



## Effect of algae or fish oil supplementation and porcine maternal stress on the adrenal transcriptome of male offspring fed a low-quality protein diet



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### ABSTRACT

Offspring adrenal function may be negatively affected *in utero* by maternal stressors such as microbial infection. Maternal supplementation with immunomodulatory compounds such as omega-3 polyunsaturated fatty acids (n-3 PUFA) may help minimize the adverse effects of maternal stress on fetal hypothalamic-pituitary-adrenal development and improve offspring health. Presently, n-3 PUFA sources are primarily fish-based, but n-3 PUFA microalgae (AL) may be an alternative. Previously, it was determined that maternal AL or fish oil (FO) supplementation to sows, in addition to maternal stress induced by *Escherichia coli* lipopolysaccharide (LPS) challenge appeared to have a greater influence on the stress response of male offspring compared to females. To further elaborate on these findings, this study assessed the effects of maternal AL or FO supplementation combined with a maternal LPS challenge on adrenal gene expression in male offspring fed a nursery diet containing low-quality protein sources. Forty-eight sows were fed gestation diets starting on gestation day (gd) 75 containing either 3.12% AL, 3.1% FO, or a control diet containing 1.89% corn oil. On gd 112, half the sows in each treatment were administered 10 µg/kg LPS *i.m.* Piglets were weaned at 21 days of age onto a common low-quality plant-based protein diet, and one week after weaning, four piglets per sow were administered 40 µg/kg LPS *i.m.* Two hours later, the piglets were euthanized to obtain adrenal tissue, and total RNA was extracted to carry out transcriptome analysis using the Affymetrix GeneChip WT Plus assay and subsequent validation by real-time PCR. Analysis revealed that adrenal steroidogenesis, fatty acid metabolism and immune function were significantly influenced by maternal diet and stress. Increased expression of immune-related genes including lymphocyte antigen 96, TLR-2 and NF-κB suggests that maternal AL supplementation may increase offspring sensitivity to inflammation after weaning. Decreased expression of lymphocyte antigen 96 in male offspring from sows receiving maternal LPS challenge also suggests a possible role of maternal stress in diminishing the offspring immune response to immune stress challenge. Increased expression of the genes encoding the 11BHS2 enzyme in offspring from sows fed FO may also reduce the magnitude of the stress response. These data provide insight to the immune and metabolic mechanisms that may be influenced by maternal diet and stress.

### 1. Introduction

The adrenal gland is important for both hormone and neurotransmitter signalling. Its main function includes the synthesis of steroid hormones within the adrenal cortex including androgens, mineralocorticoids and glucocorticoids. In addition to its role in the hypothalamic-pituitary-adrenal (HPA) axis, or stress axis, it is also involved in regulating the immune response during tissue injury or pathogen invasion, in part through the production of inflammatory mediators (Huang et al.,

2010; Mohn et al., 2011).

During fetal development, the adrenal gland may be vulnerable to adverse programming resulting from exposure to maternal stressors such as microbial infection during pregnancy (Fisher et al., 2014; Harris and Seckl, 2011). Maternal stress including simulated infection has previously been shown to alter developmental programming of both the HPA axis and the immune system in sheep and swine (Fisher-Heffernan et al., 2015; Fisher et al., 2014; Kranendonk et al., 2006; Lay et al., 2011). This may leave offspring vulnerable to inflammatory, metabolic and

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neurodegenerative diseases in adulthood (Bronson and Bale, 2014; Gur et al., 2017; Mdaki et al., 2016). Developmental programming is believed to occur through epigenetic mechanisms whereby DNA modifications, or changes in chromatin organization, result in differential expression of genes throughout development (Weaver et al., 2017). Disrupting this normal developmental process in various tissues can result in changes in immune or neuroendocrine phenotypes that may predispose the offspring to disease in neonatal and/or adult life (Harris and Seckl, 2011).

The microbe-associated molecular pattern (MAMP) lipopolysaccharide (LPS) from Gram-negative bacteria is a commonly used microbial stressor that, at sufficient concentrations, activates the HPA axis. Recognition of *Escherichia coli* LPS by sentinel immune cells occurs mainly through activation of toll-like receptor 4 (TLR-4; Gnauck et al., 2016), that in turn initiates signalling cascades leading to the production of several mediators that contribute to inflammation (Huang et al., 2010). While inflammation is an important innate effector response against pathogens, it is a non-specific response that can result in damage to the surrounding tissues (Karrow, 2006).

To help control inflammation associated with maternal stressors, the maternal diets of humans may be supplemented with immunomodulating compounds such as omega-3 polyunsaturated fatty acids (n-3 PUFA); these may provide health benefits to the offspring and may help to control inappropriate tissue programming resulting from maternal infection (Bronson and Bale, 2014; Saccone et al., 2015). Omega-3 PUFA are known for their anti-inflammatory effects, and the long-chain PUFA docosahexanoic acid (DHA) and eicosapentanoic acid (EPA) are precursors to several anti-inflammatory metabolites including resolvins and maresins (Fritsche, 2015). Maternal dietary supplementation with DHA and EPA may result in healthier offspring that are less vulnerable to disease. While most sources of DHA and EPA are derived from fish products such as fish oil (FO), alternative sources including microalgae (AL) are also being investigated; AL may prove to be a more sustainable source of n-3 PUFA (Robertson et al., 2015).

Animal models have been extensively used to investigate causes and mechanisms of fetal programming that may not be easily investigated in humans (Harris and Seckl, 2011). The swine model may provide valuable insight compared to other animal models such as rodents or sheep, because pigs are more physiologically and genetically similar to humans (Dawson et al., 2013; Roura et al., 2016). Conducting such research with pigs may also directly benefit producers, as peri-weaning morbidity and mortality are concerns for the swine industry (Campbell et al., 2013), and it may be possible to enrich maternal diets with n-3 PUFA to produce healthier piglets that are less susceptible to peri-weaning disease. This may in turn allow for the use of less-expensive ingredients, such as low-quality plant-based protein sources in nursery diets without compromising piglet growth or health (Huber et al., 2018; Skinner et al., 2014).

