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Original Research Article

Ovine hippocampal mRNA expression in offspring from dams supplemented with fishmeal and stress challenged in late pregnancy with endotoxin[1]

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ABSTRACT

Previous research has shown that adulthood disease can be attributed to stress events that occur during gestation. The objective of the present study was to determine whether maternal stress during late pregnancy, using a bacterial endotoxemia model, causes changes in hippocampal mRNA expression of candidate genes related to hypothalamic-pituitary-adrenal axis (HPAA) regulation in sheep. This study also sought to investigate whether maternal diets supplemented with fishmeal (FM) rich in omega-3 polyunsaturated fatty acids (PUFAs) offer protection to the fetus when subjected to maternal endotoxin stress. Using RT-qPCR, relative mRNA expression was assessed in both fetal lambs and 6-month-old lambs from dams supplemented with soybean meal (SM) or FM and challenged with either endotoxin or saline. From this it was found that fetal mineralocorticoid receptor (*MR*) gene expression had a tendency to be altered during endotoxin challenge, however, in the 6-month-old offspring, *MR* and glucocorticoid receptor (*GR*) gene expression were differently altered across treatment groups. These results suggest that gene regulation within the hippocampus is altered into adulthood by maternal endotoxin stress and that dietary supplementation affects outcome.

1. Introduction

Human and rodent studies have demonstrated long-term adverse health effects of maternal stress on offspring (reviewed by Monaghan and Haussmann, 2015; Reynolds, 2013). Microbial infections during human pregnancy for example, are associated with preterm birth, intrauterine growth restriction, and various neurological, metabolic and immune disorders (Dosanjh et al., 2013; Labouesse et al., 2015; Lee et al., 2015; Williamson et al., 2016; Zi et al., 2015).

Livestock also experience physiological and psychological stress that may adversely impact offspring health. Risk of respiratory disease in calves has been linked to cow morbidity during late pregnancy, milk somatic cell score and retained placenta (Lundborg et al., 2003). Mid-gestational re-grouping of sows was demonstrated to adversely affect male piglet survival, growth and reproductive morphology (Mack et al., 2014), and adrenocorticotrophic hormone (ACTH) treatment of pregnant sows was shown to exacerbate the piglets' cortisol response to mixing stress (Haussmann et al., 2000). Chicks have also been shown to have altered stress responses, high levels of reactive oxygen metabolites and short erythrocyte telomere length following administration of corticosterone at levels relevant to maternal stress (Haussmann et al., 2012). These studies indicate that effects of maternal stress on postnatal health are variable and depend on several factors including stage of gestational exposure, severity of stress, type of

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stressor, and species-specific coping strategies (reviewed by Monaghan and Haussmann, 2015).

Stress responses following perceived threats to homeostasis are characterized by activation of the hypothalamic-pituitary-adrenal axis (HPAA), ultimately resulting in the production of glucocorticoid (GC) hormones from the adrenal glands. Glucocorticoids such as cortisol can elicit a range of physiological responses, including changes in metabolism and regulation of the innate and acquired immune responses, to overcome the stressor and restore homeostasis.

Bacterial lipopolysaccharide (LPS) endotoxin is widely used in research to model Gram-negative bacterial infections. When endotoxin is administered systemically to sheep at low concentrations, an innate inflammatory response occurs, characterized by the secretion of pro-inflammatory cytokines. These cytokines activate the hypothalamus, in part by inducing prostaglandin E2 synthesis via cyclooxygenase (COX) 2, as well as the pituitary, resulting in a transient increase in ACTH and cortisol concentrations lasting several hours (Kabaroff et al., 2006a; You et al., 2008). Glucocorticoids also alter metabolism to redirect energy to support immune activities and a fever response (reviewed by Braun and Marks, 2015).

Given the wide-ranging activities of cortisol, it is important that the HPAA be tightly regulated. This is achieved partially by GC negative feedback on corticotropic releasing hormone expression within the hypothalamus, hippocampal neural signaling, and the expression of precursor protein proopiomelanocortin and synthesis of ACTH within the pituitary (reviewed by Uchoa et al., 2014). Glucocorticoid signaling is also regulated by the activities of 11-beta-hydroxysteroid dehydrogenase 1 (HSD1) and HSD2, which generally activate and inactivate GC, respectively (Uchoa et al., 2014). The ratio of high affinity mineralocorticoid receptors (MR) to low affinity glucocorticoid receptors (GR) also influences GC bioactivity by affecting tissue responsiveness to GC: MR participate in negative feedback during circadian HPAA cycling and therefore determine sensitivity to stress, whereas *GR* participate in negative feedback during stress (Zimmer and Spencer, 2014). Glucocorticoid receptors are highly expressed within the hypothalamus and hippocampus, whereas *MR* expression occurs predominantly within the hippocampus (Zimmer and Spencer, 2014). Loss of *MR* expression in the hippocampus has been associated with elevated plasma ACTH and GC levels (reviewed by Paul et al., 2015), and treatment with agonists targeting peroxisome proliferator-activated receptor (PPAR-γ), a transcription factor highly expressed within the hippocampus that is involved HPAA regulation, appears to reverse this effect (reviewed by Ulrich-Lai and Ryan, 2013).

