RESEARCH REPORT
ANIMAL SCIENCE

Title: Investigating hyperinsulinemia and increased circulating lipopolysaccharide involvement in seasonal infertility - **NPB 14-230**

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Industry summary:

Heat stress (HS) is a major economic strain for the US swine industry. In terms of reproduction, seasonal infertility is associated with HS, characterized by lengthened wean-to-estrus interval (WEI), increased spontaneous abortions and increased number of rebreeds. Unfortunately, there is unlikely to be a reduction in the strain that HS imparts to the swine industry and the negative effects of HS are likely to become more severe if climate change continues as some predict and most models forecast more extreme summer conditions in US pig-producing areas. Also, genetic selection for rapid muscle growth is thought to increase pig sensitivity to HS as these animals have a higher basal metabolic rate (i.e. greater heat load). The biological explanation for seasonal infertility is poorly understood, and until such time that a thorough understanding is gained, development of mitigating strategies will remain limited. We have discovered that heat-stressed pigs have both increased circulating insulin and lipopolysaccharide (LPS) levels. We believe that higher systemic insulin and LPS detrimentally influence ovarian biological, thereby hampering reproduction. **Our central hypothesis is that heat-induced elevated insulin and lipopolysaccharide (LPS) are causative factors in seasonal infertility**. We had two objectives in this project: **1)** To investigate ovarian impacts of hyperinsulinemia and **2)** Determine effects of increased circulating LPS on ovarian function.

Prior to this proposal, we had discovered that HS altered a number of molecular pathways in the ovary, and these alterations could all adversely impact ovarian function as well as pregnancy maintenance. We published these data in the journal *Biology of Reproduction*, a premiere journal in reproductive biology. However, we did not know the causative agents. Through the current work, we have discovered the following: **1)** HS in the presence of an insulin bolus 15 prior to tissue collection resulted in altered levels of genes and proteins in the pathway through which ovarian hormones are generated, thus, we have additional evidence that HS alters ovarian hormones which could be responsible for lengthened WEI in swine during summer months. **2)** Both HS and LPS increased a pathway that is involved in regulating both the health of the ovulated oocyte as well as ovarian hormone production. **3)** Acute LPS induced a response

in the ovary a mere 8 h after the exposure. **4)** LPS altered the machinery responsible for generation of ovarian hormones in a manner that had similarities as well as differences with HS alone. Because we had technical difficulties in counting follicles within the ovary, we extended the study to examine if there could be additional "turnover" of the chief ovarian hormone, 17β-estradiol, within the ovary during HS. We discovered this to be true, the machinery responsible for degrading 17β-estradiol in a cyclical fashion is altered by HS, indicating that this could also play a role in seasonal infertility in swine. Identification of these HS- as well as LPS-induced ovarian changes have set the stage to continue our investigations to lead to mitigation interventions to minimize seasonal infertility in swine.

Producer Take Home messages:

- HS and LPS alter a pathway in the ovary that regulates when oocyte (egg)-containing follicles are activated to grow and ovulate.
- HS and LPS affect the mechanisms by which the ovary produces key female hormones required for ovulation and maintenance of pregnancy.
- The ovarian transporter protein responsible for degrading 17β-estradiol (estrogen) in a cyclical pattern during the estrous cycle is altered by HS.

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Keywords:

Ovary, hyperthermia, lipopolysaccharide, 17β-estradiol, follicle, oocyte

Scientific Abstract:

