Impact of repeated lipopolysaccharide administration on ovarian signaling during the follicular phase of the estrous cycle in post-pubertal pigs

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ABSTRACT: Increased circulating lipopolysaccharide (**LPS**) results from heat stress (**HS**) and bacterial infection, both of which are associated with reduced female fertility. Specific effects of low-level, repeated LPS exposure on the ovary are unclear, as many studies utilize a bolus model and/or high dosage paradigm. To better understand the effects of chronic LPS exposure on ovarian signaling and function, post-pubertal gilts (n = 20) were orally administered altrenogest for 14 d to synchronize the beginning of the follicular phase of the ovarian cycle. For 5 d after synchronization, gilts (163 ± 3 kg) received IV administration of LPS (0.1 µg/kg BW, n = 10) or saline (**CT**, n = 10) 4× daily. Blood samples were obtained on days 1, 3, and 5 of LPS treatment. Follicular fluid was aspirated from dominant follicles on day 5, and whole ovarian homogenate was used for transcript and protein abundance analysis via quantitative real-time PCR and western blotting, respectively. There were no treatment differences detected in rectal temperature on any day ($P \geq 0.5$). Administering LPS increased plasma insulin ($P < 0.01$), LPS-binding protein (**LBP**; $P < 0.01$), and glucose ($P = 0.08$) on day 1, but no treatment differences thereafter were observed ($P = 0.66$). There were no treatment differences in follicular fluid concentration of LBP or 17β-estradiol ($P = 0.42$). Gilts treated with LPS had increased abundance of ovarian TLR4 protein ($P = 0.01$), but protein kinase B (**AKT**) and phosphorylated AKT (**pAKT**) were unchanged and no effect of LPS on components of the phosphatidylinositol 3 kinase (**PI3K**) pathway were observed. There was no impact of LPS on ovarian abundance of STAR or CYP19A1, nor *ESR1*, *LDLR*, *CYP19A1*, *CYP17A1*, or *3BHSD*. In conclusion, repeated, low-level LPS administration alters inflammatory but not steroidogenic or PI3K signaling in follicular phase gilt ovaries.

Key words: lipopolysaccharide, ovary, toll-like receptor 4

J. Anim. Sci. 2018.96:3622–3634
doi: 10.1093/jas/sky226

INTRODUCTION

Intestinal barrier dysfunction facilitates circulatory entry of microbial toxins, such as lipopolysaccharide (**LPS**; Amar et al., 2008; Al-Attas et al., 2009; Hawkesworth et al., 2013). In swine, heat stress (**HS**, Baumgard, 2013) and decreased feed intake (Pearce et al., 2013b) increase circulating LPS, which complexes with LPS-binding protein (**LBP**) and binds toll-like receptor 4 (**TLR4**) to elicit a cellular response (Hoshino et al., 1999). Membrane-bound protein cluster of differentiation 14 (**CD14**) and myeloid differentiation factor 2 (**MD2**) couple with TLR4 to phosphorylate the nuclear factor kappa B (**pNFKB**) rel-associated protein (**RELA**) subunit (Chow et al., 1999). Activating TLR4 also stimulates phosphatidylinositol 3-kinase (**PI3K**) signaling, which impacts primordial follicular growth (Castrillon et al., 2003; Liu et al., 2007; John et al., 2008) and steroidogenesis (Zeleznik et al., 2003; Chen et al., 2007). Lipopolysaccharide reduced primordial follicle number in cows and mice (Bromfield and Sheldon, 2013), lengthened the follicular phase (Xiao et al., 1998)

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Received April 10, 2018.
Accepted June 26, 2018.

and decreased 17β-estradiol in a variety of models such as monkeys (Xiao et al., 1998), bovine granulosa cells (Williams et al., 2008), and ovarian cortical explants (Bromfield and Sheldon, 2013). Thus, because of its widespread species effects, increased circulatory LPS is likely detrimental to porcine fertility.

Lipopolysaccharide may also be an insulin secretagogue: cattle and pigs infused with LPS have a rapid and marked increased circulating insulin (Kvidera et al., 2017a). Hyperinsulinemia can also negatively affect ovarian function and insulin-mediated ovarian signaling defects cause infertility (Poretsky et al., 1999; Brüning et al., 2000; Choi and Kim, 2010). To test the hypothesis that LPS alters circulating insulin and ovarian intracellular signaling in gilts, low-level chronic LPS exposure was administered during the estrous cycle follicular phase and ovarian molecular analyses were conducted.

MATERIALS AND METHODS

Animals

Twenty crossbred post-pubertal gilts (163 ± 3 kg) were randomly assigned to receive saline (**CT**, $n = 10$) or LPS (0.1 µg/kg BW, $n = 10$) 4× daily at 0000, 0600, 1200, and 1800 h. Animals were housed individually in a pen (56 × 221 cm) in the discovery wing at the Iowa State University Swine Nutrition Farm. Each pen contained a stainless steel feeder and a nipple drinker. Water was provided ad libitum throughout the duration of the experiment. All animals were limit fed 2.7 kg of feed consisting primarily of corn and soy to meet the NRC recommended nutrient requirements daily at 0600 h.

The study was divided into two phases: estrus synchronization (day −13 through day 0) and treatment administration (day 1 through day 5). Estrus synchronization was achieved by supplementing each gilt 15 mg altrenogest (Matrix)/day as top-dressing on their feed. On day −4, jugular catheters were surgically inserted in each animal as described previously (Sanz Fernandez et al., 2015). On day 1, altrenogest supplementation ceased and experimental IV treatment infusions began. During days 1–5, control gilts were administered 3 mL of sterile saline while LPS gilts received LPS (*Escherichia coli* O55:B5 dissolved in sterile saline; this strain was chosen as an LPS mimetic) equal to 0.1 µg/kg of initial BW administered 4× daily at 0000, 0600, 1200, and 1800 h.

Rectal temperatures were measured twice daily at 0530 and 1730 h using a digital thermometer. Blood (10 mL) was collected daily into tubes containing sodium heparin during the infusion period at 1730 h via the jugular catheter. Blood samples were centrifuged for 15 min at 1,500 × *g* at 4 °C. Plasma was removed and stored at −20 °C for further analysis. Gilts were sacrificed at 0600 h on day 6 using captive bolt penetration followed by exsanguination. Follicular fluid from ovarian dominant follicles were aspirated from one ovary and frozen on dry ice. The contralateral ovary (with intact non-aspirated dominant follicles) was flash frozen in liquid nitrogen. All animal procedures were approved by the Iowa State University Institutional Animal Care and Use Committee.

Materials

Lipopolysaccharide from *E. coli* O55:B5 (L2880), Triton x-100 (T-6878), HEPES (H3537), glycerol (G5516), NaF (S7920), EDTA (E7889), SDS (L3771), beta mercaptoethanol (M3148), Trizma Base (T1501), sodium chloride (S3014), and Ponceau S (P3504) were obtained from Sigma Aldrich (St. Louis, MO). Halt Protease and Phosphatase Inhibitor Cocktail (PI78442), Pierce Bicinchoninic acid (**BCA**) Protein Assay Kit (23227), Glycine (BP381-500), iBlot 2 Transfer Stacks (IB23001), PBS (BP665-1), Tween 20 (337), BSA (BP1605-100), dNTP (18427-013), dithiothreitol (**DTT**; Y00147), RNAse out (10777019), Superscript III (18080-093), 1st Strand Buffer (Y02321), Sodium phosphate monobasic, anhydrous (S381), and $MgCl_2$ (M33400) were obtained from ThermoFisher Scientific (Rockford, IL). Laemmli buffer (161–0737), Precision Plus Protein Kaleidoscope (161–0375), and 4–20% Mini-PROTEAN TGX Precast Protein Gels (4561096) were obtained from Bio-Rad Laboratories (Hercules, CA). RNeasy Mini Kit (74104), RNAse-free DNAse (79254), and Quantitect SybrGreen (204143) were obtained from Qiagen. Primers and Oligo dT were obtained from the Iowa State University DNA facility (Ames, IA). PCR plates (951020460) and Masterclear cap strips (951022089) were obtained from Eppendorf (Hauppauge, NY). Matrix (altrenogest) was obtained from Merck Animal Health (Madison, NJ). Signal Fire enhanced chemiluminescent (**ECL**) reagent (6883) and antibodies directed against AKT (9274), pAKT (2965), CYP19A1 (14528), STAR (8499), anti-mouse IgG, HRP-linked (7076), and anti-rabbit IgG-HRP (7074) were obtained from Cell Signaling Technology (Danvers, MA). TLR4

antibody (NB100-56566) was obtained from Novus Biologicals (Littleton, CO). Antibodies directed against AOAH D-15 (sc-163692) and mouse anti-goat IgG (sc-2354) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). LBP enzyme-linked immunosorbent assay (**ELISA**, HK503) was purchased from Hycult Biotech (Uden, Netherlands). Porcine Insulin ELISA (10-1200-01) was from Mercodia (Uppsala, Sweden).