Previously, ovine research has shown that the combination of maternal dietary n-3 PUFA supplementation and an LPS challenge can differentially modulate the offspring stress response, as characterized by fever and cortisol responses, when offspring are later exposed to an LPS challenge (Fisher-Heffernan et al., 2015; Fisher et al., 2014; You et al., 2019). In pigs, there appear to be differences in the stress response between male and female offspring, with male offspring being more sensitive than female offspring to maternal AL or FO dietary supplementation and/or maternal LPS exposure (You et al., 2019). In addition, more pronounced differences were observed among these male offspring from differing maternal treatments. To further elaborate on these findings, the aim of this study was to investigate the effects of maternal AL and FO supplementation in addition to maternal LPS challenge on the differential gene expression of the adrenal gland in male offspring that were fed a low-quality protein diet after weaning.

## 2. Methods

The animal trials were conducted at the Arkell Swine Research Station at the University of Guelph (Guelph, ON, Canada). The vitamin E and the Menhaden FO for this experiment were provided by Grand Valley Fortifiers (Cambridge, ON), and the AL (*Aurantiochytrium limacinum* biomass [AURA; CCAP 4087/2] containing 70% crude fat and 17% DHA) was provided by Alltech Inc. (Nicholasville, KY, USA). The experimental protocol (AUP # 3124) was approved by the University of Guelph Animal Care Committee and followed Canadian Council of Animal Care guidelines (CCAC, 2009).

### 2.1. Sow experimental methods

Experimental methods for the sow trial were conducted as per You et al. (2019). Briefly, forty-eight Landrace x Yorkshire cross-bred sows (12 sows per block x 4 blocks) were selected based on breeding date and parity ( $\geq 2$ ) and were randomly assigned to one of three dietary treatments: diets supplemented with 3.1% FO, 3.12% AL, or a 1.89% corn oil (CO) control diet. Sows were fed 2.5 kg per day of the assigned diets from gestation day (gd) 75, as per Lee et al. (2019b) until farrowing (approx. gd 114), and were given unlimited access to fresh water. The experimental diets were formulated to meet the estimated nutrient requirements for sows in late gestation (National Research Council, 2012). AL and FO diets were formulated based on a preliminary trial to match total DHA content, maximizing fetal PUFA enrichment, and were formulated to have similar n-6 PUFA concentrations as the CO diet (Lee et al., 2019b). Ingredient and calculated nutrient composition of maternal gestation diets are shown in Table 1.

On gd 112, eight sows per dietary treatment were challenged *i.m.* with 10  $\mu\text{g}/\text{kg}$  of LPS from *E. coli* O55:B5 (Sigma-Aldrich, Oakville, ON) dissolved in 2 mL of saline, as per Lee et al. (2019b). The remaining eight sows per dietary treatment received a 2 mL injection of saline as a control. Rectal temperature was recorded hourly up to 6 hr post LPS injection to monitor fever response, and general temperament was also observed. Sow experimental timeline is shown in Fig. 1.

### 2.2. Piglet experimental methods

Piglet experimental procedures were conducted as per You et al. (2019). Within 24 hr of birth, litters were standardized to a maximum of 12 piglets by cross-fostering within treatments and piglets were weaned at 21 days of age. At parturition, each litter was assigned an identification (ID) code, and each piglet was given a unique ID number corresponding to the litter ID code. At weaning, all of the piglets ( $n = 475$ ) were randomly mixed and sorted into 8 pens ( $n = 6/\text{pen}$ ), and were placed on a low-quality protein diet. Piglets were given unlimited access to feed and fresh water for the duration of the trial. Nursery diets were formulated to meet the estimated nutrient requirements for nursery phase 1 (National Research Council, 2012, Table 2). One week after weaning, 1 male offspring per litter ( $n = 48$ ,  $n = 8/\text{maternal treatment}$ ) was randomly selected and received a 40  $\mu\text{g}/\text{kg}$  *i.m.* injection of LPS from *E. coli* O55:B5. After the LPS challenge (2 hr), the pigs were euthanized by administration of 0.3 mL/kg pentobarbital by cardiac puncture. Immediately following euthanasia, adrenal tissue was collected from the piglets and snap frozen in liquid nitrogen. Samples were then stored at  $-80^\circ\text{C}$  until RNA isolation and microarray analysis could be conducted. The piglet experimental timeline is shown in Fig. 1.

### 2.3. Microarray analysis

Following the LPS challenge, adrenal tissue was selected from 24 of the male offspring ( $n = 4/\text{maternal gestation dietary treatment}$  and

**Table 1**  
Formulations of sow experimental diets.

Days on feed	3.12% AL <sup>a</sup>	3.1% FO <sup>b</sup>	CO <sup>c</sup>
	39	39	39
<b>Ingredient Composition (%)</b>			
Corn (NRC <sup>d</sup> ; 8.3% CP <sup>e</sup> )	74.28	74.06	77.06
Corn Oil	1.55	1.79	1.89
Fish Oil <sup>f</sup>	–	3.10	–
Microalgae <sup>g</sup>	3.12	–	–
Soybean Meal	17.38	17.38	17.38
L-Lysine	0.07	0.07	0.07
Salt	0.40	0.40	0.40
Limestone	1.52	1.52	1.52
Mono-Cal Phosphate	1.17	1.17	1.17
Vitamin/Mineral Premix <sup>h</sup>	0.50	0.50	0.50
Vitamin E <sup>f</sup>	0.02	0.02	0.02
<b>Calculated Nutrient content<sup>i</sup></b>			
ME, kcal/kg	3097	3153	3090
CP (%)	14.48	14.61	14.46
SID <sup>j</sup> Lysine (%)	0.64	0.65	0.64
SID Methionine + Cysteine (%)	0.43	0.44	0.43
SID Threonine (%)	0.45	0.46	0.45
SID Tryptophan (%)	0.13	0.13	0.13
<b>Analyzed nutrient content</b>			
Dry matter (%)	86.98	88.17	87.53
CP (%)	14.34	15.03	14.44
Phosphorus (%)	0.57	0.58	0.54
Sodium (%)	0.18	0.17	0.17
Calcium (%)	0.81	0.78	0.88
Potassium (%)	0.65	0.67	0.65
Magnesium (%)	0.14	0.14	0.13
Eicosapentanoic acid (EPA; mg/g)	0.19	3.49	0.08
Docosahexanoic acid (DHA; mg/g)	3.76	3.16	0.03
Total omega-3 PUFA <sup>k</sup> (mg/g)	5.43	10.21	1.70
Ratio n-3:n-6 <sup>l</sup>	0.21	0.37	0.07