Heat stress (HS) during hot summer months causes seasonal infertility in swine, characterized by longer wean to estrus interval, increased numbers of rebreeds and spontaneous abortion. We previously discovered alterations to ovarian molecular signaling pathways that regulate follicle and oocyte viability as well as steroid hormone production in pre-pubertal gilts that were heat-stressed (35°C) for 7 or 35 d. We have demonstrated that heat-stressed pigs have increased systemic insulin as well as lipopolysaccharide, thus this project investigated the impacts of both of these physiological perturbations on ovarian function. Ovaries were collected from pre-pubertal gilts who underwent one of the following experimental paradigms: 1) pre-pubertal gilts (n = 6/treatment) were exposed to HS or thermoneutral (TN) conditions for 5 d, in the presence of hyperinsulinemia (0.1 U.kg/BW) 15 min prior to euthanasia; 2) pre-pubertal gilts (n = 6/treatment) were exposed to a bolus of LPS (10 μg/kg) and euthanasia performed 8 h later. Ovaries from both groups of animals were processed for total RNA or protein isolation, followed by quantitative reverse transcriptase PCR (qRT-PCR) or western blotting to measure alterations to RNA and protein abundance, respectively, due to experimental treatment (HS or LPS). Hyperinsulinemia during HS increased ($P < 0.05$) mRNA encoding *LHR*, *STAR*, *CYP11A1*, *HSD3B1*, *CYP17A1* and *CYP19A1* and also increased ($P < 0.05$) protein abundance of pAKT, CYP19A1 and tended ($P = 0.07$) to increase CYP11A1 protein. No impact on mRNA encoding *INSR*, *IRS1*, *cKIT*, *AKT1*, *FOXO3* or *LDLR* or protein abundance of IRS1 or AKT was observed. Increased ($P < 0.05$) ovarian SULT1E1 and ABCC1 protein was induced by HS, proteins that degrade 17β-estradiol were observed, a novel finding that could explain deranged estrous cyclicity during HS. A bolus of LPS induced ($P < 0.05$) mRNA encoding *FOXO3*, *LDLR*, *STAR*, and *CYP11A1* but reduced ($P < 0.05$) *LHR*, *CYP17A1* and *CYP19A1*. Increased ($P < 0.05$) protein abundance of TLR4 and pAKT were observed. Additionally TLR4, the ligand for LPS, was demonstrated to be increased ($P < 0.05$)

in ovaries from pre-pubertal gilts who experienced cyclical HS for 5 d. Taken together, these data support that the ovary is responsive to increased LPS, which could partially explain altered reproduction due to HS in swine. Also, hyperinsulinemia during HS abrogated some of the previously observed ovarian changes due to HS alone, thus new avenues for investigation have been opened and these data further our biological understanding of ovarian influences culpable for seasonal infertility.

Introduction:

Annual economic losses to global animal agriculture due to thermal (heat) stress (**HS**) surpass billions of dollars. In the United States, summer-induced decreased production is well-documented in every aspect of animal agriculture. This heat-induced economic burden is due to poor sow reproductive performance, increased morbidity, mortality, suboptimal growth, inefficient nutrient utilization, decreased carcass value and carcass processing problems (1). We have recently estimated (in discussion with Dr. Steve Pollman) seasonal infertility in swine costs the US economy $420 million; and the Iowa economy $60 million, based on the number of sows and the 2013 price of grain. The fiscal losses occur despite recent advances in cooling systems, barn management, and aggressive implementation of other heat abatement strategies.

The US swine industry experiences seasonal infertility (conception rates) and impaired reproductive performance (embryonic death), particular during July, August and September (2, 3). For example, 28 day pregnancy rates reach their lowest levels from August to October, and consequently reduced farrowing rates occur in November and December. The deleterious effects of HS on pig reproduction variables are likely to increase in the future if climate change continues as some predict and as genetic selection for lean tissue accretion enhances the animal's sensitivity to HS (4). Consequently, climate change threatens the global protein food supply chain and compromises the competiveness of the US hog industry (5).

We have demonstrated that heat-stressed gilts have increased circulating insulin levels, despite their reduced feed intake and normal blood glucose levels (6). This is very unusual as typically animals consuming less feed have reduced blood insulin concentrations. We have confirmed that increased insulin occurs in other heat-stressed species, including growing steers (7) and lactating cows (8), and this may be a conserved mammalian response to HS (9). We hypothesize that heat-induced elevated insulin may be responsible for seasonal infertility and poor sow performance during the summer months.

Heat-stressed animals redistribute blood to the periphery in an attempt to maximize radiant heat dissipation ultimately resulting in loss of intestinal barrier function. We have repeatedly demonstrated this in acutely and chronically heat-stressed pigs (10) and have recently shown that HS increases (>2 fold) circulating LPS within 6 hours (11, 12). LPS is detrimental for reproductive parameters, as it is thought to inducing preterm labor and contributing to premature embryonic loss (13-15): both phenomena observed in heat-stressed pigs. LPS decreases expression of the progesterone and luteinizing hormone (LH) receptors (13, 16), and since progesterone is essential for maintenance of pregnancy and LH is required for ovulation, these support our hypothesis that LPS contributes to seasonal infertility. LPS I.V. infusion to cows also reduced corpus luteum (CL) function and size (17), reduced circulating progesterone (17) and accelerated activation of the primordial follicle pool (18). As noted earlier, deregulated and uninhibited activation of primordial follicle growth will ultimately result in premature follicle depletion. LPS exposures during the prenatal period can also have negative fertility consequences, including early puberty onset (19, 20) and decreased follicular reserve (18, 21). We propose that heat-induced increased LPS as a key culprit responsible for reduced reproductive efficiency.