The maternal cortisol response to endotoxin challenge is heightened during late pregnancy (Kabaroff et al., 2006b), likely due to increased fetal cortisol output and reduced placental HSD2 activity as term approaches (Clarke et al., 2002). This suggests heightened susceptibility of the fetus to maternal stress during the last stage of pregnancy. Studies conducted by Fisher et al. (2010, 2014) support this hypothesis by demonstrating that programming of the fetal HPAA may be altered in response to endotoxin-induced maternal stress, likely due to rapid developmental changes occurring within the HPAA at this time (Li et al., 2013). This group has also shown that maternal supplementation with fishmeal or soybean, rich in omega-3 (n-3) and omega-6 fatty acids respectively, influenced offspring sensitivity to maternal endotoxin-induced stress in an ovine model (Fisher et al., 2014).

Fishmeal supplementation in ovine diets through gestation and lactation has previously been shown to cause polyunsaturated fatty acid (PUFA) enrichment in the offspring (Fisher et al., 2014; Or-Rashid et al., 2012). Throughout lactation, lambs acquired PUFA through the dam's milk; these PUFA can be incorporated into cells in the mammary gland via fatty acid translocase activity and can be secreted as milk fat globules (McManaman, 2014). Plasma samples from offspring at birth (Fisher et al., 2014) indicate that certain n-3 PUFA can also enter the maternal circulation and cross the placental barrier into fetal circulation (Haggarty, 2004; Or-Rashid et al., 2012). Although n-3 PUFA are readily degraded in the rumen, the use of dietary ingredients such as feather meal can be used to bypass the rumen, and this has been shown to allow considerable maternal PUFA enrichment (Or-Rashid et al., 2009). These fatty acids likely cross the placenta by simple diffusion as non-esterified fatty acids or are assisted by fatty acid binding proteins (Haggarty, 2004). Those PUFA that cross the placental barrier may affect the offspring's response to stressors both in utero and after birth.

The purpose of this study was to measure changes in the expression of *MR*, *GR*, *HSD1*, *HSD2*, *COX1* and *2*, *IL-1*, and *PPAR-*γ genes within the hippocampus of late gestation lambs and 6-month old offspring following a maternal endotoxin challenge. These genes were chosen due to their roles in HPAA activation and regulation. The hippocampus was chosen as a target tissue because of its involvement in feedback signaling to the HPAA, and has been previously reported as highly sensitive to inflammatory stress during development (Zhang and Praag, 2015; Zimmer and Spencer, 2014).

2. Materials and methods

All experimental procedures were approved by the Animal Care Committee of the University of Guelph in accordance with the Canadian Council for Animal Care.

2.1. Experiment one

Twenty-four cross-bred Rideau-Arcott ewes were used in a randomized complete block design (Fig. 1). On day 100 of gestation (gd), ewes were allocated to a control diet containing 4% soybean meal (SM) based on a dry matter basis (rich in n-6 PUFA; $n = 12$) or a diet containing 4% fishmeal (FM) based on a dry matter basis (rich in n-3 PUFA; $n = 12$). Ewes were ultrasound checked in order to determine litter size and to balance treatments groups. Ewes were fed twice a day with a total amount of 2.64 kg feed/day during gestation. Nutrient requirements were based on both the weight and age of the ewes and were calculated from the Cornell Net Carbohydrate and Protein System for sheep (Cornell University, Ithaca, NY). Detailed dietary composition tables can be found in (Fisher-Heffernan et al., 2015).