<sup>a</sup> AL, microalgae.<sup>b</sup> FO, fish oil.<sup>c</sup> CO, 1.89% corn oil control.<sup>d</sup> NRC, National Research Council.<sup>e</sup> CP, crude protein.<sup>f</sup> Fish oil and Vitamin E provided by Grand Valley Fortifiers (Cambridge, ON, CA).<sup>g</sup> Algae provided by Alltech Inc. (Nicholasville, KY, USA) and supplied as dried biomass containing 15.8% CP, 70% CF and 17% DHA.<sup>h</sup> Supplied per kg of diet: vitamin A, 12,000 IU as retinyl acetate; vitamin D3, 1200 IU as cholecalciferol; vitamin E, 48 IU as DL- $\alpha$ -tocopherol acetate; vitamin K, 3 mg as menadione; vitamin B12, 0.03 mg; pantothenic acid, 18 mg; riboflavin, 6 mg; choline, 600 mg; folic acid, 2.4 mg; niacin, 30 mg; thiamine, 18 mg; pyridoxine, 1.8 mg; biotin, 200  $\mu$ g; Cu, 18 mg as CuSO<sub>4</sub>·5H<sub>2</sub>O; Fe, 120 mg as FeSO<sub>4</sub>; Mn, 24 mg as MnSO<sub>4</sub>; Zn, 126 mg as ZnO; Se, 0.36 mg as FeSeO<sub>3</sub>; I, 0.6 mg as KI; DSM.<sup>i</sup> Calculated on the basis of the NRC (2012) ingredient values.<sup>j</sup> SID, standardized ileal digestible.<sup>k</sup> PUFA, polyunsaturated fatty acids.<sup>l</sup> Ratio n-3:n-6, ratio of omega-3 PUFA to omega-6 PUFA.

maternal LPS status). The adrenal gland was selected for microarray analysis due to previously observed differences in the offspring cortisol response following LPS challenge (You et al., 2019). Total RNA was

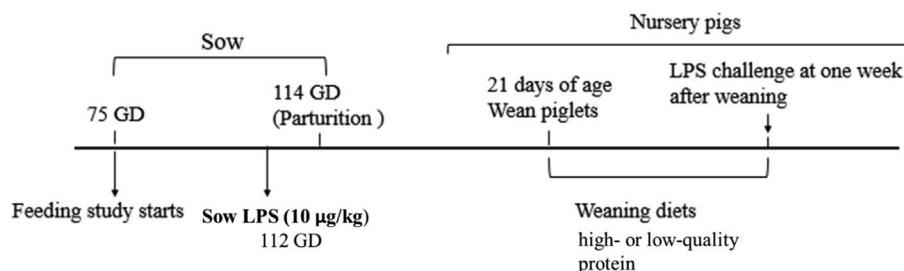
**Table 2**  
Formulation of offspring experimental diets.

Diet	Low Quality
<b>Days on feed</b>	7
<b>Ingredient Composition (%)</b>	
Corn (NRC <sup>a</sup> ; 8.3% CP <sup>b</sup> )	45.28
Wheat	15.00
Fat, Animal/vegetable blend	5.00
Soybean meal	30.00
L- Lysine	0.37
L- Methionine	0.15
L- Tryptophan	0.02
L- Threonine	0.24
Salt	0.50
Limestone	1.30
Monocalcium Phosphate	1.52
Mineral/Vitamin premix <sup>c</sup>	0.60
Vitamin E <sup>d</sup>	0.02
<b>Calculated nutrient content<sup>e</sup></b>	
ME, kcal/kg	3471
CP (%)	20.85
SID <sup>f</sup> Lysine (%)	1.21
SID Methionine + Cysteine (%)	0.71
SID Threonine (%)	0.86
SID Tryptophan (%)	0.24
<b>Analyzed nutrient content (%)</b>	
Dry matter	87.08
CP	21.18
Phosphorus	0.63
Calcium	0.87
Sodium	0.18
Potassium	0.89
Magnesium	0.17

<sup>a</sup> NRC, National Research Council.<sup>b</sup> CP, crude protein.<sup>c</sup> Supplied per kg of diet: vitamin A, 12,000 IU as retinyl acetate; vitamin D3, 1200 IU as cholecalciferol; vitamin E, 48 IU as DL- $\alpha$ -tocopherol acetate; vitamin K, 3 mg as menadione; vitamin B12, 0.03 mg; pantothenic acid, 18 mg; riboflavin, 6 mg; choline, 600 mg; folic acid, 2.4 mg; niacin, 30 mg; thiamine, 18 mg; pyridoxine, 1.8 mg; biotin, 200  $\mu$ g; Cu, 18 mg as CuSO<sub>4</sub>·5H<sub>2</sub>O; Fe, 120 mg as FeSO<sub>4</sub>; Mn, 24 mg as MnSO<sub>4</sub>; Zn, 126 mg as ZnO; Se, 0.36 mg as FeSeO<sub>3</sub>; I, 0.6 mg as KI; DSM.<sup>d</sup> Vitamin E provided by Grand Valley Fortifiers (Cambridge, ON, CA).<sup>e</sup> Calculated on the basis of the NRC (2012) ingredient values.<sup>f</sup> SID, standardized ileal digestible.

extracted from 20–30 mg of adrenal tissue using the Qiagen RNeasy extraction kit (Qiagen, Valencia, CA, USA) as per the manufacturer's specifications. Following RNA extraction, the RNA pellet was re-suspended in 40  $\mu$ L of nuclease-free water.

RNA quality and quantity were then assessed using a Nanodrop 1000 spectrophotometer (Thermo Scientific, NanoDrop Products, Wilmington, DE, USA). RNA integrity was assessed using an Agilent 2100 bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA), as per manufacturer's protocol. Samples were prepared for microarray analysis using Applied Biosystems' GeneChip WT plus reagent kit (Applied Biosystems, Foster City, CA, USA) as per manufacturer's instructions.

**Fig. 1.** Schematic timeline of events for animal trials. GD, gestation day; LPS, Lipopolysaccharide. Adapted from You et al. (2019).

Following sample preparation, samples were loaded onto Affymetrix Porcine Gene 1.0 ST GeneChips™ for hybridization following the manufacturer's protocol. GeneChips were then incubated in a hybridization oven for 16 hr at 65 °C and were spun at a speed of 60 rpm.

After 16 hr of incubation, hybridization cocktail was removed from the GeneChips, 160 µL of holding buffer was added, and the GeneChips were loaded into a fluidics station (Affymetrix GeneChip fluidics station 450) for washing and staining using the provided wash and staining solutions. GeneChips were then analyzed using the Affymetrix GeneChip Scanner 3000.