Stated Objectives from original proposal

Deciphering ovarian impacts of hyperinsulinemia and characterizing effects of increased circulating LPS on ovarian function.

Materials & Methods:

Animals:

Hyperinsulinemia during HS experiment:

Crossbred gilts (35±4 kg) housed in constant climate controlled rooms in individual pens were exposed to thermal neutral (TN) conditions (21.4 ± 0.6°C; 23-26% humidity; n = 6) with pair feeding to the HS gilts or HS conditions (31.6 ± 4°C; 17-26% humidity; n = 6) with *ad libitum* feed intake for 5 d. On day 3 of the experimental phase, measurement of glucose requirement during TN or HS was performed. On day 5 of the experimental phase, gilts were anesthetized with an intravenous (i.v.) bolus of insulin (0.1 U.kg/BW), followed 15 min later by euthanasia and ovary collection. The specific animal study details have been published by the Baumgard group (22).

LPS acute exposure study:

Crossbred gilts (35±4 kg) were housed in constant climate controlled rooms in individual pens with *ad libitum* feed intake. Feed was removed the night before LPS exposure, thus there was no dissimilar feed intake. Gilts received a bolus of saline or LPS (10 μg/kg BW) administered i.v. and euthanasia performed 8 h later.

Gilts were euthanized, one ovary was stored at -80^{o}C and the other ovary was fixed in 4% paraformaldehyde.

RNA isolation: Ovaries were stored in RNA*later* solution at -80°C. Total RNA was isolated using an RNeasy Mini kit and concentrated using an RNeasy MinElute kit. RNA was eluted using 14 μL of RNase-free water. RNA concentration was determined using an ND-1000 Spectrophotometer (λ = 260/280nm; NanoDrop technologies, Inc., Wilmington, DE).

First strand cDNA synthesis and real-time polymerase chain reaction (PCR): Total RNA was reverse transcribed into cDNA utilizing the Superscript III One-Step RT-PCR System. Two microliters of diluted cDNA (1:50) was amplified using an Eppendorf Mastercycler using a Quantitect™ SYBR Green PCR kit. A typical cycling program consisted of a 15 min hold at 95°C and 45 cycles of: denaturing at 95°C for 15 sec, annealing at 58°C for 15 sec, and extension at 72°C for 20 sec at which point data will be acquired. Product melt conditions were determined using a temperature gradient from 72°C to 99°C with a 1°C increase at each step.

Protein Isolation: Pools of whole ovarian protein homogenates were prepared from cultured ovaries via homogenization in tissue lysis buffer. Briefly, homogenized samples were placed on ice for 30 min, followed by two rounds of centrifugation at 10,000 rpm for 15 min. Supernatant was collected and sample stored at -80^{o}C until further use. Protein was quantified using a standard BCA protocol on a 96-well assay plate. Emission absorbance values were detected with a λ= 540nm excitation on a Synergy™ HT Multi-Detection Microplate Reader using KC4™ software (Bio-Tek® Instruments Inc., Winooski, VT).

Western Blot Analysis: SDS-PAGE was used to separate protein homogenates which were subsequently transferred to nitrocellulose membranes. Briefly, membranes were blocked for 1-4 h with shaking at 4^{o}C in 5% milk in Tris-buffered saline (TBS) with Tween-20 (TTBS). Membranes were incubated with primary antibody in 5% milk in TTBS for 1 h at 4^{o}C. Membranes were washed with TTBS three times for 10 min. HRP-conjugated secondary antibody was added for 1h at room temperature. Membranes were again washed in TTBS, followed by a single wash for 10 min in TBS. Western blots were detected using chemiluminescence (ECL plus reagent) and exposed to X-ray film. Densitometry of the appropriate bands was performed using NCBI Image J software.

Statistical Analysis: All data were statistically analyzed using GraphPad Prism software. Comparison of two treatments was performed using T-test; comparison of more than two treatments was performed by ANOVA. A *P*-value < 0.05 was considered significantly different.