On gd 131 and 132, half the ewes from each dietary group were subjected to an endotoxin challenge with a 2 mL i.v. bolus of 1.2 μg/kg body weight of LPS from *Escherichia coli* 055:B5 (Sigma–Aldrich, Oakville, Ontario) dissolved in saline, or a 2 mL bolus of saline for control (CON). Twenty-four hours post endotoxin challenge, the ewes were slaughtered at the University of Guelph meat abattoir. The fetal lambs (FM + CON, $n = 8$; FM + LPS, $n = 10$; SM + CON, $n = 8$; SM + LPS, $n = 10$) were immediately obtained from the abattoir, and the hippocampus was isolated and snap frozen in liquid nitrogen. The tissues were stored in −80 °C conditions until analysis could be performed. Incidence of single vs. multiple lambs is shown in Table 1. Gender of fetal lambs was not recorded at time of tissue collection and is therefore not accounted for.

2.2. Experiment two

Ewes were treated as described above; however, the endotoxin challenge was performed on gd 135 (Fig. 2). Before the endotoxin challenge, ewes were ultrasound scanned to determine litter size and to balance the treatment groups accordingly. In order to ensure adequate milk supply to the reared lambs, ewes were allowed to

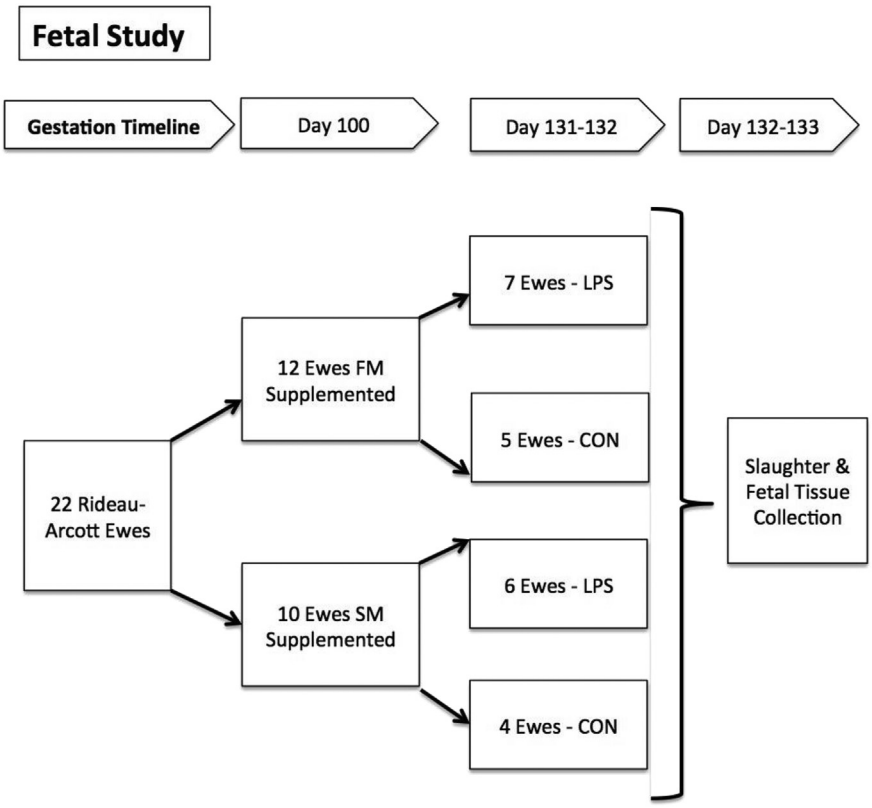

Fig. 1. Schematic time line of events for the fetal tissue collection trial. FM = fishmeal; SM = soybean meal; LPS = lipopolysaccharide; CON = control.

Table 1
Type of births across treatment groups for experiment one.

Treatment	Single	Twins	Triplets
SM + CON	0	8	0
SM + LPS	2	6	2
FM + CON	1	6	2
FM + LPS	1	6	3

SM = soybean meal; CON = control; LPS = lipopolysaccharide; FM = fishmeal.

raise a maximum of 2 lambs that remained with their dam until 50 days of age. Dietary supplements were continued through lactation at a rate of 3.90 kg of feed/day. Once lambs were weaned, they were housed in groups indoors at the OMAFRA Ponsonby General Animal Facility. All lambs were fed the same diet of lamb grower and hay *ad libitum*. Health of the lambs was assessed by monitoring monthly weight gain and this was not found to differ throughout the trial as indicated in Fisher et al. (2014). Type of birth (singleton vs. multiple) are illustrated in Table 2. At 6 months of age, female lambs (FM + CON, $n = 5$; FM + LPS, $n = 7$; SM + CON, $n = 7$; SM + LPS, $n = 5$) were transported to the University of Guelph meat abattoir where they were slaughtered using captive bolt and exsanguination. Hippocampal tissues were immediately collected and snap frozen in liquid nitrogen. All samples were stored in −80 °C conditions until analysis could be performed.