Protein Isolation and Western Blotting

Ovaries were incubated in tissue lysis buffer (1% Triton x-100, 50 mM HEPES, 150 mM NaCl, 10% glycerol, 50 mM NaF, 2 mM EDTA, 1% SDS, containing 1X protease and phosphatase inhibitors), homogenized, and incubated on ice for 30 min. Tissue homogenates were centrifuged at 10,621 × g at 4 °C for 15 min. The supernatant was transferred to a fresh tube and centrifuged at 10,621 × g at 4 °C for 15 min. The supernatant was transferred to a fresh tube and stored at −80 °C until the time of analysis. The concentration of protein samples was determined using a BCA assay. Protein samples were diluted to 4 µg/µL in sterile water with 1X Laemmli buffer, incubated at 95 °C for 5 min and separated on a Mini-PROTEAN TGXTM 4–20% precast gel followed by transfer to a nitrocellulose membrane utilizing the iBlot 2 Dry Transfer System. Protein (40 µg) was loaded per lane; equal protein loading and transfer quality were confirmed by Ponceau S staining. Membranes were washed in PBS with 0.2% Tween-20 (**PBST**) and were incubated in 5% BSA in PBST for 1–3 h at room temperature with gentle rocking action. Membranes were incubated with primary antibodies, as listed in Table 1, overnight at 4 °C with rocking, washed three times in PBST for 10 min, incubated with the appropriate secondary antibody (Table 1) for 1 h at room temperature with rocking, followed by three washes of 10 min in PBST. Membranes were incubated with ECL reagent for 3 min before imaging using a ChemiImager 5500 (Alpha Innotech, San Leandro, CA) with AlphaEaseFC software (v3.03 Alpha Innotech). Appropriately sized bands were identified and densitometric analysis performed using ImageJ software (NCBI). Densitometric analysis of Ponceau S staining was performed to confirm equal protein loading and for normalization of specific proteins of interest. Negative controls for all antibodies included primary antibody only, secondary antibody only, and primary antibody species-specific IgG with secondary antibody, to demonstrate antibody specificity.

Follicular Fluid Analysis

Follicular fluid was aspirated from dominant ovarian follicles at time of tissue collection and pooled per animal. Centrifugation at 2,415 relative centrifugal force to remove cellular debris was performed to remove cellular debris and fluid was diluted in 1% BSA buffer (1 g BSA, 0.12 g sodium phosphate monobasic, anhydrous, 0.88 g sodium chloride, 0.01 g sodium azide, 100 mL dd H_2O, at 7.0–7.2 pH) in serial dilutions: 1:10, 1:100, 1:1,000, and 1:10,000. E_2 concentration in follicular fluid was measured by radioimmunoassay as previously described (Perry et al., 2004).

RNA Isolation and Polymerase Chain Reaction Analysis

Ovaries were powdered using a mortar and pestle and RNA extracted using an RNeasy Mini Kit following the manufacturer's protocol, including the optional on-column DNAse digestion step. Concentration and quality of RNA were measured using a ND-1000 Spectrophotometer (λ = 260/280 nm). Complementary DNA was synthesized using total RNA (200 ng), 1 µL oligo dT, 1 µL dNTP, and water to a total volume of 13 µL. Samples were heated to 65 °C for 5 min then placed on ice until addition of master mix. Master mix was

Table 1. Antibody dilutions used for protein detection

Protein	Primary antibody	Primary dilution	Secondary antibody	Secondary dilution
AKT	Rabbit anti-AKT	1:1000	Goat anti-rabbit IgG HRP-linked	1:1000
pAKT	Rabbit anti-pAKT	1:500	Goat anti-rabbit IgG HRP-linked	1:1000
TLR4	Mouse anti-TLR4	1:500	Horse anti-mouse IgG HRP-linked	1:1000
AOAH	Goat anti-AOAH	1:100	Mouse anti-goat IgG HRP-linked	1:10000
STAR	Rabbit anti-STAR	1:1000	Goat anti-rabbit IgG HRP-linked	1:1000
CYP19A1	Rabbit anti-CYP19A1	1:500	Goat anti-rabbit IgG HRP-linked	1:1000

Abbreviations: AKT = RAC-alpha serine/threonine-protein kinase; AOAH = acyloxyacyl hydrolase; CYP19A1 = cytochrome P450 isoform 19A; HRP = horseradish peroxidase; IgG = Immunoglobulin G; pAKT = phosphorylated AKT; STAR = steroidogenic acute regulatory protein; TLR4 = toll-like receptor 4.

composed of 4 µL 1st Strand Buffer, 1 µL 0.1 mM DTT, 1 µL RNAse Out, and 1 µL Superscript III. Seven microliters of master mix were added to each sample and then heated to 50 °C for 60 min and then 70 °C for 15 min. Samples were stored at −20 °C until use. Complementary DNA was diluted (1:50) and amplified using a QuantiTect SYBR Green PCR Kit on an Eppendorf MasterCycler RealPlex 4 and primers specific for *TLR4*, *AOAH*, *FOXO3*, *CYP19*, *LDLR*, *LHR*, *CYP17A1*, *AKT1*, *ESR1*, *INSR*, *IRS1*, *STAR*, and *HSD3B1* (see Table 2 for primer sequences). The primers for *TLR4* and *MD2* (Moue et al., 2008), *MYD88* (Tohno et al., 2007), *NFkB* (Ross et al., 2010), *AOAH* (Mani et al., 2013) have been previously reported in the literature. The remainder was from Nteeba et al. (2015). The primers for *INSR*, *IRS1*, *CYP17*, and *CYP19* were designed in house. The PCR consisted of a 15 min hold at 95 °C followed by 40 cycles of: denaturing at 95 °C for 15 s, annealing for 5 s (see Table 2 for annealing temperatures), and extension at 72 °C for 30 s. A melting gradient of 72 °C to 99 °C at 1 °C increase per step was performed to confirm amplification of a single PCR product and to identify primer dimer formation. Technical controls to ensure that genomic DNA amplification was absent included use of DNase in the RNA isolation, and during PCR we omitted cDNA template or the forward/reverse primers. We also included reactions containing the master mix or H_2O only. Relative fold change was calculated using the 2-ΔΔCT method normalized to *GAPDH*. The results are presented as mean fold difference ± the SE relative to the lowest abundance sample within each gene.

Statistical Analysis

Feed intake, rectal temperature, insulin, glucose, follicle diameter, and plasma LBP were analyzed utilizing the PROC MIXED of SAS (Cary, NC). Fixed effects were treatment, day of infusion, and treatment × day, with day set as a repeated measure. Baseline values from before the start of infusion were used as covariates for each parameter.

Western blot, PCR, follicular fluid LBP, and follicular fluid E_2 data were all analyzed using unpaired two-tailed *t*-tests in GraphPad Prism. Statistical significance was set at $P < 0.05$ with a tendency for difference considered if the P value was below 0.1.

RESULTS

Chronic LPS Exposure Did Not Affect Rectal Temperature or Feed Intake

There was no difference in daily rectal temperatures between CT- and LPS-treated gilts (Figure 1A), thus the animals were not displaying a febrile response. There was additionally no treatment difference ($P = 0.94$) in feed intake at any time point (Figure 1B).