Results:

Objective 1: Deciphering ovarian impacts of hyperinsulinemia

Ovaries were obtained from gilts that received either saline or insulin (0.1 U.kg/BW) through an indwelling jugular catheter 15 min prior to euthanasia. The insulin IV bolus was designed to mimic acute hyperinsulinemia that occurs during HS (while not causing hypoglycemia).

Experiment 1.1. Investigation of impact of hyperinsulinemia on follicle numbers in gilt ovaries.
Technical difficulty with this aspect of the proposal was experienced. Due to the heterogeneous nature of ovarian morphology, we had difficulty in sorting the smaller primordial follicles from the larger follicles. In addition, we have since discovered through another NPB funded project that at the age of the animals from which ovaries were collected, there was significant variation in the ovarian status of these animals so a direct comparison was no valid without the confounding effects of proximity to puberty. We have preserved the ovary samples so that if a marker of porcine primordial follicles becomes available, we can return to these samples.

Experiment 1.2. Determination of hyperinsulinemia's role in HS-induced altered ovarian PI3K and steroidogenic mRNA and protein expression.
Total ovarian mRNA was isolated and converted to cDNA. Using gene-specific primers that we had previously validated in porcine ovaries, we amplified a number of genes that have important roles in activation of follicles to grow, viability of the oocyte, as well as steroid hormone production (17β-estradiol and progesterone). Importantly, these gilts were pre-pubertal and thus the hormone that is being made in small amounts in these ovaries would predominate towards 17β-estradiol as the animal matures towards puberty. We discovered that during HS in the presence of hyperinsulinemia, that there was no impact on mRNA encoding the insulin receptor (*INSR*), the INSR substrate 1 (*IRS1*), *cKIT*, *AKT1*, *FOXO3*, or *LDLR* ($P > 0.05$). Though lack of an impact of HS was observed, these data are of interest, since in the absence of exogenous insulin, we previously found that the mRNA encoding the *INSR, IRS1*, AKT1 and FOXO3 were increased by HS (23). In this project, we also observed that genes responsible for producing proteins that convert cholesterol to 17β-estradiol were increased by HS with accompanying hyperinsulinemia. These data are in agreement with our previous findings and provide mechanistic data in that the presence of exogenous insulin does not abrogate the impact of HS on genes involved in steroid synthesis.

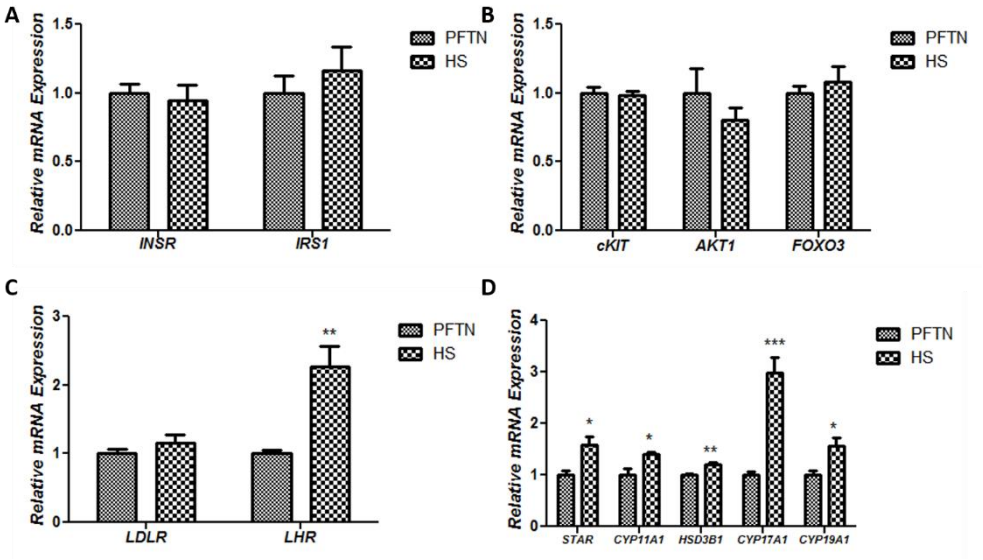

Figure 1. Gene specific effects of HS on expression level. Total ovarian mRNA was isolated from gilts who received exposure to TN (PFTN) or HS conditions in the presence of HI. Quantitative reverse transcriptase polymerase chain reaction was utilized to amplify genes involved in (A) insulin signaling, (B) follicle viability, (C) luteinizing hormone response, and (D) steroid synthesis. Data are presented as relative fold change and significant difference from the PFTN control is indicated by * $P < 0.01$; ** $P < 0.01$ and *** $P < 0.001$.