2.3. RNA extraction and reverse transcription

Total RNA was extracted using the TRIzol method (Invitrogen, Burlington, Ontario). Briefly, 1 mL of TRIzol was added to 50 to 100 mg of hippocampus tissue. Tissue was homogenized and incubated at room temperature for 5 min to allow dissociation of the nucleoprotein complex. Chloroform was then added at 200 μL and the tube was shaken for 15 s prior to incubation at room temperature for 5 min. The sample was then centrifuged at 12,000 × *g* for 15 min at 4 °C. The aqueous phase containing the RNA was removed and added to a new micro-centrifuge tube, then 500 μL of iso-propanol was added and incubated at room temperature for 10 min followed by centrifugation for 10 min at 4 °C. The supernatant was then removed from the tube leaving the RNA pellet, which was washed twice with 1 mL of 75% ethanol by vortexing to ensure the pellet was floating and then centrifuged at 4 °C. The RNA pellet was allowed to air dry for approximately 10 min and was then re-suspended in 40 μL of DEPC water and incubated on a heat block at 57 °C for 10 min. RNA quality was assessed using the Nanodrop 8000 (Thermo Scientific, USA) spectrophotometer. Samples were then stored at −80 °C until cDNA reverse transcription could be performed.

RNA was reverse transcribed to cDNA using SuperScript III reverse transcriptase (Invitrogen, Burlington, Ontario). A sample mixture containing 1 μL of oligo$(DT)_{12\text{-}18}$, primer, 1 μL of 10 nmol/L dNTP mix, 10 μL of DECP water, and 1 μL of 1 μg/μL RNA in a 0.2 mL tube was incubated at 65 °C for 5 min. These samples were then immediately put on ice for 1 min. The following was then added to each sample: 4 μL 10xRT buffer, 1 μL DTT, 1 μL DEPC water, and 1 μL of reverse transcriptase; the samples were then incubated at 50 °C for 45 min. The reaction was terminated by increasing the temperature to 70 °C for 15 min. The samples were stored at −20 °C until further analysis could be performed.

2.4. Real-time PCR analysis of hippocampus gene expression

Real-time PCR was performed to assess the mRNA expression of hippocampal *MR*, *GR*, *HSD1*, *HSD2*, *COX1* and COX*2*, *IL-1*, and *PPAR-γ*

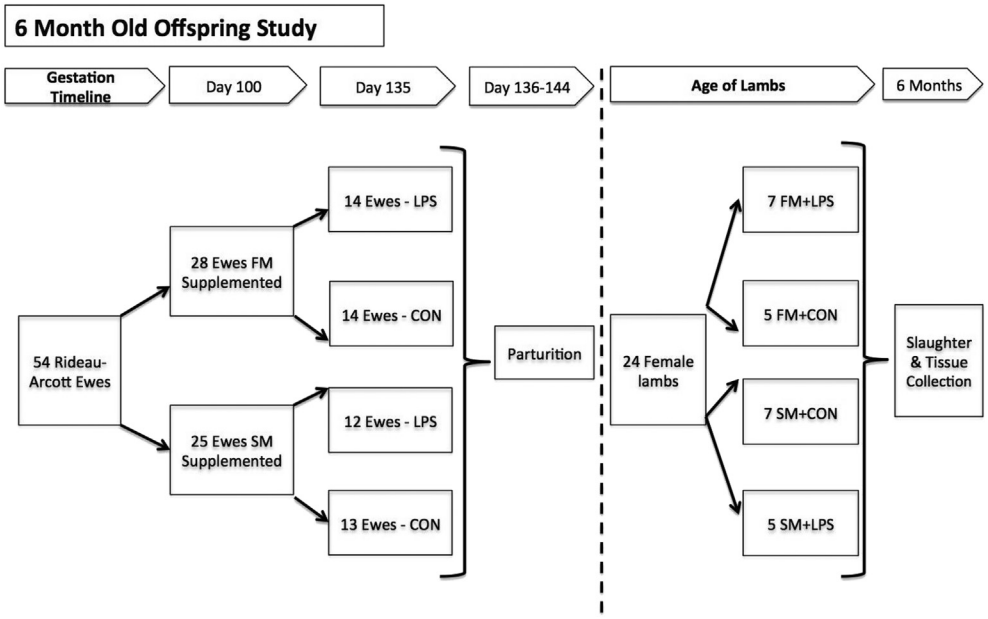

Fig. 2. Schematic time line of events for the 6-month old offspring tissue collection trial. FM = fishmeal; SM = soybean meal; LPS = lipopolysaccharide; CON = control.