Table 2. Primer sequences used for gene amplification

Gene	Forward primer sequence (5′ - 3′)	Reverse primer sequence (5′ - 3′)	Annealing temperature (°C)
INSR	TGTCATCAATGGGCAGTTTG	ATCAGGTTCCGAACAGTTGC	48.0
IRS1	CTATGCCAGCATCAGCTTCC	GGAGGATTTGCTGAGGTCAT	51.0
AKT	ATCGTGTGGCAGGATGTGTA	CTGGCCGAGTAGGAGAACTG	53.0
TLR4	CTCTGCCTTCACTACAGAGA	CTGAGTCGTCTCCAGAAGAT	53.0
NFkB	CCCATGTAGACAGCACCACCTATGAT	ACAGAGGCTCAAAGTTCTCCACCA	54.0
MYD88	GGCAGCTGGAACAGACCAA	GGCAGGACATCTCGGTCAGA	54.0
MD2	CCACCTTGTTTTCTTCCATATTTACTG	CATCAGAGGAATTGCAGATCCA	53.0
AOAH	TCAGGGGGACAGAAATATGG	CCAGAATCACGCAGAATCAC	48.0
ESR1	AGCACCCTGAAGTCTCTGGA	TGTGCCTGAAGTGAGACAGG	54.0
LDLR	GAGTTGGCTTTTGCTCTGCT	GGGTTTTGGTGAATGAATGG	48.0
LHR	CATGGCACCGATCTCTTTCT	CGGAATGCCTTTGTGAAAAT	48.0
CYP19A1	GGAGCTTGGGGTTAATGGAT	GGGAAGGATGCTCTTTGATG	51.0
CYP17A1	ACCCAGCTCATCTCACCATC	GCGCTCCTTGATCTTCACTT	54.0
STAR	TTGGAAGAGACGGATGGAAG	CCCACATTCCTGCTATTGCT	51.8

Abbreviations: *AKT* = RAC-alpha serine/threonine-protein kinase; *AOAH* = acyloxyacyl hydrolase; *CYP17A1* = cytochrome P450 isoform 17A; *CYP19A1* = cytochrome P450 isoform 19A; *ESR1* = estrogen receptor alpha; *FOXO3* = forkhead protein box O3; *HSD3B1* = 3 beta-hydroxysteroid dehydrogenase; *INSR* = insulin receptor; *IRS1* = insulin receptor substrate 1; *LDLR* = low-density lipoprotein receptor 1; *LHR* = luteinizing hormone receptor; *MD2* = myeloid differentiation factor 2; *MYD88* = myeloid differentiation primary response 88; *NFKB* = nuclear factor kappa B; *STAR* = steroidogenic acute regulatory protein; *TLR4* = toll-like receptor 4.

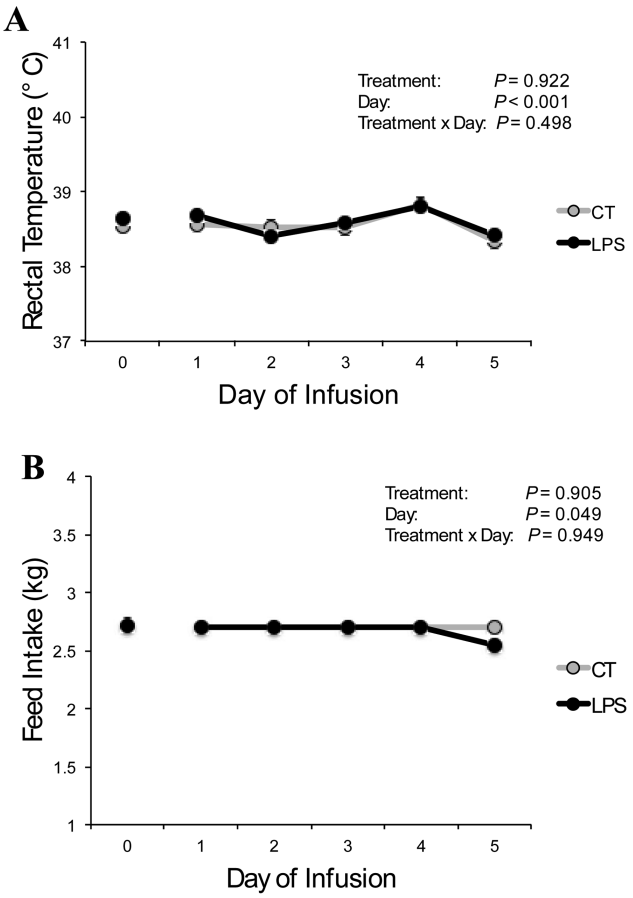

Figure 1. Impact of chronic lipopolysaccharide (LPS) infusion on rectal temperature and feed intake. Post-pubertal gilts received an equivolume bolus of saline (CT; $n = 4$) or LPS ($n = 6$; 0.1 µg/kg) four times daily (0000 h, 0600 h, 1200 h, 1800 h) for 5 d. (A) Rectal temperature was recorded twice daily (0530 h, 1730 h). Line graph represents average daily rectal temperature per treatment ± SEM. (B) Feed intake was recorded daily. Line graph represents ADFI per treatment ± SEM. Control animals are represented by the gray line, LPS-infused animals are represented by the black line.

Influence of LPS on Circulating Insulin and Glucose

After 24 h of LPS infusion, there was an initial increase (119%; $P < 0.01$) in circulating insulin relative to controls, but no treatment differences in insulin were detected on days 3 and 5 (Figure 2A). Infusing LPS tended to increase (16%; $P = 0.08$) blood glucose on day 1 but this difference was resolved by day 3 (Figure 2B). The glucose:insulin ratio (a crude measure of insulin sensitivity) was decreased ($P = 0.02$) on day 1 in LPS-infused pigs, but there were no treatment differences in this ratio from day 3 onward (Figure 2C).

LPS Infusion Did Not Impact Insulin-Mediated Intracellular Ovarian Signaling

There were no treatment differences detected ($P \geq 0.21$) in ovarian transcript abundance of the insulin receptor (***INSR***), insulin receptor substrate 1 (***IRS1***), RAC-alpha serine/threonine-protein kinase (***AKT***), or forkhead protein box O3 (***FOXO3***; Table 3). Also, no treatment difference ($P \geq 0.13$) was observed in the protein abundance of AKT, phosphorylated AKT (**pAKT**), or the pAKT:AKT ratio in post-pubertal gilts (Figure 4 and Table 4). Ponceau S staining of total protein confirmed no difference in the amount of protein per lane of the separating gel (Tables 5 and 6).

LPS Infusion Did Not Alter Follicular Size and Ovarian 17β-Estradiol in Follicular Fluid

There was no treatment difference ($P = 0.64$) detected in the diameter of ovulatory follicles between groups, nor was there any impact of experimental treatment on the volume of follicular fluid obtained per ovary ($P = 0.95$; Tables 5 and 6). No treatment difference was detected in 17β-estradiol concentrations in follicular fluid aspirated from the dominant follicles ($P = 0.42$; Tables 5 and 6).

Impact of LPS on Ovarian Steroidogenic Enzyme mRNA and Protein Abundance

Lipopolysaccharide exposure did not affect ($P \geq 0.68$) mRNA abundance for estrogen receptor alpha (***ESR1***), low-density lipoprotein receptor 1 (***LDLR***), or luteinizing hormone receptor (***LHR***). Transcript abundance of cytochrome P450 (**CYP**) isoform 19A (*CYP19A1*), CYP isoform 17A (*CYP17A1*), steroidogenic acute regulatory protein (***STAR***), and 3 beta-hydroxysteroid dehydrogenase (*HSD3B1*) were unchanged ($P \geq 0.69$) by LPS (Table 3). Furthermore, ovarian protein levels of CYP19A1 and STAR did not differ ($P \geq 0.62$) between LPS and CT gilts (Figure 4 and Table 4). There was no difference in the amount of protein loaded between samples as verified by Ponceau S staining of total protein (Tables 5 and 6).

Effect of Chronic LPS Infusion on Blood and Follicular Fluid LBP Level

Circulating LBP was increased (149%; $P = 0.006$) relative to CT on day 1 of LPS exposure but no treatment differences were detected from day 3 onward (Figure 3A). There was no difference in LBP levels in follicular fluid aspirated from dominant follicles of CT- and LPS-exposed gilts on day 5 (Figure 3B).