We next proceeded to protein quantification. Total ovarian protein was isolated and western blotting performed to determine impacts of HS in the presence of hyperinsulinemia on ovarian protein abundance. IRS1 was quantified but no change was observed in animals that received a hyperinsulinemic stimulus, regardless of whether HS conditions were present (Figure 2). We had previously noted increased IRS1 level in heat-stressed animals in the absence of exogenous insulin, thus these data provide insight into mechanisms that could be involved during HS.

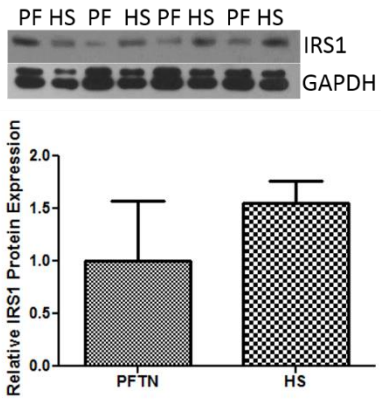

Figure 2. HS during HI does not increase ovarian IRS1 protein. Total ovarian protein from gilts who received exposure to TN (PF) or HS conditions in the presence of HI was separated via SDS-PAGE and western blotting to detect IRS1 performed. Quantification revealed no difference between treatments in IRS1 protein level.

We have previously noted increased AKT during HS in the porcine ovary. We determined any impact of hyperinsulinemia during TN or HS conditions and found no change in total ovarian AKT, but increased phosphorylated AKT with a concomitant increase in the pAKT:AKT ratio (Figure 3). These data are in agreement with our previous findings in the heat-stressed gilt ovary (23), and are not overridden by insulin presence.

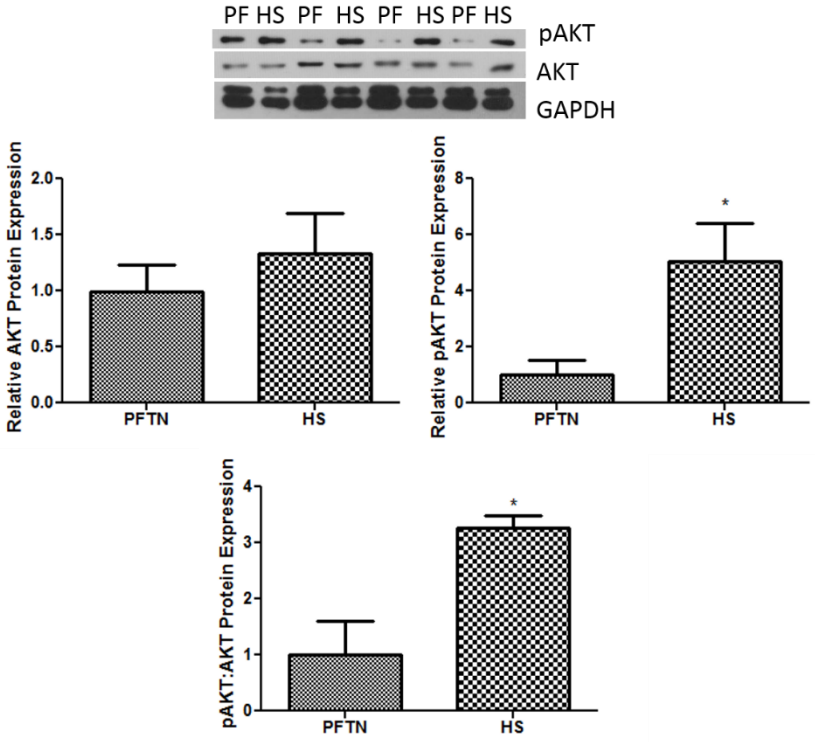

Figure 3. HS increases ovarian pAKT protein. Total ovarian protein from gilts who received exposure to TN (PF) or HS conditions in the presence of HI was separated via SDS-PAGE and western blotting to detect AKT or pAKT protein abundance performed. Quantification revealed no difference between treatments in total ovarian AKT, but increased pAKT due to HS. * indicates $P < 0.05$.