Table 2
Type of birth across treatment groups for experiment two.

Treatment	Single	Twins	Triplets
SM + CON	0	4	3
SM + LPS	0	4	1
FM + CON	1	3	1
FM + LPS	1	3	3

SM = soybean meal; CON = control; LPS = lipopolysaccharide; FM = fishmeal.

Table 3
Primer sequences used in the real-time PCR analysis to measure hippocampal mRNA expression.

Gene	Sequence (5′-3′)	Product size, bp
GR[1] (Fwd)	GCCCAAACCCTTTACTTTCAC	224
GR[1] (Rev)	CTCCCAACCCTTGACTTTTTC	
MR[2] (Fwd)	TCCAAAGGATGGCCTCAAAA	73
MR[2] (Rev)	ATCTTTCTCAGCTCCTTGATGTAATTT	
GAPDH[1] (Fwd)	TAACTTCTGTGCTGTGCCAGCC	103
GAPDH[1] (Rev)	TTAAAAGCAGCCCTGGTGACC	
HSD1[2] (Fwd)	ATCCCTGTCTGATGGCTTTT	98
HSD1[2] (Rev)	TGGTCTGAATTCCTCATTCG	
HSD2[2] (Fwd)	AGCAGGAGATGCCCGTTTC	67
HSD2[2] (Rev)	GCAATGCCAAGGCTGCTT	
COX1[1] (Fwd)	TCATGCGTCTGGTACTCACAG	157
COX1[1] (Rev)	ATTGCTTCTTCCCTTTGGTGT	
COX2[1] (Fwd)	GGTGTGAAAGGGAGGAAAGAG	118
COX2[1] (Rev)	AAATTGATGGGTGAAGTGCTG	
PPAR-γ[1] (Fwd)	ACCAAAGTGCAATCAAAGTGG	127
PPAR-γ[1] (Rev)	AGTGGAACCCTGACGCTTTAT	
IL-1[1] (Fwd)	CGTGCAGTCAGTAAAATGCAA	111
IL-1[1] (Rev)	AGCTCATGCAGAACACCACTT	

GR = glucocorticoid receptor; *MR* = mineralocorticoid receptor; *GAPDH* = reference gene; *HSD1* = 11-beta-hydroxysteroid dehydrogenase 1; *HSD2* = 11-beta-hydroxysteroid dehydrogenase 2; *COX1* = cyclooxygenase 1; *COX2* = cyclooxygenase 2; *PPAR-γ* = peroxisome proliferator-activated receptor; *IL-1* = interleukin 1β.
[1] Primers designed using Primer 3.0.
[2] Sloboda et al. (2008).

and the reference gene *GAPDH*. The *GR*, *COX1*, *COX2*, *IL-1* and *PPAR-γ* primers were designed using Primer 3.0, and the primers for hippocampal *MR*, *HSD1*, *HSD2* were designed elsewhere (Sloboda et al., 2008), and are reported in Table 3. All PCR reactions were carried out in 25 μL reaction volume which included 12.5 μL of Platinum SYBR Green qPCR SuperMix −UDG with ROX (Invitrogen, Burlington, Ontario), 0.25 μL of 10 μmol/L forward primer, 0.25 μL of 10 μmol/L reverse primer, 11 μL of DEPC water, and 1 μL of cDNA template. The cDNA samples from both fetal and adult sheep were amplified under the following cycle conditions: 50 °C for 3 min, 95 °C for 5 min, 95 °C for 15 s, annealing temperature for 30 s and 72 °C for 30 s. The annealing temperatures were as follows: 60 °C for *MR*, *GR*, *HSD1*, and *COX2*, and 61 °C was used for *HSD2*, *COX1*, *IL-1*, *PPAR-γ* and *GAPDH*. All samples were run in triplicate and relative CT values were obtained by comparing all samples to a pooled standard curve. A standard curve was constructed using two-fold serial dilutions of standards specific for each transcript. All the genes of interest were compared with the internal control *GAPDH*. Results were expressed as a ratio of the relative CT value of gene of interest to the relative CT value of *GAPDH*. Melting curve analyses demonstrated a single PCR product for each gene of interest, and this was confirmed by gel electrophoresis. The intra- and inter-plate coefficients of variation were <1% for all genes of interest.