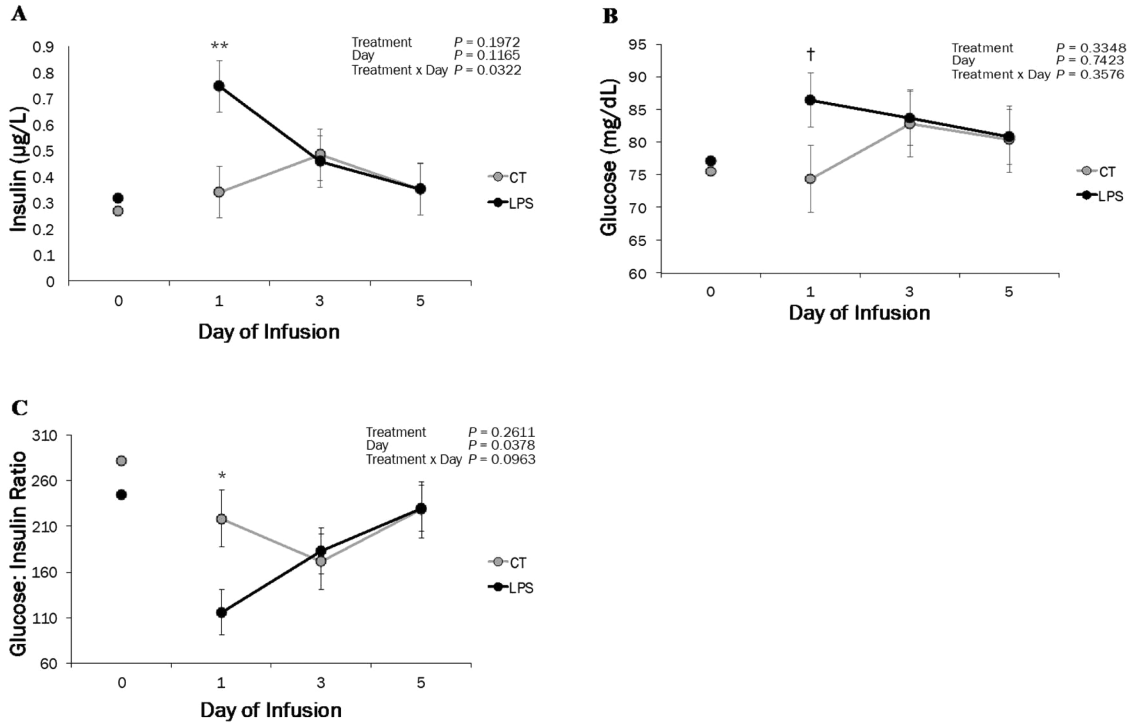

Figure 2. Influence of repeated LPS infusion on circulating insulin and glucose. Post-pubertal gilts received an equivolume bolus of saline (CT; $n = 4$) or LPS ($n = 6$; 0.1 µg/kg) four times daily (0000 h, 0600 h, 1200 h, 1800 h) for 5 d. Blood samples were taken daily at 1730 h. Graphs represent average daily blood (A) insulin, (B) glucose, and (C) glucose:insulin concentration per treatment ± SEM. $^{\dagger}P < 0.1$, $^{*}P < 0.05$, and $^{**}P < 0.01$ at a given time point. Control animals are represented by the grey line, LPS-infused animals are represented by the black line.

Table 3. Impact of LPS infusion on mRNA encoding genes involved in insulin, steroidogenic, and TLR-mediated intracellular signaling

		Relative gene level ± SEM		
Pathway	Gene	CT	LPS	*P* value
Insulin-mediated	*INSR*	20.25 ± 14.87	12.94 ± 3.61	0.77
	IRS1	16.16 ± 14.75	2.25 ± 0.38	0.62
	AKT	2.15 ± 1.15	1.99 ± 0.42	0.80
	FOXO3	2.01 ± 0.98	1.96 ± 0.76	0.94
Steroidogenesis	*ESR1*	2.84 ± 1.59	3.31 ± 1.13	0.70
	LHR	12.53 ± 8.76	20.35 ± 11.01	0.68
	LDLR	4.44 ± 2.70	3.52 ± 1.26	0.92
	CYP19A1	3.29 ± 0.88	11.33 ± 8.93	0.69
	CYP17A1	4.23 ± 2.34	2.85 ± 0.94	0.81
	HSD3B1	3.85 ± 0.75	3.79 ± 1.78	0.70
	STAR	26.73 ± 0.91	14.54 ± 12.0	0.91
TLR4	*TLR4*	2.14 ± 0.59	6.49 ± 4.34	0.38
	NFkB	1.77 ± 0.76	2.13 ± 0.32	0.50
	MYD88	2.03 ± 0.65	3.52 ± 1.73	0.48
	MD2	2.53 ± 0.77	3.59 ± 1.26	0.59
	AOAH	1.29 ± 0.17	3.76 ± 1.45	0.25

Values represent mean fold changes ± SEM. Abbreviations: *AKT* = RAC-alpha serine/threonine-protein kinase; *AOAH* = acyloxyacyl hydrolase; *CYP17A1* = cytochrome P450 isoform 17A; *CYP19A1* = cytochrome P450 isoform 19A; *ESR1* = estrogen receptor alpha; *FOXO3* = forkhead protein box O3; *HSD3B1* = 3 beta-hydroxysteroid dehydrogenase; *INSR* = insulin receptor; *IRS1* = insulin receptor substrate 1; *LDLR* = low-density lipoprotein receptor 1; *LHR* = luteinizing hormone receptor; *MD2* = myeloid differentiation factor 2; *MYD88* = myeloid differentiation primary response 88; *NFKB* = nuclear factor kappa B; *STAR* = steroidogenic acute regulatory protein; *TLR4* = toll-like receptor 4.

Table 4. Effect of LPS infusion on relative abundance of proteins involved in insulin, steroidogenic, and TLR-mediated intracellular signaling

		Relative protein level ± SEM		
Pathway	Protein	CT	LPS	*P* value
Insulin-mediated	AKT	1.00 ± 0.21	0.98 ± 0.15	0.95
	pAKT	1.00 ± 0.06	0.70 ± 0.12	0.13
	pAKT:AKT	1.07 ± 0.18	0.76 ± 0.15	0.24
Steroidogenesis	STAR	1.00 ± 0.83	0.83 ± 0.30	0.92
	CYP19A1	1.00 ± 0.08	1.07 ± 0.09	0.62
TLR4	TLR4	1.00 ± 0.36	2.34 ± 0.25	0.02*
	AOAH	1.00 ± 0.20	1.15 ± 0.08	0.44

Values represent relative protein abundance ± SEM. Abbreviations: *AKT* = RAC-alpha serine/threonine-protein kinase; *AOAH* = acyloxyacyl hydrolase; *CYP19A1* = cytochrome P450 isoform 19A; *pAKT* = phosphorylated AKT; *STAR* = steroidogenic acute regulatory protein; *TLR4* = toll-like receptor 4.

*$P < 0.05$.

Table 5. Quantification of total protein loading on western blot gels by ponceau S staining

	Relative protein level ± SEM		
Protein of interest	CT	LPS	*P* value
AKT/pAKT	24.10 ± 0.71	23.13 ± 0.49	0.30
STAR	24.96 ± 0.85	26.17 ± 0.99	0.46
CYP19A1	23.29 ± 0.70	23.74 ± 0.91	0.76
TLR4	24.96 ± 0.85	26.17 ± 0.99	0.46
AOAH	40.79 ± 0.89	39.19 ± 0.31	0.09

Values represent total protein staining ± SEM on the gels used to measure the specific protein of interest. Abbreviations: *AKT* = RAC-alpha serine/threonine-protein kinase; *AOAH* = acyloxyacyl hydrolase; *CYP19A1* = cytochrome P450 isoform 19A; *pAKT* = phosphorylated AKT; *STAR* = steroidogenic acute regulatory protein; *TLR4* = toll-like receptor 4.

Impact of LPS Exposure on Ovarian TLR4 Pathway Activation

Lipopolysaccharide exposure did not impact ($P \geq 0.25$) ovarian transcript abundance of *TLR4*, *NFkB*, myeloid differentiation primary response 88 (***MYD88***), *MD2*, or Acyloxyacyl hydrolase (***AOAH***) in LPS gilts (Table 3). At the protein level, LPS-exposed gilts had increased (134%; $P = 0.02$) abundance of ovarian TLR4 compared to controls and there was no difference in ovarian AOAH protein level between groups ($P = 0.44$; Figure 4 and Table 4). As indicated in Tables 5 and 6, there was no difference in the amount of protein loaded on the separating gel.

DISCUSSION

Increased circulating LPS is associated with various physiological states including HS (Baumgard, 2013), reduced feed intake (Pearce et al., 2013), and obesity (Amar et al., 2008; Al-Attas et al., 2009; Hawkesworth et al., 2013). Studies investigating mechanistic impacts of LPS have typically utilized acute, high-concentration exposure paradigms, in which LPS administration is delivered either as a bolus or multiple injections over a short period of time (i.e., 6–24 h). Physiological states that result in increased circulating LPS due to reduced intestinal integrity and increased intestinal permeability, however, elicit a chronic, low-level endogenous LPS exposure, thus acute administration paradigms may not accurately reflect the natural dynamics of in vivo endotoxin appearance.

Lipopolysaccharide activates the innate immune system via the TLR4 pathway (Hoshino et al., 1999), which culminates in RELA phosphorylation (Chow et al., 1999) and production of various proinflammatory cytokines (Lu et al., 2008). Previous in vitro studies have demonstrated that LPS upregulates cytokine IL-6 and chemokine IL-8 production in cultured bovine granulosa cells, increases meiotic failure in bovine cumulus-oocyte complexes (Bromfield and Sheldon, 2011), and reduces the primordial follicle pool in vivo in mice and in vitro in cultured bovine ovarian cortical explants (Bromfield and Sheldon, 2013). Heat stress increases circulating LPS in pigs (Pearce et al., 2013a, 2013b) and though there have been a number of studies examining fertility impacts of LPS, these experiments have been performed in nonporcine species, thus the contribution of LPS to infertility in swine remains unclear. Additionally, as exposure paradigms were short in nature and high in LPS concentrations, they largely are unreflective of in vivo chronic exposure. Furthermore, most studies do not account for metabolism or detoxification of the LPS by the immune system, nor examine other physiological changes that may impact the ovary. Thus, our objectives were 2-fold: to chronically expose post-pubertal gilts to low-level LPS and

to determine systemic as well as ovarian molecular and steroidogenic responses to LPS administration.