These data represent continuation of characterization of ovarian physiological changes in response to HS. As previously noted, HS increases ovarian PI3K signaling, potentially negatively affecting oocyte viability and the rate of recruitment from the follicular reserve, ultimately resulting in infertility.

We evaluated the effect on CYP11A1, one of the initiating proteins catalyzing conversion of cholesterol to 17β-estradiol and found an increase in the HS HI females (Figure 4; $P = 0.077$). In addition, hyperthermia increased ovarian CYP19A1, which converts testosterone to 17β-estradiol (Figure 4). Aberrant levels of 17β-estradiol would impact display of behavioral estrus, as well as potentially affect ovulation. Again these data are in agreement with our previous findings and strongly support that HS alters ovarian steroid production.

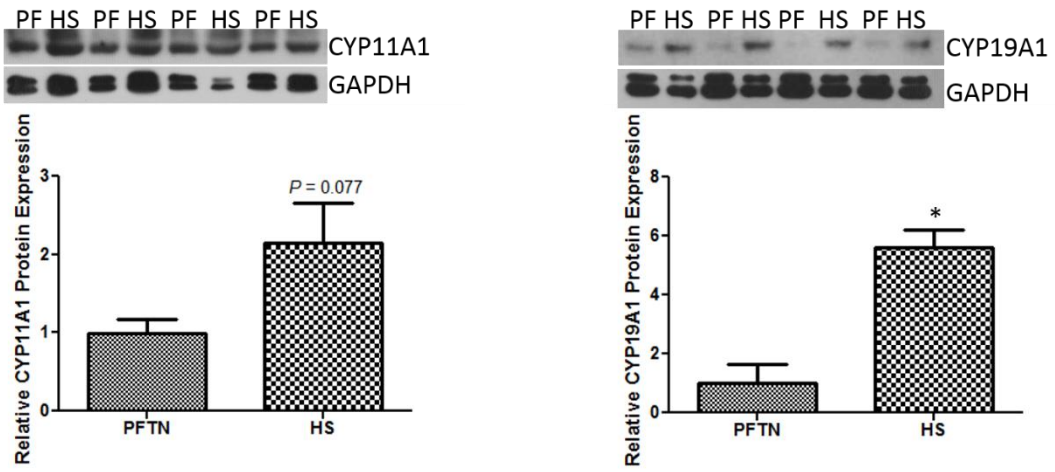

Figure 4. HS increases ovarian CYP11A1 and CYP19A1 protein. Total ovarian protein from gilts who received exposure to TN (PF) or HS conditions in the presence of HI was separated via SDS-PAGE and western blotting to detect CYP11A1 or CYP19A1 protein abundance performed. Quantification revealed increased ovarian CYP11A1 and CYP19A1. * indicates $P < 0.05$.

Due to our difficulties with follicle counting, we extended the study to interrogate whether HS is altering the rate at which 17β-estradiol is degraded within the ovary. During the normal estrous cycle, the ovary requires removal and "turnover" of 17β-estradiol to ensure that cyclical steroid synthesis can move from 17β-estradiol predominance to progesterone and subsequently to a nadir in both hormones. We knew from the literature that 17β-estradiol can be sulfonated by sulfotransferase SULT1E1, and that sulfonated 17β-estradiol (E-S) represents a substrate to multidrug resistance protein 1 (ABCC1). We had access to ovarian protein samples from post-pubertal synchronized gilts, thus we analyzed both SULT1E1 and ABCC1 protein levels in heat-stressed gilt ovaries. We needed to perform these measurements in cyclical gilts to ensure that adequate levels of 17β-estradiol to evaluate this question. We found that both ovarian SULT1E1 and ABCC1 protein were elevated by HS. What this translates to is that 17β-estradiol may be degraded too quickly in the ovaries of heat-stressed pigs. Whether the increase in 17β-estradiol production proceeds increased 17β-estradiol degradation or vice versa remains unclear but this is a hugely novel finding, and opens an avenue for future investigation as regards ovarian causes of seasonal infertility.