#### 2.4. Real-time polymerase chain reaction (RT-PCR) analysis

Total RNA (1 µg) from the adrenal gland was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription (RT) Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Real-time PCR was performed in triplicate for the genes 11BHSD1, 11BHSD2, TLR-2, LY96 and PPARG using commercially available porcine TaqMan Assays (Applied Biosystems) and the 7500 fast RT-PCR System (Applied Biosystems). In a 96-well plate, 5 µL of 1:10 cDNA samples were loaded in triplicate for each gene, and were combined with 1 µL of primers, 10 µL of TaqMan™ Fast advanced master mix and 4 µL of nuclease-free water for a total reaction volume of 20 µL. Samples were held for 2 min at 50 °C and for 10 min at 95 °C followed by 40 amplification cycles at 95 °C for 15 s, and at 60 °C for 60 s.  $\beta$ -actin was selected as a house keeping gene to account for variation in the PCR. The relative quantification was expressed as a ratio of the target gene to the control gene using the delta-delta cycle threshold method as described in Zhang et al. (2018).

#### 2.5. Data analysis

Raw data were obtained from the GeneChip scanner in the form of a.CEL file. These data were initially analyzed using transcriptome analysis console 4.0 (TAC 4.0; Applied Biosystems). Data were normalized to a control treatment and were set to detect fold-differences of <-1.2 or >1.2; a P-value of <0.05 was considered significant. The fold-difference cut-offs of <-1.2 and >1.2 were selected to capture genes where a small increase or decrease in expression may have a pronounced physiologic effect. The list of Gene IDs, gene symbols and fold-differences were obtained for differentially expressed genes with a significant P-value, and data underwent secondary analysis using Qiagen Ingenuity Pathway Analysis software (IPA; Ingenuity Systems Inc., Redwood City, CA, USA) to determine the canonical pathways, biological functions and proteins involved. Project datasets were created using the gene IDs, fold-change and P-values obtained from TAC 4.0 analysis, and were then analyzed using the IPA core expression analysis. Pathways, functions and proteins with a P-value of <0.05 and an activation z-score of <-2.0 or >2.0 were considered significant.

PCR results were analyzed using PROC GLIMMIX of SAS version 9.4. A multiple means comparison with a Tukey adjustment was conducted, similar to Karrow et al. (2017), and the statistical model included the fixed effects of maternal gestation dietary treatment, maternal LPS status, and their interaction, and included the random effect of block. Significant differences are reported where  $P < 0.05$  and statistical trends are reported where  $0.05 \leq P \leq 0.1$ .

### 3. Results

Due to RNA quality following isolation and quantification, only 19 out of 24 samples could be used for the transcriptome analysis ( $n = 4$  for FO + CON,  $n = 3$  for FO + LPS, AL + CON, AL + LPS, CO + CON and CO + LPS). The raw data from the microarray analysis are available in the NCBI Gene Expression Omnibus (accession number GSE135782).

#### 3.1. Microarray analysis of differentially expressed adrenal genes

Of the 25,470 genes on the GeneChip Porcine Gene 1.0 ST array, a range of 449 to 2335 genes were differentially expressed, depending on the treatment selected for data normalization (Table 3). The factorial design of the maternal treatments allowed for the investigation of the effects of maternal LPS challenge within a maternal dietary treatment, as well as the effects of a maternal dietary treatment within an LPS treatment on the male offspring.

#### 3.2. Lipid metabolism

When investigating the effect of maternal diet and LPS treatments on the male offspring, lipid metabolism was one of the top enriched pathways across treatments (Table 4). A total of 13 genes ( $P < 0.05$ ) were enriched in pigs from the maternal AL + LPS treatment compared to CO + LPS, and 58 genes ( $P < 0.05$ ) were enriched in pigs from the maternal AL + CON treatment compared to FO + CON. Genes that were found to be differentially expressed included fatty acid elongase (ELOVL7), PPAR- $\gamma$ , acyl-CoA dehydrogenases, acyl-CoA synthetases, phospholipase A2, arachidonate lipoxygenase, prostaglandin synthases and prostaglandin reductase, as outlined in Table 4.

#### 3.3. Steroidogenesis

The expression of adrenal genes involved in steroidogenesis were also enriched when investigating the effect of maternal diet and LPS treatments (Table 5). These genes included the cytochrome P450 enzymes CYP1A1, CYP1A2, CYP4F2 and CYP39A1, and hydroxysteroid dehydrogenases HSD17B8, HSD11B1, HSD11B2, HSDL1 and HSDL2 (Table 5).

#### 3.4. Immune function

When investigating the effect of maternal diet and LPS treatments on the male offspring, immune response was also one of the top enriched pathways across treatments (Table 6). The expression of several immune-related genes was higher in pigs from dams fed diets supplemented with AL and FO compared to those fed CO, and in pigs from sows that were

**Table 3**

Overview of differentially expressed male offspring adrenal genes in pigs that were fed a low-quality protein diet and were from sows supplemented with AL, FO or CO then challenged with LPS or saline ( $n = 4$  for FO + CON,  $n = 3$  for all other treatments).

Treatment Comparison	Number of Genes			% of total genes
	Up-regulated	Down-regulated	Total	
AL <sup>a</sup> +LPS <sup>b</sup> vs AL + CON <sup>c</sup>	1211	1114	2325	9.13
FO <sup>d</sup> +LPS vs FO + CON	352	521	873	3.43
CO <sup>e</sup> +LPS vs CO + CON	283	183	466	1.83
AL + LPS vs CO + LPS	492	383	875	3.44
AL + CON vs CO + CON	266	319	585	2.30
FO + LPS vs CO + LPS	393	376	769	3.02
FO + CON vs CO + CON	241	208	449	1.76
AL + LPS vs FO + LPS	729	737	1466	5.76
AL + CON vs FO + CON	207	315	522	2.05

<sup>a</sup> AL, microalgae.

<sup>b</sup> LPS, lipopolysaccharide.

<sup>c</sup> CON, saline control.

<sup>d</sup> FO, fish oil.

<sup>e</sup> CO, corn oil.



**Table 4**

Differentially expressed male offspring adrenal genes pertaining to lipid metabolism in pigs that were fed a low-quality protein diet and were from sows supplemented with AL, FO or CO then challenged with LPS or saline (n = 4 for FO + CON, n = 3 for all other treatments).