To test our objectives, post-pubertal gilts were infused with 0.1 µg LPS/kg BW four times daily via a jugular catheter during their follicular phase of the estrous cycle. Selecting the LPS dose was based upon estimates from our previous LPS models (Kvidera et al., 2017a, 2017b; Horst et al., 2018) and was anticipated to create a chronic and mild inflammation without an overt febrile response. After 5 d of LPS infusion, the ovaries were collected immediately prior to ovulation; an experimental design that facilitated follicular fluid collection to assess the environmental matrix with greatest proximity to the oocyte. None of the animals exhibited a fever throughout the trial, thus we could directly assess the effect of LPS without the confounding effects of elevated body temperature. Additionally, ensuring both treatment groups consumed a similar amount of feed eliminated confounding effects of dissimilar feed intake due to LPS exposure. As has been demonstrated in a number of studies (Waldron et al., 2006; Rhoads et al., 2009; Baumgard and Rhoads, 2013; Kvidera et al., 2017b), LPS acted as an insulin secretagogue and an acute increase in blood insulin was observed without concomitant hypoglycemia. During LPS infusions, muscle and adipose tissues become refractory to insulin action and hepatic glucose export increases; a coordinated metabolic scenario which is employed to ensure adequate glucose delivery to the immune system (Calder, 1995; Kvidera et al, 2017b) Using the plasma glucose:insulin ratio as an indicator, we did note a decrease in insulin sensitivity on day 1. Interestingly, resolution of these differences by day 3 of infusion indicates that the pigs developed rapid LPS tolerance. The specific mechanisms by which this tolerance was mediated were not examined in this study. Regardless, acute changes in insulin indicate successful establishment of a porcine model to study follicular effects of chronic low-level LPS exposure.

Table 6. Influence of LPS infusion on dominant follicle diameter, follicle fluid volume, or E_2 concentration

Follicular parameter	CT	LPS	*P* value
Diameter (mm)	7.67 ± 0.62	7.25 ± 0.57	0.64
Volume (mL)	3.93 ± 0.64	3.98 ± 0.65	0.95
E_2 in fluid (ng/mL)	18.85 ± 14.02	39.90 ± 17.76	0.42

Values represent mean values ± SEM. Abbreviations: E_2 = 17β-estradiol; LPS = lipopolysaccharide.

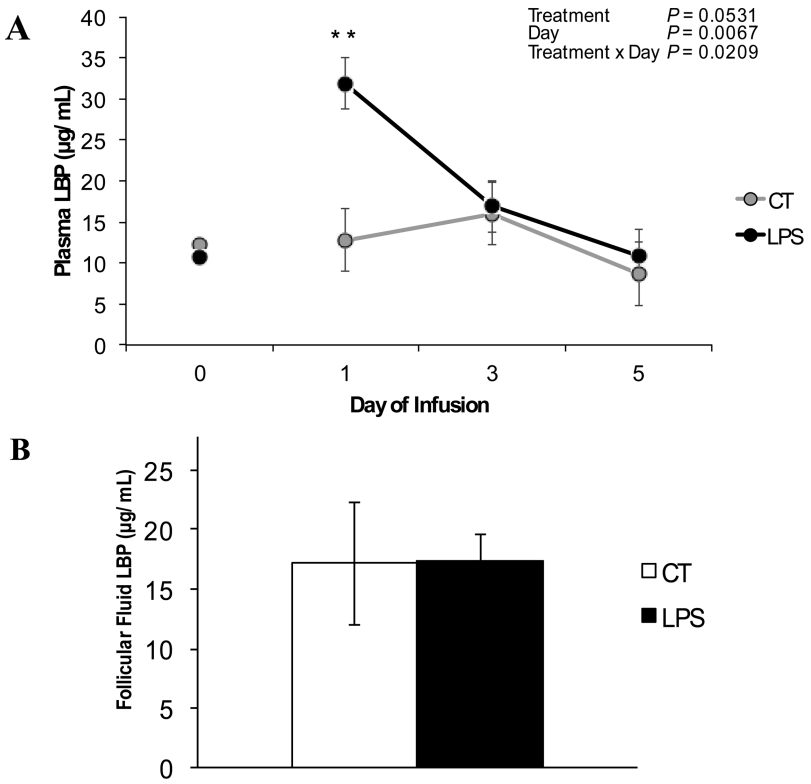

Figure 3. Impact of chronic lipopolysaccharide (LPS) infusion on blood and follicular fluid LPS-binding protein (LBP) level. Post-pubertal gilts received an equivolume bolus of saline (CT; $n = 4$) or LPS ($n = 6$; 0.1 µg/kg) four times daily (0000 h, 0600 h, 1200 h, 1800 h) for 5 d. (A) Blood samples were taken daily at 1730 h. Line graph represents average daily blood LBP level per treatment ± SEM. **Difference between treatments ($P < 0.01$). (B) At time of tissue collection, follicular fluid was aspirated and the bar chart represents the average concentration of LBP in follicular fluid per treatment ± SEM. No difference between experimental treatment in follicular fluid LBP was observed ($P > 0.05$ between treatments).

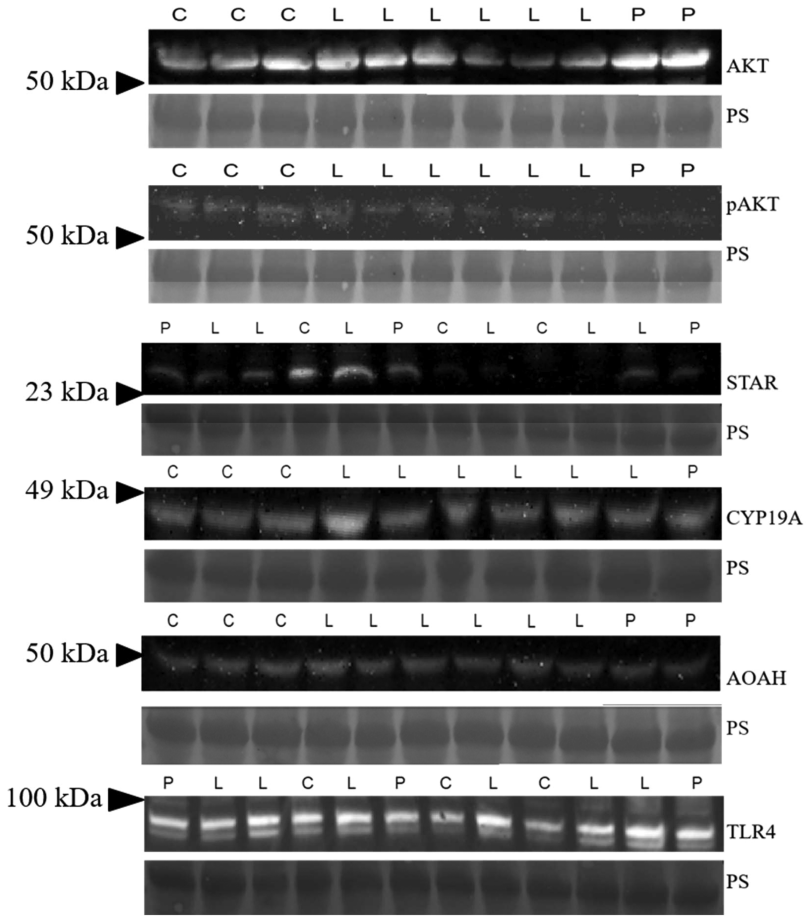

Figure 4. Impact of chronic lipopolysaccharide (LPS) infusion on ovarian protein abundance. Ovarian AKT, pAKT, STAR, CYP19A1, AOAH, and TLR4 protein abundance were quantified using western blotting from whole ovarian protein homogenates isolated from post-pubertal gilts that received equivolume saline (C) or LPS (L; 0.1 µg/kg) four times daily (0000 h, 0600 h, 1200 h, 1800 h) for 5 d. A pooled (P) control sample was included for comparison between gels. Equal protein loading was confirmed by Ponceau S (PS) staining. Arrows indicate location of corresponding molecular weight marker with the corresponding marker sizes.