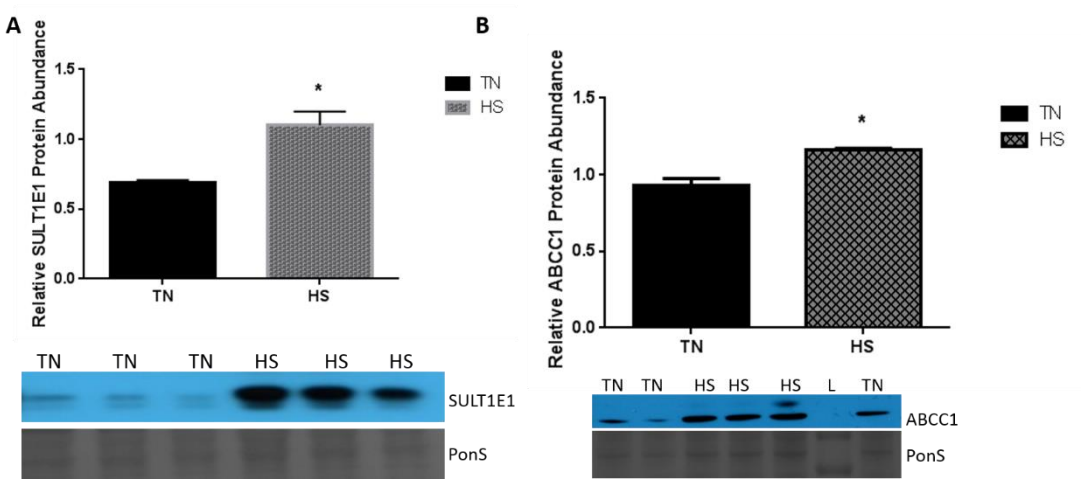

Figure 5. HS increases ovarian SULT1E1 and ABCC1 protein. Total ovarian protein from synchronized post-pubertal gilts who received exposure to TN (PF) or HS was separated via SDS-PAGE and western blotting to detect (A) SULT1E1 or (B) ABCC1 protein abundance performed. Quantification revealed increased ovarian SULT1E1 and ABCC1. * indicates $P < 0.05$.

Objective 2: Characterizing effects of increased circulating LPS on ovarian function

Ovaries were obtained from gilts 8 h after receiving either saline or LPS (10 µg/kg BW) through an indwelling jugular catheter. The LPS infusion is designed to mimic acute metabolic endotoxemia that occurs during HS.

Increased circulating LPS is observed in HS pigs (10, 24) and studies in non-porcine species has demonstrated a range of negative effects of increased LPS on female reproduction. Bovine ovarian cortical explants exposed to LPS had reduced number of primordial follicles, concomitant with increased atresia of the ovarian reserve (18). Similarly, mice exposed to LPS *in vivo* had reduced primordial follicle number which was described as a TLR4-mediated effect, since *Tlr4*$^{-/-}$ mice were refractory to LPS-mediated primordial follicle depletion (18). LPS also causes corpus luteum regression by inducing the production of PGF2α (25, 26). LPS administration causes delayed ovulation, and lengthens the time to CL formation and sufficient P_4 production (27, 28). Thus, LPS could have a causative role in seasonal infertility due to HS. To determine if the receptor for LPS, TLR4, is present in the pig ovary and increased by HS, we performed western blotting on total ovarian protein to detect TLR4. Ovarian TLR4 was induced 8 h after a single LPS injection, demonstrating the ovarian responsiveness to systemic LPS (Figure 6).

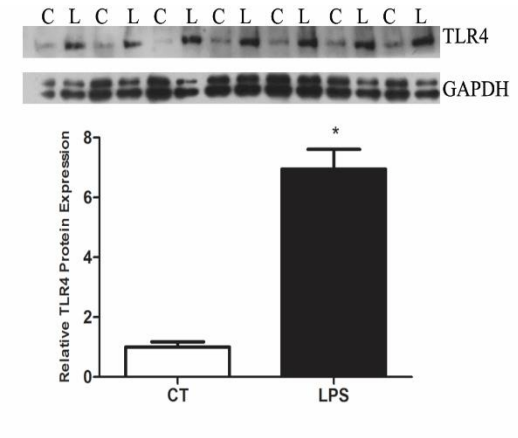

Figure 6. LPS increases ovarian TLR4 protein. Total ovarian protein from gilts who received exposure to saline (CT) or LPS was separated via SDS-PAGE and western blotting to detect TLR4 protein abundance performed. Quantification revealed increased ovarian TLR4, evidence that the ovary quickly responds to systemic LPS. * indicates $P < 0.05$.