Treatment Comparison	Gene	Description	Fold change	P-value
AL <sup>a</sup> +LPS <sup>b</sup> vs AL + CON <sup>c</sup>	ACADL	Acyl-CoA Dehydrogenase Long-chain	-1.74	0.005
	ECHDC1	Ethylmalonyl-CoA decarboxylase 1	-1.63	0.022
	ACSL3	Acyl-CoA synthetase long-chain family 3	-1.62	0.015
	ACADM	Acyl-CoA Dehydrogenase medium-chain	-1.51	0.047
	PLA2G16	Phospholipase A2 group 16	-1.47	0.004
	ECHS1	Enoyl CoA hydratase short chain 1	1.48	0.046
	FO <sup>d</sup> +LPS vs. FO + CON	ACSL5	Acyl-CoA synthetase long-chain family 5	-1.22
PTGIS		Prostaglandin I synthase	1.30	0.036
CO <sup>e</sup> +LPS vs. CO + CON	LIPE	Lipase	1.22	0.031
	ELOVL7	ELOVL fatty acid elongase 7	1.28	0.040
	FFAR2	Free-fatty acid receptor 2	1.34	0.040
AL + LPS vs CO + LPS	PPARG	Peroxisome proliferator-activated receptor gamma	-1.54	0.015
	ACACA	Acetyl-CoA carboxylase alpha	1.30	0.039
AL + CON vs. CO + CON	ALOXE3	Arachidonate Lipoxygenase 3	-1.42	0.007
FO + LPS vs. CO + LPS	PLA2G16	Phospholipase A2 group 16	1.22	0.024
	PLA2G12A	Phospholipase A2 group 12A	1.51	0.006
	PTGR2	Prostaglandin reductase 2	1.53	0.046
	PTGES2	Prostaglandin E synthase 2	1.76	0.004
FO + CON vs. CO + CON	ALOXE3	Arachidonate Lipoxygenase 3	-1.27	0.042
	LPCAT2	Lysophosphatidylcholine acyltransferase 2	1.38	0.016
AL + LPS vs FO + LPS	ACADL	Acyl-CoA Dehydrogenase Long-chain	-1.53	0.012
	ACADSB	Acyl-CoA Dehydrogenase Short/Branched chain	-1.40	0.049
	PLA2G16	Phospholipase A2 group 16	-1.39	0.013
	PPARG	Peroxisome proliferator-activated receptor gamma	-1.39	0.046
	ECHDC3	Enoyl CoA hydratase domain containing 3	-1.64	0.014
AL + CON vs. FO + CON	PLA2G7	Phospholipase A2 group 7	1.59	0.033

<sup>a</sup> AL, microalgae.

<sup>b</sup> LPS, lipopolysaccharide.

<sup>c</sup> CON, saline control.

<sup>d</sup> FO, fish oil.

<sup>e</sup> CO, corn oil.

challenged with LPS compared to those that received saline as a control. Commonly enriched pathways included cell-mediated immune response, antibody-mediated immune response, hypersensitivity response, free-radical scavenging, inflammatory response and immune cell trafficking. Commonly enriched molecules included LY96 (MD-2), TLR-2, MYD88, NF-κB, IL-1R and IL-6R (Table 6).

### 3.5. Real-time PCR validation of several differentially expressed adrenal genes

The target genes HSD11B1, HSD11B2, LY96, TLR2 and PPARG showing significant fold-change in expression among treatments following microarray analysis were further validated using RT-PCR. The fold-differences in expression were similar for each of these genes between treatments with the exception of TLR-2 for the comparison of FO + LPS vs. CO + LPS; the correlation between microarray and RT-PCR analyses was 0.90 (Fig. 2).

**Table 5**

Differentially expressed male offspring adrenal genes pertaining to steroidogenesis in pigs that were fed a low-quality protein diet and were from sows supplemented with AL, FO or CO then challenged with LPS or saline (n = 4 for FO + CON, n = 3 for all other treatments).

Treatment Comparison	Gene	Description	Fold change	P-value
AL <sup>a</sup> +LPS <sup>b</sup> vs AL + CON <sup>c</sup>	AIG1	Androgen-induced 1	-1.82	0.019
	HSDL2	Hydroxysteroid Dehydrogenase like 2	-1.77	0.011
	HSD11B1	11β Hydroxysteroid dehydrogenase type 1	-1.67	0.030
	AVPR1	Arginine vasopressin receptor 1	-1.62	0.015
	HSDL1	Hydroxysteroid Dehydrogenase like 1	1.24	0.039
FO <sup>d</sup> +LPS vs FO + CON	MRAP2	Melanocortin 2 receptor accessory protein 2	-1.58	0.025
	CYP39A1	Cytochrome P450 39A1	-1.44	0.017
	CYP1a2	Cytochrome P450 1A2	1.21	0.039
	CYP4F2	Cytochrome P450 4F2	1.42	0.040
	CRHBP	Corticotropin releasing hormone binding protein	1.44	0.047
AL + LPS vs CO <sup>e</sup> +LPS	CYP2J2L	Cytochrome P450 2J2 like	1.52	0.035
AL + CON vs CO + CON	ADRA2A	Adrenoreceptor alpha 2A	-1.27	0.025
	ADRBK2	β-Adrenergic receptor kinase 2	1.40	0.008
FO + LPS vs CO + LPS	GLCCI1	Glucocorticoid induced 1	1.45	0.003
	CYP2J2L	Cytochrome P450 2J2 like	1.42	0.022
FO + CON vs CO + CON	ADRBK2	β-Adrenergic receptor kinase 2	1.21	0.023
	HSD11B2	11β Hydroxysteroid dehydrogenase type 2	1.71	0.023
AL + LPS vs FO + LPS	HSD11B2	11β Hydroxysteroid dehydrogenase type 2	-1.57	0.019
	HSDL2	Hydroxysteroid dehydrogenase like 2	-1.49	0.038
	HSDL1	Hydroxysteroid dehydrogenase like 1	1.22	0.041
AL + CON vs FO + CON	CYP1A1	Cytochrome P450 1A1	1.31	0.020
	HSD17B8	17β Hydroxysteroid Dehydrogenase type 8	-1.31	0.033

<sup>a</sup> AL, microalgae.

<sup>b</sup> LPS, lipopolysaccharide.

<sup>c</sup> CON, saline control.

<sup>d</sup> FO, fish oil.

<sup>e</sup> CO, corn oil.