Insulin binds the α-subunits activating the INSR tyrosine kinase in the β-subunits, with subsequent auto-phosphorylation and recruitment of substrate adaptors including the INSR substrate (**IRS** 1–4) proteins. Tyrosine-phosphorylated IRS then displays binding sites for numerous signal transduction partners including the PI3K/Protein kinase B (**PKB/AKT**) signaling pathway. The PI3K pathway is involved in a myriad of cellular processes, including regulating primordial follicle growth activation via phosphorylation of AKT and FOXO3 (John et al., 2008). Dysregulated primordial follicular activation is undesirable since those activated follicles will not necessarily proceed toward ovulation; instead, they will undergo atresia and be prematurely removed from the ovary. In a murine progressive obesity model, decreased numbers of primordial and primary follicles with increases in the populations of secondary and pre-antral follicles compared to lean controls have been demonstrated (Nteeba et al., 2014). When obesity is induced with a high-fat diet model in mice, altered ovarian PI3K component gene expression with increases in *Irs1* and *Kitlg* along with decreases in *Akt* and *Foxo3* (Nteeba et al., 2013) were observed; both important regulators of primordial follicle activation (Castrillon et al., 2003; Liu et al., 2007; John et al., 2008). Though other studies have reported alterations to primordial follicle numbers due to LPS treatment (Bromfield and Sheldon, 2013), we detected no difference in relative abundance of AKT or pAKT, or alterations in mRNA abundance of *INSR*, *IRS1*, *AKT*, or *FOXO3*. Our previous work in pre-pubertal gilts exposed to HS for 7 or 35 d demonstrated increased ovarian *AKT*, *FOXO3*, and pAKT compared to gilts in thermal neutral conditions, and increased ovarian abundance of *IR*, *IRS1*, and pIRS1 (Nteeba et al., 2015). These differences could indicate specific effects of HS relative to LPS exposure, systemic, and ovarian tolerance to LPS, or could to differential physiological responses to LPS between the pre- and post-pubertal ovary.

As a soluble acute phase protein, LBP binds LPS in serum (Grube et al., 1994; Schumann, 2011). While LPS can activate the TLR4 complex while unbound (Triantafilou and Triantafilou, 2002), when bound to LBP it has an increased rate of binding to CD14 (Hailman et al., 1994). In human granulosa–luteal cell culture, LPS exposure caused increased LBP production; interestingly, over 90% of the cells positive for LBP also contained detectable 3βHSD (Sancho-Tello et al., 1992), suggesting that the granulosa–luteal cells, not immune cells, were producing LBP. We observed increased plasma LBP on day 1 but not day 3 or 5, which is consistent with the temporal pattern of acute phase proteins (Epstein et al., 1999). Baseline porcine serum LBP concentrations of approximately 10 µg/mL have been reported (Barbé et al., 2011), similar to the levels observed in our study. We did not observe differences in LBP concentrations between treatments in follicular fluid at day 5, however, given that follicular fluid arises from components of serum this finding was not surprising (Shalgi et al., 1973). It may be worthwhile in future experiments to assess whether increased LBP occurs in follicular fluid after 24 h of exposure, as was observed in the serum samples in this study.

Acyloxyacyl hydrolase cleaves the lipid A moiety from LPS (Munford and Erwin, 1992), thereby rendering the modified LPS less likely to bind TLR4 (Munford and Hall, 1986). Lipopolysaccharide-binding protein binds LPS in a conformation that increases the susceptibility of the toxin to cleavage by AOAH (Gioannini et al., 2007). Acyloxyacyl hydrolase is produced by macrophages and has primarily been observed in the liver, though studies have established that it can also be produced in the spleen and lungs (Munford, 2005). Interestingly, we did discover ovarian AOAH protein, which is novel, but did not observe a difference in ovarian abundance of AOAH between groups. There may have been increased AOAH in other tissues, such as the liver, as supported by the temporal response of LBP or ovarian AOAH activity could have been altered which are potential avenues for future exploration.

We discovered that the ovary responded to chronic LPS exposure through increasing TLR4 protein abundance. Others have shown that *TLR4* increases in bovine granulosa cells after LPS exposure (Shimizu et al., 2012); however, studies characterizing the effects of ovarian LPS exposure have mainly focused on molecules downstream of TLR4 (Bromfield and Sheldon, 2011; Price et al., 2012; Price and Sheldon, 2013), not on TLR4 abundance. We did not, however, observe changes to the mRNA abundance of *TLR4*, *NFkB*, *MyD88*, *MD2*, or *AOAH*, which could be attributable to tissue collection at a single time point; thus, we may have missed observable increases in ovarian mRNA at earlier times in the exposure paradigm. It could also be the case that protein abundance or activity of these TLR4 downstream factors was altered, though that was outside the scope of the current study. Since we have recently demonstrated the localization of TLR4 protein to the mural granulosa cells of antral follicles in the porcine ovary (Dickson et al., 2018), we consider increased TLR4 protein to occur in ovarian tissue, rather than be due to increased abundance of circulating immune cells, though that is certainly a possibility for consideration.

Administrating LPS alters serum steroid hormone concentrations in rhesus monkeys (Xiao et al., 1999), sheep (Battaglia, 1997), rats (Shakil et al., 1994), pigs (Cort et al., 1986), goats (Fredriksson et al., 1985), and mice (Aisemberg et al., 2013). In an in vitro model, LPS decreases progesterone production by theca cells by reducing STAR, which is responsible for transport of cholesterol into the inner mitochondrial matrix; the rate-limiting step in steroidogenesis (Falck, 1960; Jaiswal et al., 2014; Magata et al., 2014). Interestingly, we saw no differences in STAR or CYP19A1 protein or mRNA. This could be the result of differences in LPS dosage, route of administration, or length of administration. Additionally, though the concentrations were in the expected range (Babalola and Shapiro, 1988), we did not observe any difference in 17β-estradiol concentrations in the follicular fluid. Thus, we did not observe altered endocrine production or steroidogenesis in this experimental model of chronic, low-level LPS exposure.

Taken together, these data demonstrate that the ovaries of post-pubertal gilts are responsive to chronic low-level LPS exposure, and that systemic tolerance to LPS exposure develops. Some limitations to the current study are that tissues were collected at a single time point, so the possibility of a temporal pattern of protein and/or mRNA induction at different points in the exposure period cannot be discounted. A single bacterial LPS source was employed in this experiment, which serves as a proxy for LPS exposure, but also does not fully represent the milieu of bacterial strains resident in the intestine that may enter circulation during HS or an off-feed event, which may amplify effects observed in our study. Additionally, we chose to perform whole ovarian mRNA and protein analysis rather than to focus on specific cellular fractions within the organ, and this work would add in the future

to these studies. This model does however serve to demonstrate the responsivity of the ovary-to-LPS exposure at a level that is representative of chronic endotoxemia that occurs in humans and domestic species and has relevance to bacterial infection, HS, off-feed events, and other insults, including medicinal, that negatively impact intestinal integrity.

LITERATURE CITED

Aisemberg, J., C. A. Vercelli, M. V. Bariani, S. C. Billi, M. L. Wolfson, and A. M. Franchi. 2013. Progesterone is essential for protecting against LPS-induced pregnancy loss. LIF as a potential mediator of the anti-inflammatory effect of progesterone. PLoS One. 8:e56161. doi:10.1371/journal.pone.0056161

Al-Attas, O. S., N. M. Al-Daghri, K. Al-Rubeaan, N. F. da Silva, S. L. Sabico, S. Kumar, P. G. McTernan, and A. L. Harte. 2009. Changes in endotoxin levels in T2DM subjects on anti-diabetic therapies. Cardiovasc. Diabetol. 8:20. doi:10.1186/1475-2840-8-20

Amar, J., R. Burcelin, J. B. Ruidavets, P. D. Cani, J. Fauvel, M. C. Alessi, B. Chamontin, and J. Ferriéres. 2008. Energy intake is associated with endotoxemia in apparently healthy men. Am. J. Clin. Nutr. 87:1219–1223. doi:10.1093/ajcn/87.5.1219

Babalola, G. O., and B. H. Shapiro. 1988. Correlation of follicular steroid hormone profiles with ovarian cyclicity in sows. J. Reprod. Fertil. 84:79–87. doi:10.1530/jrf.0.0840079

Barbé, F., K. Atanasova, and K. Van Reeth. 2011. Cytokines and acute phase proteins associated with acute swine influenza infection in pigs. Vet. J. 187:48–53. doi:10.1016/j.tvjl.2009.12.012

Battaglia, D. F., J. M. Bowen, H. B. Krasa, L. A. Thrun, C. Viguié, and F. J. Karsch. 1997. Endotoxin inhibits the reproductive neuroendocrine axis while stimulating adrenal steroids: a simultaneous view from hypophyseal portal and peripheral blood. Endocrinology. 138:4273–4281. doi:10.1210/endo.138.10.5449

Baumgard, L. H., and R. P. Rhoads. 2013. Effects of heat stress on postabsorptive metabolism and energetics. Annu. Rev. Anim. Biosci. 1:311–337. doi:10.1146/annurev-animal-031412-103644

Bromfield, J. J., and I. M. Sheldon. 2011. Lipopolysaccharide initiates inflammation in bovine granulosa cells via the TLR4 pathway and perturbs oocyte meiotic progression in vitro. Endocrinology. 152:5029–5040. doi:10.1210/en.2011-1124.