2.5. Statistical analysis

Statistical analysis of gene expression data was carried in a 2 by 2 factorial arrangement with diet (FM vs. SM) and endotoxin status (LPS vs. CON) as the factors. The analysis accounted for maternal diet, maternal endotoxin status treatment and accounted for multiple births and siblings. The CT values for *MR*, *GR*, *HSD1* and *HSD2* were used to determine the ratio of *GR:MR* and *HSD1:HSD2* within each treatment. The CT values obtained from fetal and 6 month-old samples were then log transformed and residual plots were examined and showed no evidence of variance heterogeneity after transformation. The mixed model procedure from SAS (version 9.2) was used to analyze all CT values from the genes and gene ratios of interest. Significant differences over time were reported at $P < 0.05$ and trends over time were indicated by $P < 0.1$. Polynomial contrasts were used to assess changes in mRNA expression including interactions with treatment, diet, and gender when available.

3. Results

3.1. Fetal hippocampus gene expression

In the fetal hippocampus, there was a trend towards significance in *MR* expression with lower expression of *MR* in SM + LPS compared with SM + CON ($P = 0.09$) and FM + LPS ($P = 0.08$) treatments (Fig. 3A and B). There were no other differences in fetal gene expression observed in any of the treatment groups or across gender, and no differences were observed in the *GR:MR* ratios (Fig. 3C).

3.2. Hippocampus gene expression of female lambs at 6 months of age

Hippocampal *GR* gene expression was greater in the FM + CON treatment compared with all other treatments, greater in the FM + LPS treatment compared with the SM + LPS treatment ($P < 0.01$, Fig. 4A), and differences between the SM treatments were not observed. Mineralocorticoid receptor expression was also greater in the FM + CON treatment compared with all other treatments, lower in the FM + LPS treatment compared with the SM + LPS treatment ($P < 0.01$, Fig. 4B), and differences between the SM treatments were not observed. No significant differences were noted in the ratios of *GR* to *MR* (Fig. 4C), or for any of the other candidate genes.

4. Discussion

It is now widely accepted that maternal stress throughout gestation can adversely alter programming of offspring genes during development. Researchers are now trying to identify the mechanisms of action involved in these re-programming events to understand their health implications and to determine effective preventative and treatment strategies. The focus of this study was to investigate the effects of maternal supplementation with FM or SM on both fetal and offspring hippocampal *GR*, *MR*, *HSD1*, *HSD2*, *COX1*, *COX2*, *IL-1* and *PPAR-γ* expression following a simulated bacterial infection during late gestation using endotoxin. To our knowledge, this is the first study to examine the effect of FM or SM supplementation on the expression of these genes in the offspring hippocampus following a simulated maternal infection.

Based on the candidate genes measured in this study, it would appear as though the fetal hippocampus was responsive to maternal endotoxin-induced stress. There was a trend in *MR* expression; with lower levels observed in the SM + LPS treatment compared with both the FM + LPS and SM + CON treatments. This trend appeared to reflect a dietary treatment effect, where *MR* expression increased in response to maternal endotoxin challenge in the FM treatment, whereas *MR* expression was decreased in the SM treatment. These differences in *MR* expression could potentially affect subsequent circadian cycling of the HPAA, as hippocampal *MR* are thought to play a key role in feedback control of basal HPAA activity, especially during the night cycle where cortisol concentrations are lowest and *MR* are most highly occupied (reviewed by Berardelli et al., 2013). Decreased *MR* expression could therefore cause higher circadian levels of cortisol which could negatively affect related physiological processes. Conversely, higher *MR* expression could lead to lower circulating cortisol, and this could also cause an inappropriate response when faced with a stressor. These results must be interpreted with caution however, since this change in gene expression occurred within 24 h of the maternal stress challenge, and may be within a physiological range that does not adversely affect fetal development.

In order to assess whether or not fetal responsiveness to maternal endotoxin stress had long-lasting effects, gene expression was also assessed in the hippocampus from female offspring at 6 months of age. Since these offspring were transported for at least 30 min to the abattoir for slaughter during the early morning, it should be noted that they were acutely stressed at the time of tissue collection. Thus, the gene expression results reported herein may be different from expression levels at the same time of day under non-stress conditions. In these animals, the FM + CON treatment had the greatest expression of both hippocampal *GR* and *MR* compared with all other treatment groups. Also, the FM + LPS offspring differed from SM + LPS offspring with greater *GR* expression and lower *MR* expression. Interestingly, we previously reported higher serum cortisol concentrations in these same animals from the FM + LPS treatment compared with all other treatment groups at weaning, and at 5.5 months of age in response to endotoxin challenge (Fisher et al., 2014). Additionally, for male and, to a lesser degree, female offspring, there was a greater cortisol response in the SM + LPS treatment compared with the SM + CON treatment in response to an ACTH challenge, which was performed 24-h post weaning (Fisher et al., 2014). Differences in *GR* expression could explain these previously reported high cortisol levels measured in the offspring of stressed ewes. Since the hippocampus plays a substantial role in down-regulating the cortisol response through the negative feedback loop, a decrease in *GR* expression may result in a prolonged or increased cortisol response.