## 4. Discussion

This study is the first to investigate the effects of maternal AL and FO supplementation in combination with maternal LPS challenge in late gestation on adrenal mRNA expression in swine. The animals in this study were previously used to assess acute-phase response to an LPS challenge (You et al., 2019). The results from this study showed differential effects of maternal gestation dietary treatment and maternal LPS status, leading to the hypothesis that the maternal treatments would also influence the adrenal transcriptome of the offspring. Previous differences in the acute-phase response were also noted between male and female offspring, with maternal treatment more profoundly influencing male offspring compared to female offspring (You et al., 2019). Therefore, it was expected that differences in gene expression in adrenal tissue would be more pronounced within male offspring from differing maternal treatments, and hence, males were selected for the present study.

### 4.1. Lipid metabolism

Several genes that are involved in lipid metabolism were found to be differentially expressed. This includes the expression of phospholipase A2 (PLA2) group 16. The expression of PLA2 was higher in offspring from

**Table 6**

Differentially expressed male offspring adrenal genes pertaining to immune function in pigs that were fed a low-quality protein diet and were from sows supplemented with AL, FO or CO then challenged with LPS or saline (n = 4 for FO + CON, n = 3 for all other treatments).

Treatment Comparison	Gene	Description	Fold change	P-value	
AL <sup>a</sup> +LPS <sup>b</sup> vs AL + CON <sup>c</sup>	CD180	CD180	-2.55	0.029	
	IFNGR1	Interferon gamma receptor 1	-2.26	0.023	
	SLA-DRA	MHC II SLA-DRA	-1.78	0.010	
	LY96	Lymphocyte antigen 96	-1.62	0.035	
	CD83	CD83	-1.61	0.034	
	MYD88	MyD-88	-1.57	0.010	
	SLA-DQA1	MHC II SLA-DQA1	-1.55	0.034	
	IL10	Interleukin-10	-1.46	0.018	
	IL1RL1	Interleukin-1 receptor like 1	1.28	0.022	
	NFKB2	Nuclear factor kappa B2	1.47	0.001	
	IL17B	Interleukin 17b	1.76	0.007	
	SLA-DRB1	MHC II SLA-DRB1	1.85	0.021	
	FO <sup>d</sup> +LPS vs FO + CON	LY96	Lymphocyte antigen 96	-1.40	0.045
		HP	Haptoglobin	1.81	0.012
		CO <sup>e</sup> +LPS vs CO + CON	SLA-DRB1	MHC II SLA-DRB1	-1.66
AL + LPS vs CO + LPS	LY96	Lymphocyte antigen 96	1.55	0.036	
	LY96	Lymphocyte antigen 96	-1.66	0.029	
	SLA-DQA1	MHC II SLA-DQA1	-1.50	0.044	
	CD34	CD34	-1.42	0.004	
	NFKB2	Nuclear factor kappa B2	1.26	0.036	
	SLA-DRB1	MHC II SLA-DRB1	1.77	0.022	
	HP	Haptoglobin	2.02	0.040	
	SLA-DOB	MHC II SLA-DOB	2.60	0.010	
	AL + CON vs CO + CON	SLA-DRB1	MHC II SLA-DRB1	-1.74	0.009
		SLA-DOA	MHC II SLA-DOA	1.34	0.035
		IL2RG	Interleukin-2 receptor gamma	1.43	0.017
	FO + LPS vs CO + LPS	TLR2	Toll-like receptor 2	1.50	0.004
		LY96	Lymphocyte antigen 96	1.51	0.044
		TLR2	Toll-like receptor 2	-1.33	0.045
		IL1RL1	Interleukin-1 receptor like 1	-1.29	0.004
SLA-DRB1		MHC II SLA-DRB1	1.47	0.040	
FO + CON vs CO + CON		SLA-DRB1	MHC II SLA-DRB1	-2.07	0.006
		NOD1	Nucleotide-binding oligomerization domain-containing protein 1	-1.36	0.037
		TLR9	Toll-like receptor 9	1.25	0.037
		LY96	Lymphocyte antigen 96	1.47	0.029
		SLA-DQA1	MHC II SLA-DQA1	-1.84	0.005
		CD83	CD83	-1.75	0.009
		SLA-DRA	MHC II SLA-DRA	-1.58	0.024
		CD34	CD34	-1.42	0.001
		IL12B	Interleukin 12b	1.29	0.036
		IL1RL1	Interleukin 1 receptor like1	1.34	0.004
	IL23R	Interleukin 23 receptor	1.49	0.014	
	IL17B	Interleukin 17b	1.56	0.017	
	AL + LPS vs FO + LPS	TLR2	Toll-like receptor 2	1.39	0.015

<sup>a</sup> AL, microalgae.

<sup>b</sup> LPS, lipopolysaccharide.

<sup>c</sup> CON, saline control.

<sup>d</sup> FO, fish oil.

<sup>e</sup> CO, corn oil.

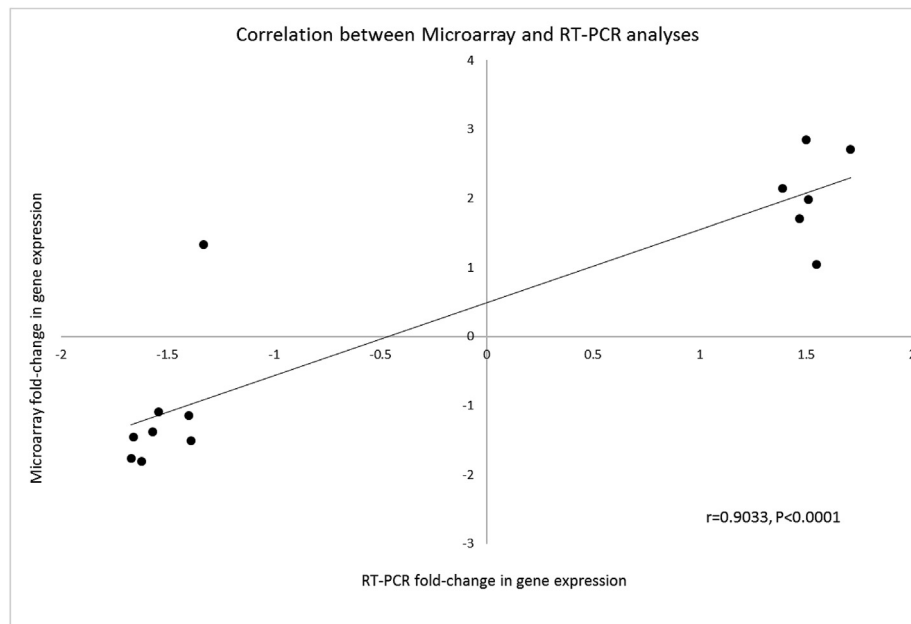
FO + LPS sows compared to offspring from AL + LPS and CO + LPS sows. PLA2 is responsible for hydrolysis of fatty acids from membrane phospholipids, indicating increased lipid metabolism and lipolysis (Liu et al., 2018). Due to the increased concentrations of EPA and DHA in the offspring from FO sows at the time of sampling (You et al., 2019), there would likely be increased EPA and DHA available for metabolism by lipoxygenase and cyclooxygenase enzymes, resulting in increased production of anti-inflammatory metabolites (Duncan et al., 2008; Fritsche, 2015). It is interesting to note that the increased expression of PLA2 group 16 occurred in offspring from sows challenged with LPS; this may suggest a role of maternal stress in preferentially using lipid stores as a source of energy.