Bromfield, J. J., and I. M. Sheldon. 2013. Lipopolysaccharide reduces the primordial follicle pool in the bovine ovarian cortex ex vivo and in the murine ovary in vivo. Biol. Reprod. 88:98. doi:10.1095/biolreprod.112.106914

Brüning, J. C., D. Gautam, D. J. Burks, J. Gillette, M. Schubert, P. C. Orban, R. Klein, W. Krone, D. Müller-Wieland, and C. R. Kahn. 2000. Role of brain insulin receptor in control of body weight and reproduction. Science 289:2122–2125. doi:10.1126/science.289.5487.2122

Calder, P. C. 1995. Fuel utilization by cells of the immune system. Proc. Nutr. Soc. 54:65–82. doi:10.1079/PNS19950038

Castrillon, D. H., L. Miao, R. Kollipara, J. W. Horner, and R. A. DePinho. 2003. Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. Science. 301:215–218. doi:10.1126/science.1086336

Chen, Y.-J., P.-W. Hsiao, M.-T. Lee, J. I. Mason, F.-C. Ke, and J.-J. Hwang. 2007. Interplay of PI3K and cAMP/PKA signaling, and rapamycin-hypersensitivity in TGF 1 enhancement of FSH-stimulated steroidogenesis in rat ovarian granulosa cells. J. Endocrinol. 192:405–419. doi:10.1677/JOE-06-0076

Choi, K., and Y. B. Kim. 2010. Molecular mechanism of insulin resistance in obesity and type 2 diabetes. Korean J. Intern. Med. 25:119–129. doi:10.3904/kjim.2010.25.2.119

Chow, J. C., D. W. Young, D. T. Golenbock, W. J. Christ, and F. Gusovsky. 1999. Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. J. Biol. Chem. 274:10689–10692. doi:10.1074/jbc.274.16.10689

Cort, N., H. Kindahl, and S. Einarsson. 1986. The effect of a gram-negative bacterial endotoxin and cloprostenol on the plasma levels of 15-keto-13,14-dihydro-PGF2α, progesterone, oestradiol-17β, oestrone sulphate and luteinizing hormone in non-pregnant and 60-day-pregnant gilts. Anim. Reprod. Sci. 10:147–162. http://linkinghub.elsevier.com/retrieve/pii/0378432086900266 (accessed June 7, 2017).

Dickson, M. J., C. L. Hager, A. Al-Shaibi, P. Q. Thomas, L. H. Baumgard, J. W. Ross, and A. F. Keating. 2018. Impact of heat stress during the follicular phase on porcine ovarian steroidogenic and phosphatidylinositol-3 signaling. J. Anim. Sci. 50011. doi:10.1093/jas/sky144/4976453

Epstein, F. H., C. Gabay, and I. Kushner. 1999. Acute-phase proteins and other systemic responses to inflammation. N. Engl. J. Med. 340:448–454. doi:10.1056/NEJM199902113400607

Falck, B. 1960. Site of production of oestrogen in rat ovary as studied in micro-transplants. Acta Physiol. Scand. 47:1–101. doi:10.1111/j.1748-1716.1960.tb01823.x

Fredriksson, G., H. Kindahl, and L.-E. Edqvist. 1985. Endotoxin-induced prostaglandin release and corpus luteum function in goats. Anim. Reprod. Sci. 8:109–121. http://linkinghub.elsevier.com/retrieve/pii/0378432085900776 (accessed June 10, 2017).

Gioannini, T. L., A. Teghanemt, D. Zhang, P. Prohinar, E. N. Levis, R. S. Munford, and J. P. Weiss. 2007. Endotoxin-binding proteins modulate the susceptibility of bacterial endotoxin to deacylation by acyloxyacyl hydrolase. J. Biol. Chem. 282:7877–7884. doi:10.1074/jbc.M605031200

Grube, B. J., C. G. Cochane, R. D. Ye, C. E. Green, M. E. McPhail, R. J. Ulevitch, and P. S. Tobias. 1994. Lipopolysaccharide binding protein expression in primary human hepatocytes and HepG2 hepatoma cells. J Biol Chem. 269:8477–8482. http://www.ncbi.nlm.nih.gov/pubmed/7510687 (accessed June 7, 2017).

Hailman, E., H. S. Lichenstein, M. M. Wurfel, D. S. Miller, D. A. Johnson, M. Kelley, L. A. Busse, M. M. Zukowski, and S. D. Wright. 1994. Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14. J. Exp. Med. 179:269–77. http://www.ncbi.nlm.nih.gov/pubmed/7505800 (accessed April 11, 2016).

Hawkesworth, S., S. E. Moore, A. J. Fulford, G. R. Barclay, A. A. Darboe, H. Mark, O. A. Nyan, and A. M. Prentice. 2013. Evidence for metabolic endotoxemia in obese and diabetic Gambian women. Nutr. Diabetes. 3:e83. doi:10.1038/nutd.2013.24

Horst, E. A., S. K. Kvidera, E. J. Mayorga, C. S. Shouse, M. Al-Qaisi, M. J. Dickson, J. Ydstie, H. A. Ramirez Ramirez, A. F. Keating, D. J. Dickson, et al. 2018. Effect of chromium on bioenergetics and leukocyte dynamics following immunoactivation in lactating Holstein cows. J. Dairy Sci. 101:5515–5530. doi:10.3168/jds.2017-13899

Hoshino, K., O. Takeuchi, T. Kawai, H. Sanjo, T. Ogawa, Y. Takeda, K. Takeda, and S. Akira. 1999. Cutting edge: toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. J. Immunol. 162:3749–52. http://www.jimmunol.org/content/jimmunol/162/7/3749.full.pdf (accessed May 14, 2017).

Jaiswal, S. K., N. J. Siddiqi, and B. Sharma. 2014. Carbofuran induced oxidative stress mediated alterations in Na^+-K^+-ATPase activity in rat brain: amelioration by vitamin E. J. Biochem. Mol. Toxicol. 28:320–7. doi:10.1002/jbt.21783

John, G. B., T. D. Gallardo, L. J. Shirley, and D. H. Castrillon. 2008. Foxo3 is a PI3K-dependent molecular switch controlling the initiation of oocyte growth. Dev. Biol. 321:197–204. http://linkinghub.elsevier.com/retrieve/pii/S0012160608010087 (accessed July 5, 2016).

Kvidera, S. K., E. A. Horst, M. Abuajamieh, E. J. Mayorga, M. V. S. Fernandez, and L. H. Baumgard. 2017a. Glucose requirements of an activated immune system in lactating Holstein cows. J. Dairy Sci. 100:2360–2374. http://linkinghub.elsevier.com/retrieve/pii/S0022030216309213 (accessed March 7, 2017).

Kvidera, S. K., E. A. Horst, E. J. Mayorga, M. V. Sanz-Fernandez, M. Abuajamieh, and L. H. Baumgard. 2017b. Estimating glucose requirements of an activated immune system in growing pigs. J. Anim. Sci. 95:5020–5029. doi:10.2527/jas2017.1830

Liu, L., S. Rajareddy, P. Reddy, C. Du, K. Jagarlamudi, Y. Shen, D. Gunnarsson, G. Selstam, K. Boman, and K. Liu. 2007. Infertility caused by retardation of follicular development in mice with oocyte-specific expression of Foxo3a. Development. 134:199–209. http://www.ncbi.nlm.nih.gov/pubmed/17164425 (accessed March 7, 2017).

Lu, Y., W. Yeh, and P. S. Ohashi. 2008. LPS/TLR4 signal transduction pathway. Cytokine. 42(2):145–151. doi:10.1016/j.cyto.2008.01.006

Magata, F., M. Horiuchi, A. Miyamoto, and T. Shimizu. 2014. Lipopolysaccharide (LPS) inhibits steroid production in theca cells of bovine follicles in vitro: distinct effect of LPS on theca cell function in pre- and post-selection follicles. J. Reprod. Dev. 60:280–287. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4139502&tool=pmcentrez&rendertype=abstract (accessed September 1, 2016).

Mani, V., A. J. Harris, A. F. Keating, T. E. Weber, J. C. M. Dekkers, and N. K. Gabler. 2013. Intestinal integrity, endotoxin transport and detoxification in pigs divergently selected for residual feed intake. J. Anim. Sci. 91:2141–2150. http://www.animalsciencepublications.org/publications/jas/abstracts/91/5/2141 (accessed June 15, 2016).