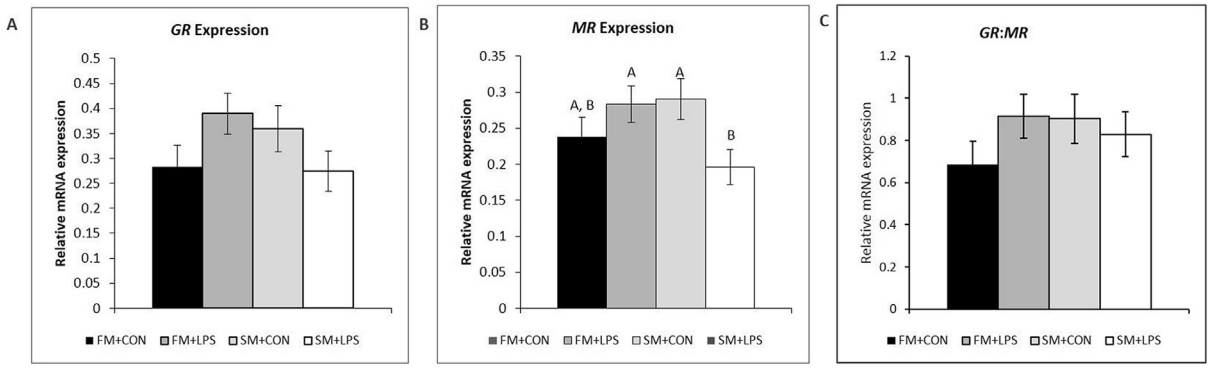

Fig. 3. Expression of fetal hippocampus glucocorticoid receptor (*GR*) (A), mineralocorticoid receptor (*MR*) (B), and the ratio of *GR* to *MR* (*GR:MR*) (C) relative to the *GAPDH* reference gene. Offspring were born to dams supplemented with fishmeal and challenged with saline (FM + CON, $n = 9$) or endotoxin (FM + LPS, $n = 10$), or offspring born to dams supplemented with soybean meal and challenged with saline (SM + CON, $n = 8$) or endotoxin (SM + LPS, $n = 10$). $^{A, B}$ Differing letters above bars indicate trends ($P < 0.1$). FM = fishmeal; SM = soybean meal; LPS = lipopolysaccharide; CON = control.

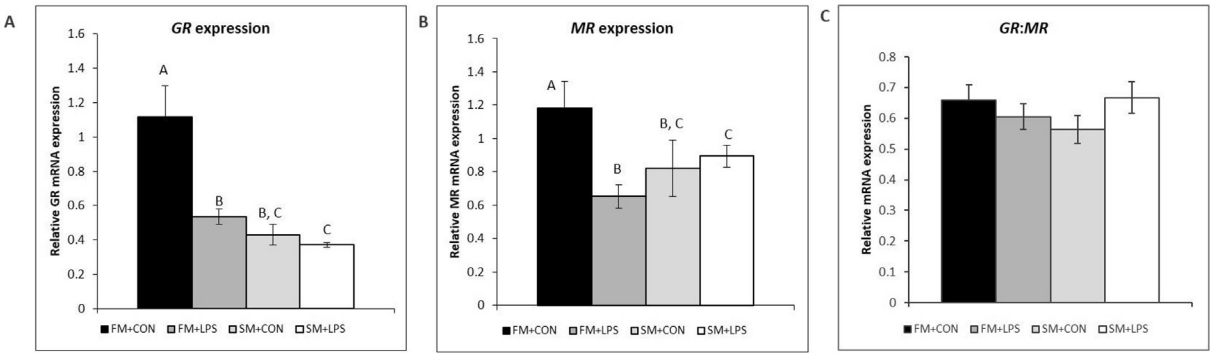

Fig. 4. Expression of 6-month old offspring hippocampus glucocorticoid receptor (*GR*) (A), mineralocorticoid receptor (*MR*) (B), and the ratio of *GR* to *MR* (C) relative to the *GAPDH* reference gene. Offspring were born to dams supplemented with fishmeal and challenged with saline (FM + CON, $n = 5$) or endotoxin (FM + LPS, $n = 7$), or offspring born to dams supplemented with soybean meal and challenged with saline (SM + CON, $n = 7$) or endotoxin (SM + LPS, $n = 5$). Data are reported as least squared means ± SE. $^{A, B, C}$ Differing letters above bars indicate significant differences ($P < 0.05$). FM = fishmeal; SM = soybean meal; LPS = lipopolysaccharide; CON = control.

