altered immune function but the response is less marked than that observed for other husbandry practices.

Study 4

- Acute dietary restriction decreased oestradiol ($P<0.01$) and IGF-1 ($P<0.01$) in follicular fluid and mRNA for follicle stimulating hormone receptor FSHR ($P<0.01$) in granulosa cells but increased mRNA for IGFBP2 ($P<0.05$) in theca cells of the newly selected dominant follicle.
- This only led to anovulation when dietary restriction also decreased mRNA for CYP19A1 ($P<0.05$), IGF2 ($P<0.01$) and IGF1R ($P<0.05$) in granulosa cells and LHCGR ($P<0.05$) in theca cells of ovarian follicles.
- These results suggest that the catabolic environment induced by dietary restriction may ultimately cause anovulation by reducing oestradiol synthesis, FSH-responsiveness and IGF signalling in granulosa, and LH-responsiveness in theca cells of dominant follicles.

5. Opportunity/Benefit:

The results of this research have significantly extended our understanding of nutritional effects on reproduction, particularly of the underlying biological mechanisms, by which negative energy balance modulate reproductive function. It indicates that some animals have the biological capacity to be more tolerant or sensitive to an energy deficit. The identification, through genetic selection possibly using systemic IGF-1 measurements, of such tolerant animals could be used as a strategy to counteract the detrimental effects of NEB, and increase overall reproductive performance in cattle. Because of the central role of reproductive performance in determine productive and economic efficiency in Irish seasonal calving dairy and beef herds, the results of this study are of immediate and practical relevance to beef and dairy farmers.

6. Dissemination:

National Conferences and seminars
Presented at the Agricultural Research Forum.

Open Day Farmer Discussion Groups
The results of these studies have informed the presentations at Beef Open in Grange and have been incorporated into presentations at Discussion Group and Farmer and industry seminars.

Main publications:

Walsh, S.W., Matthews, D., Browne, J.A., Forde, N., Crowe, M.A., Mihm, M., Diskin, M., Evans, A.C. (2012). Acute dietary restriction in heifers alters expression of genes regulating exposure and response to gonadotrophins and IGF in dominant follicles. *Anim Reprod Sci.*133:43-51.
Walsh, S.W., Mehta, J.P., McGettigan, P.A., Browne, J.A., Forde, N., Alibrahim, R.M., Mulligan, F.J., Loftus, B., Crowe, M.A., Matthews, D., Diskin, M., Mihm, M., Evans, A.C. (2012) Effect of the metabolic environment at key stages of follicle development in cattle: focus on steroid biosynthesis. *Physiol Genomics*, 44:504-17.
Matthews, D. (2012). Investigating variation in reproductive responses to diet restriction in heifers. Ph.D. Thesis University, College Dublin.
Matthews, D., Kenny, D.A., Morris, D.G., Waters, S., Wylie, A.R.G. and Diskin, M.G. (2011). Effect of short term diet restriction on follicle wave dynamics, incidence of anovulation, metabolic hormones and metabilite profiles. In: Agricultural Research Forum, Tullamore, Co. Offally, 14-Mar, p. 170.

Popular publications:

Kenny, D.A. and Diskin, M.G. (2010). Factors affecting the reproductive efficiency of beef cow herds. Irish Charolais News 50-52.

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