The expression of PPAR- $\gamma$  was found to be lower in offspring from the AL + LPS sows compared to those from both the FO + LPS and CO + LPS sows. PPAR- $\gamma$  is involved in lipid metabolism in both adipose tissue and in immune cells such as dendritic cells and macrophages (Ahmadian et al., 2013). PPAR- $\gamma$  is also involved in the immune response; inhibiting the production of pro-inflammatory cytokines, by limiting the transcription of inflammatory genes, and promoting the differentiation of anti-inflammatory immune cell phenotypes (Ahmadian et al., 2013; Martin, 2010). The decreased expression of PPAR- $\gamma$  in offspring from maternal AL + LPS sows may therefore indicate reduced lipid metabolism in offspring, and therefore they may be more susceptible to inflammation, as potentially indicated by the increased cortisol response in these pigs following LPS challenge (You et al., 2019).

#### 4.2. Steroidogenesis

LPS administration can directly stimulate the production of the steroid hormones cortisol and aldosterone in the adrenal glands (Huang et al., 2010; Vakharia and Hinson, 2005). Based on findings from previous studies and the differences observed in the cortisol and fever responses in these same offspring following LPS challenge (You et al., 2019), it was anticipated that genes involved in steroidogenesis would be differentially expressed. The enzymes 11- $\beta$ -hydroxysteroid dehydrogenase type 1 and 2 (11BHSD1/2) are responsible for activating and inactivating cortisol, respectively (Alikhani-Koopaei et al., 2004). The expression of the 11BHSD enzymes can be affected by maternal stress; stress has been associated with reduced placental expression of 11BHSD2 (Marques et al., 2015), allowing more cortisol to cross the placenta and enter fetal circulation. Expression of the 11BHSD enzymes within the adrenal cortex has also been characterized in rats and in humans (Maz-zocchi et al., 1998; Shimojo et al., 1996); however, the effects of stress on adrenal 11BHSD expression are not well characterized (Zallocchi et al., 2004). In the present study, expression of 11BHSD2 was higher in offspring from FO sows compared to offspring from AL and CO sows, suggesting that maternal FO supplementation may reduce the magnitude of cortisol response from the adrenal gland in offspring following a stress challenge. However, results from the cortisol analysis do not agree with this observation (You et al., 2019), which may be due to the time of tissue sampling; expression of 11BHSD2 may be upregulated in offspring from sows fed the FO diet in attempt to attenuate the cortisol response following LPS challenge, possibly resulting in a quicker resolution of the stress response.

The primary cytochrome P450 enzymes involved in steroidogenesis in the adrenal gland were not found to be differentially expressed amongst treatments. Similarly, a previous study by Hazard et al. (2008) found that the expression of genes involved in adrenal steroidogenesis were not differentially expressed following acute ACTH challenge, but may be differentially expressed when pigs are subjected to a chronic ACTH challenge. Therefore, the lack of significant findings in this area may be attributed to the acute nature of the LPS challenge in this study.



**Fig. 2.** Correlation between fold-change in gene expression from microarray and real-time polymerase chain reaction (RT-PCR) in the adrenal gland of male offspring fed a low-quality protein diet from dams fed diets supplemented with microalgae, fish oil or a control diet and were challenged with lipopolysaccharide from *Escherichia coli*, or saline as a control in late gestation.

#### 4.3. Immune function

The enrichment of several immune-related pathways was observed within the adrenal gland. While the major function of the adrenal gland is the production of hormones and the release of catecholamines, there is also evidence that the adrenal gland is involved in the immune response. The adrenal gland can be directly activated by LPS through TLR-4 signalling (Mohn et al., 2011; Vakharia and Hinson, 2005). This results in the production of cortisol, aldosterone and other mediators including cytokines such as IL-1, nitric oxide and prostaglandins (Martinez Calejman et al., 2011; Mohn et al., 2011; Vakharia and Hinson, 2005). Collectively, these findings indicate that the adrenal gland has a role in the immune and stress responses to LPS, and these can occur in an ACTH-independent manner.

In the present study, the expression of lymphocyte antigen 96 (LY96), also known as MD-2, was differentially expressed in most treatments; MD-2 is a co-receptor required for recognition of LPS by TLR-4 on endothelial and immune cells (Gnauck et al., 2016). The expression of MD-2 was higher in pigs from sows supplemented with AL and FO receiving saline compared to pigs from sows fed the CO control diets and was higher in pigs from AL + CON and FO + CON sows compared to those from the same diets that received LPS. This suggests that the combination of maternal AL or FO supplementation and LPS challenge may decrease offspring sensitivity to future stressors, including LPS, although this appears to contradict the fever and cortisol responses observed in these offspring following LPS challenge (You et al., 2019). While MD-2 expression is essential for the LPS signalling cascade, there are many other molecules involved; the increased expression of MD-2 alone may not be sufficient to alter the offspring sensitivity to an LPS challenge, as may be reflected in the fever and cortisol responses to LPS. The differential expression of MD-2 also suggests that the male offspring from the maternal FO and AL dietary treatments may be more sensitive to future microbial stress compared to offspring from the maternal CO dietary treatment, and this finding is reflected in the fever response of offspring following LPS challenge (You et al., 2019) as well as cytokine concentrations in pigs fed AL following LPS challenge (Lee et al., 2019a).