Additionally, for the FM + LPS treatment, lower hippocampal *MR* expression could also contribute higher plasma ACTH and GC levels (reviewed by Paul et al., 2015). Conversely, high *GR* and *MR* expression in offspring from the FM + CON treatment suggests that these animals may have quick resolution during circadian GC cycling (i.e., *MR*) and stress (i.e., *GR*), as there is a greater likelihood of cortisol binding to these receptors and subsequently down-regulating the HPAA through enhanced negative feedback signaling. This could be advantageous when dealing with day-to-day stressors, and should be explored at the protein level in future studies.

It was somewhat surprising that differences in hippocampal *GR* and *MR* expression were not observed between the SM + LPS and SM + CON treatments at 6 months of age, as previous ovine and rodent studies have shown that administration of synthetic GC alters *GR* and *MR* expression into adulthood (Levitt et al., 1996; Sloboda et al., 2008). However, fetal programming studies have historically reported variable results when examining changes in *GR* and *MR* expression, likely attributed to different life stages, the type and severity of stress, species and gender, and specific tissues (Bloomfield et al., 2003; Dean and Matthews, 1999; Henry et al., 1994; Levitt et al., 1996; Lingas and Matthews, 2001; Sloboda et al., 2008). In support of these observations, we previously did not observe differences in cortisol levels between the SM + LPS and SM-CON treatments when female lambs were weaned or endotoxin challenged at 5.5 months of age, but differences were observed for male and female lambs in response to ACTH challenge performed 24 h post weaning (Fisher et al., 2014).

It was interesting to observe that FM + LPS offspring had greater *GR* expression in the hippocampus compared with SM + LPS offspring, since these FM + LPS offspring had a greater cortisol response than the SM + LPS offspring at weaning and after endotoxin challenged at 5.5 months of age, but not in response to ACTH challenge performed 24 h post weaning (Fisher et al., 2014). Future analysis of the hippocampus transcriptome, as well as that of the hypothalamus, pituitary and adrenals by RNA sequencing may shed more light onto these treatment differences; however, these studies are costly to perform and beyond the scope of the present study.

There are several limitations to consider with the present study. The small sample size, and inability to retain male lambs at the research station, made it impossible to investigate gender-associated changes in the cortisol response previously reported by Fisher et al. (2014). Also, it was also not possible to compare changes in gene expression between the fetal lambs and the 6-month-old offspring because: the maternal endotoxin challenge occurred on different days and the fetal lambs were likely more influenced by the maternal dietary treatments. Since the lambs in experiment two remained with their dams until weaning at 50 days of age, there is the possibility that changes in gene expression observed in this study resulted from postnatal rather than prenatal effects. However, phenotypic differences were previously reported concerning the HPAA (Fisher et al., 2014) and immune responses (Fisher-Heffernan et al., 2015) across these different treatments and n-3 PUFA analyses showed no differences across treatments at 4.5 months of age (Fisher-Heffernan et al., 2015). Considering these results, and the fact that the present study was carried out at 180 days of age, we are confident that the changes in gene expression presented herein can be attributed to the maternal endotoxin stress event during late gestation. Lastly, this study involved only the analysis of mRNA expression, and should be followed-up at the protein level, despite good correlations being reported between *GR* and *MR* gene and GR and MR protein expression (Zimmer and Spencer, 2014).

5. Conclusion

This study provided a starting point for understanding the mechanisms involved in fetal programming and how maternal supplementation with FM or SM affects hippocampal gene expression. This study demonstrated differences in *GR* and *MR* expression in the hippocampus of offspring born to FM supplemented dams. The fetal SM + LPS lambs displayed less *MR* expression compared with their SM + CON and FM + LPS counterparts, and the 6-month old FM + CON offspring had greater expression of hippocampal *GR* and *MR* compared with all other treatments. With further studies, it will be possible to determine if the enhanced stress response reported in the FM + LPS offspring is beneficial or detrimental to offspring health.

Conflict of interest

The authors declare that there are no conflicts of interest.

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