Moue, M., M. Tohno, T. Shimazu, T. Kido, H. Aso, T. Saito, and H. Kitazawa. 2008. Toll-like receptor 4 and cytokine expression involved in functional immune response in an originally established porcine intestinal epitheliocyte cell line. Biochim. Biophys. Acta - Gen. Subj. 1780:134–144. http://linkinghub.elsevier.com/retrieve/pii/S0304416507002693 (accessed February 11, 2017).

Munford, R. S. 2005. Detoxifying endotoxin: time, place and person. J. Endotoxin Res. 11:69–84. doi:10.1179/096805105X35161

Munford, R. S., and A. L. Erwin. 1992. Eukaryotic lipopolysaccharide deacylating enzyme. In: Dennis, E. A., D. A. Vance, editors. Methods in Enzymology. Vol. 209. Academic Press, Inc., Cambridge, MA. p. 485–492. http://linkinghub.elsevier.com/retrieve/pii/007668799209059C (accessed March 11, 2016).

Munford, R., and C. Hall. 1986. Detoxification of bacterial lipopolysaccharides (endotoxins) by a human neutrophil enzyme. Science. 234:203–205. doi:10.1126/science.3529396

Nteeba, J., S. Ganesan, and A. F. Keating. 2014. Progressive obesity alters ovarian folliculogenesis with impacts on pro-inflammatory and steroidogenic signaling in female mice. Biol. Reprod. 91:1–25. doi:10.1095/biolreprod.114.121343

Nteeba, J., J. W. Ross, J. W. Perfield II, and A. F. Keating. 2013. High fat diet induced obesity alters ovarian phosphatidylinositol-3 kinase signaling gene expression. Reprod. Toxicol. 42:68–77. doi:10.1016/j.reprotox.2013.07.026

Nteeba, J., M. V. Sanz-Fernandez, R. P. Rhoads, L. H. Baumgard, J. W. Ross, and A. F. Keating. 2015. Heat stress alters ovarian insulin-mediated phosphatidylinositol-3 kinase and steroidogenic signaling in gilt ovaries. Biol. Reprod. 92:148. doi:10.1095/biolreprod.114.126714

Pearce, S. C., V. Mani, R. L. Boddicker, J. S. Johnson, T. E. Weber, J. W. Ross, R. P. Rhoads, L. H. Baumgard, and N. K. Gabler. 2013a. Heat stress reduces intestinal barrier integrity and favors intestinal glucose transport in growing pigs. PLoS One. 8:e70215. doi:10.1371/journal.pone.0070215

Pearce, S. C., V. Mani, T. E. Weber, R. P. Rhoads, J. F. Patience, L. H. Baumgard, and N. K. Gabler. 2013b. Heat stress and reduced plane of nutrition decreases intestinal integrity and function in pigs. J. Anim. Sci. 91:5183–5193. doi:10.2527/jas.2013-6759

Perry, G. A., M. F. Smith, and T. W. Geary. 2004. Ability of intravaginal progesterone inserts and melengestrol acetate to induce estrous cycles in postpartum beef cows. J. Anim. Sci. 82:695–704. doi:10.2527/2004.823695x

Poretsky, L., N. A. Cataldo, Z. Rosenwaks, and L. C. Giudice. 1999. The insulin-related ovarian regulatory system in health and disease. Endocr. Rev. 20:535–582. doi:10.1210/edrv.20.4.0374

Price, J. C., J. Cronin, and I. M. Sheldon. 2012. Toll-like receptor expression and function in the COV434 granulosa cell line. Am. J. Reprod. Immunol. 68:205–17. doi:10.1111/j.1600-0897.2011.01103.x

Price, J. C., and I. M. Sheldon. 2013. Granulosa cells from emerged antral follicles of the bovine ovary initiate inflammation in response to bacterial pathogen-associated molecular patterns via Toll-like receptor pathways. Biol. Reprod. 89:119. doi:10.1095/biolreprod.113.110965

Rhoads, M. L., R. P. Rhoads, M. J. VanBaale, R. J. Collier, S. R. Sanders, W. J. Weber, B. A. Crooker, and L. H. Baumgard. 2009. Effects of heat stress and plane of nutrition on lactating Holstein cows: I. Production, metabolism, and aspects of circulating somatotropin. J. Dairy Sci. 92:1986–1997. doi:10.3168/jds.2008-1641

Ross, J. W., M. D. Ashworth, D. Mathew, P. Reagan, J. W. Ritchey, K. Hayashi, T. E. Spencer, M. Lucy, and R. D. Geisert. 2010. Activation of the transcription factor,

nuclear factor kappa-B, during the estrous cycle and early pregnancy in the pig. Reprod. Biol. Endocrinol. 8:39. doi:10.1186/1477-7827-8-39

Sancho-Tello, M., T.-Y. Chen, T. K. Clinton, R. Lyles, R. F. Moreno, L. Tilzer, K. Imakawa, and P. F. Terranova. 1992. Evidence for lipopolysaccharide binding in human granulosa-luteal cells. J. Endocrinol. 135:571–NP. doi:10.1677/joe.0.1350571

Sanz Fernandez, V. M., J. S. Johnson, M. Abuajamieh, S. K. Stoakes, J. T. Seibert, L. Cox, S. Kahl, T. H. Elsasser, J. W. Ross, S. Clay Isom, et al. 2015. Effects of heat stress on carbohydrate and lipid metabolism in growing pigs. Physiol. Rep. 3:e12315–e12315. doi:10.14814/phy2.12315

Schumann, R. R. 2011. Old and new findings on lipopolysaccharide-binding protein: a soluble pattern-recognition molecule. Biochem. Soc. Trans. 39:989–993. doi:10.1042/BST0390989

Shakil, T., A. Snell, and S. A. Whitehead. 1994. Effects of lipopolysaccharide and cyclosporin on the endocrine control of ovarian function. J. Reprod. Fertil. 100:57–64. doi:10.1530/jrf.0.1000057

Shalgi, R., P. Kraicer, A. Rimon, M. Pinto, and N. Soferman. 1973. Proteins of human follicular fluid: the blood-follicle barrier. Fertil. Steril. 24:429–434. http://linkinghub.elsevier.com/retrieve/pii/S0015028216397308 (accessed June 7, 2017).

Shimizu, T., K. Miyauchi, K. Shirasuna, H. Bollwein, F. Magata, C. Murayama, and A. Miyamoto. 2012. Effects of lipopolysaccharide (LPS) and peptidoglycan (PGN) on estradiol production in bovine granulosa cells from small and large follicles. Toxicol. In Vitro. 26:1134–42. doi:10.1016/j.tiv.2012.06.014

Tohno, M., T. Shimazu, H. Aso, Y. Kawai, T. Saito, and H. Kitazawa. 2007. Molecular cloning and functional characterization of porcine myd88 essential for TLR signaling. Cell. Mol. Immunol. 4:369–376.

Triantafilou, M., and K. Triantafilou. 2002. Lipopolysaccharide recognition: CD14, TLRs and the LPS-activation cluster. Trends Immunol. 23:301–304. Available from http://linkinghub.elsevier.com/retrieve/pii/S1471490602022330 (accessed June 7, 2017).

Waldron, M. R., A. E. Kulick, A. W. Bell, and T. R. Overton. 2006. Acute experimental mastitis is not causal toward the development of energy-related metabolic disorders in early postpartum dairy cows. J. Dairy Sci. 89:596–610. doi:10.3168/jds.S0022-0302(06)72123-3

Williams, E. J., K. Sibley, A. N. Miller, E. A. Lane, J. Fishwick, D. M. Nash, S. Herath, G. C. England, H. Dobson, and I. M. Sheldon. 2008. The effect of *Escherichia coli* lipopolysaccharide and tumour necrosis factor alpha on ovarian function. Am. J. Reprod. Immunol. 60:462–473. doi:10.1111/j.1600-0897.2008.00645.x

Xiao, E., L. Xia-Zhang, A. Barth, J. Zhu, and M. Ferin. 1998. Stress and the menstrual cycle: relevance of cycle quality in the short- and long-term response to a 5-day endotoxin challenge during the follicular phase in the rhesus monkey. J. Clin. Endocrinol. Metab. 83(7):2454–2460. doi:10.1210/jcem.83.7.4926

Xiao, E., L. Xia-Zhang, and M. Ferin. 1999. Stress and the menstrual cycle: short- and long-term response to a five-day endotoxin challenge during the luteal phase in the rhesus monkey. J. Clin. Endocrinol. Metab. 84:623–626. doi:10.1210/jcem.84.2.5448

Zeleznik, A. J., D. Saxena, and L. Little-Ihrig. 2003. Protein kinase B is obligatory for follicle-stimulating hormone-induced granulosa cell differentiation. Endocrinology. 144:3985–3994. doi:10.1210/en.2003-0293