TLR-2 expression was also found to be differentially expressed within offspring from differing maternal treatments, with increased expression

in pigs from sows fed AL compared to pigs from sows fed FO and CO, all of which received saline injections. TLR-2 is typically involved in the recognition of MAMPs from Gram-positive bacteria, heterodimerizing with either TLR-1 or TLR-6 (Conti et al., 2014; Peddireddy et al., 2017), whereas MAMPs from Gram-negative bacteria are typically recognized by TLR-4. Certain studies have shown a role of TLR-2 in the immune response to LPS, indicating that TLR-2 activation may be required for TLR-4 signalling induced by LPS in certain tissues (Conti et al., 2014; Good et al., 2012; Huang et al., 2010). Studies have also reported that TLR-2 knockout mice have a decreased magnitude of immune response following LPS challenge (Good et al., 2012). Therefore, the increased expression of TLR-2 in offspring from AL + CON sows may further contribute to increased sensitivity of these offspring to future microbial stressors resulting in the activation of both the TLR-2 and TLR-4 pathways. TLR-2 and TLR-4 signalling pathways can also be activated by alarmins; endogenous molecules that indicate tissue or cellular injury (Yang et al., 2017). Differential programming of the TLR-2 and TLR-4 pathways may therefore differentially affect alarmin signalling; resulting in offspring being increasingly susceptible to inflammation and potentially increasing the risk of inflammatory disease (Roh and Sohn, 2018).

The expression of the transcription factor NF- $\kappa$ B was also differentially modulated by maternal treatments; NF- $\kappa$ B expression was increased in pigs from the AL sows that were challenged with LPS compared to their saline controls and compared to sows from the CO control diet that were challenged with LPS. NF- $\kappa$ B is released at the end of the LPS signalling cascade (Martinez Calejman et al., 2011), whereby phosphorylation of  $\kappa$ B results in its' dissociation from NF- $\kappa$ B, allowing NF- $\kappa$ B to translocate into the nucleus, bind to response elements on DNA and induce the transcription of several pro-inflammatory cytokines (Gnauck et al., 2016). Studies have shown that NF- $\kappa$ B expression increases within the adrenal gland in rats following LPS stimulation (Medicherla et al., 2002), and that n-3 PUFA can reduce NF- $\kappa$ B expression in macrophages with or without exposure to LPS (Allam-Ndoul et al., 2016; Novak et al., 2003). Contrary to previous research, in the present study, the higher expression of NF- $\kappa$ B resulting from the combination of maternal AL supplementation and maternal LPS challenge may predispose offspring to inflammation in later life and increase sensitivity to future stressors. This was also

reflected in the offspring fever response following LPS challenge (You et al., 2019), as well as cytokine concentrations following LPS challenge in pigs fed diets supplemented with AL (Lee et al., 2019a). This may be attributed to the nature of the algae supplement used, as algae species are known to possess bioactive compounds including carotenoids and beta-glucans, as well as antinutritive factors (Yaakob et al., 2014), that may influence the effects of the n-3 PUFA on the offspring.

The results from the microarray analysis give insight into how these molecular mechanisms may contribute to differences in phenotype and what those differences might mean to swine producers. While increasing the immune response may help protect against invading microbes, increased immune response also increases energy requirements for immune function (Rauw, 2012); in growing animals this might mean a trade-off between growth and immune function (Van Der Most et al., 2011). An increased cortisol response following exposure to stressors may provide short-term advantages, but chronic or excessive stress responses can dampen the immune response and can leave offspring vulnerable to disease (Dhabhar, 2014). Changes in the expression of genes involved in the immune and cortisol responses may indicate that offspring from sows fed AL during gestation may have an increased immune response to stressors after weaning. This was highlighted by the increased cortisol response following LPS challenge in offspring from the AL + LPS sows compared to those from the CO + LPS sows (You et al., 2019). Growth performance in these offspring was not affected in the nursery phase (Lee et al., 2019b); however, there may be negative effects of a heightened immune response on overall growth in the growing or finishing phases in these pigs. This warrants further investigation to validate the use of AL and FO in maternal gestation diets.

One potential limitation of this study is that microarray analysis may underestimate the fold-change in mRNA transcripts (Hazard et al., 2008), and therefore it is possible that important immune-related or steroidogenic genes were not found to be differentially expressed when in fact they were. Additionally, mRNA was extracted from the whole adrenal gland, and it is therefore possible that expression of certain genes were increased within the adrenal cortex and decreased within the adrenal medulla, or vice versa, resulting in no overall changes in expression as suggested by Murani et al. (2011).

It is also of note that microarray analysis was only conducted on adrenal tissue of male offspring; differences between male and female offspring stress and immune responses have been characterized in response to maternal stress (Fisher-Heffernan et al., 2015; Fisher et al., 2014). Therefore, microarray analysis of adrenal tissue from female offspring may reveal different effects of either maternal dietary supplementation, or maternal LPS status than were presently observed. Finally, it is important to consider that the tissues analyzed were from pigs fed a low-quality protein diet. While the pigs in question were only fed the low-quality diet for 1 week, it was previously observed that the offspring nursery diet quality affected the concentrations of the cytokines IL-6, IL-10 and IL-1ra following offspring LPS immune challenge (Lee et al., 2019b). Offspring fed the low-quality protein diet may therefore be more vulnerable to inflammation and disease than those fed a high-quality protein diet. It is therefore possible that the results of the microarray analysis were also influenced by the low-quality protein diet, and any anti-inflammatory effects of maternal AL or FO supplementation may be more pronounced in offspring fed a high-quality protein diet. It is also worth noting that only a portion of the swine genome is annotated (Liu et al., 2017); thus, it is possible as further elucidation of gene ontology occurs, further insight into the results obtained from microarray analysis of the adrenal gland will provide a more comprehensive view of the mechanisms at play in these offspring.

## 5. Conclusions

Gene expression within the adrenal gland of male offspring fed a low-quality protein diet was influenced by both maternal diet (FO and AL) and maternal LPS status. Adrenal steroidogenesis, lipid metabolism and

immune function pathways were significantly influenced. Increased expression of immune-related genes including lymphocyte antigen 96, TLR-2 and NF- $\kappa$ B suggests that maternal AL supplementation may increase offspring sensitivity to inflammation after weaning. Decreased expression of lymphocyte antigen 96 in male offspring from sows receiving maternal endotoxin challenge also suggests a possible role of maternal stress in diminishing the offspring immune response to immune and/or stress challenges. Increased expression of the genes encoding the 11BHS2 enzyme in offspring from sows fed FO may also reduce the magnitude of the stress response. These data provide insight into immune and metabolic mechanisms that may be influenced by maternal diet and maternal stress. Future analysis of gene expression in the adrenal gland of female offspring and offspring fed a high-quality protein diet, or gene expression of other endocrine tissues may provide more insight into the effects of maternal AL and FO supplementation and maternal LPS challenge at the genome level.

## Declaration of competing interest

The authors declare no conflicts of interest.

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