We next determined any impact of LPS on ovarian insulin and PI3K signaling. We discovered that there was no effect of increased circulating LPS on ovarian IRS1 protein (Figure 7). In contrast, ovarian pAKT was dramatically increased due to LPS injection (Figure 8). Thus, increased PI3K signaling during HS may result from increased circulating LPS.

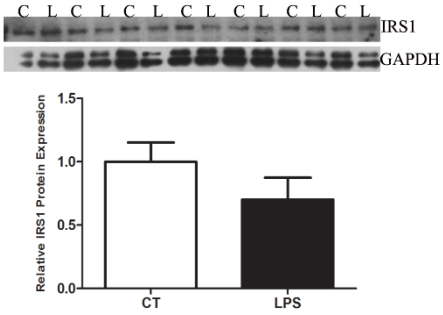

Figure 7. Acute LPS exposure does not impact ovarian IRS1 protein. Total ovarian protein from gilts who received a single injection of saline (CT) or LPS was separated via SDS-PAGE and western blotting to detect IRS1 protein abundance performed. Quantification revealed no impact of acute LPS exposure on ovarian IRS1 protein.

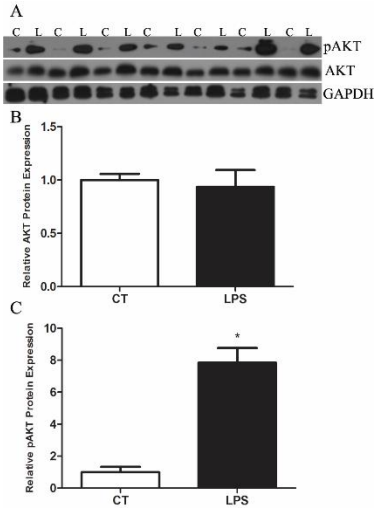

Figure 8. Acute LPS exposure does not impact ovarian total AKT but increases pAKT protein. Total ovarian protein from gilts who received a single injection of saline (CT) or LPS was separated via SDS-PAGE and western blotting to detect AKT or pAKT protein abundance performed. Quantification revealed that acute LPS exposure does not impact total ovarian AKT protein but increases pAKT. * indicates $P < 0.05$.

We next investigated whether acute increased LPS affected the abundance of mRNA encoding genes involved in PI3K activation or steroidogenesis and we found no evidence of transcriptional activation of *cKIT* or *AKT1* (Figure 9A). Thus, increased pAKT may be due to post-translational modification rather than increased transcription. We determined that STAR, CYP11A1 were increased by LPS, yet CYP17A1 and CYP19A1 were decreased, potentially indicating a local feedback loop being activated (Figure 9C).

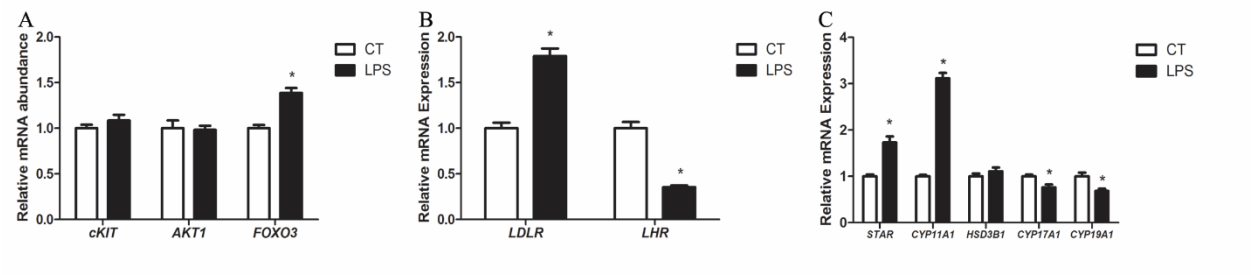

Figure 9. Acute LPS exposure affects mRNA abundance of genes involved in folliculogenesis and steroidogenesis. Total ovarian RNA was isolated from gilts who received a single injection of saline (CT) or LPS, and qRT-PCR performed to quantify levels of (A) *cKIT*, *AKT1*, *FOXO3;* (B) *LDLR*, *LHR*; and (C) *STAR*, *CYP11A1*, *HSD3B1*, *CYP17A1*, *CYP19A1*. * indicates $P < 0.05$.

To next decipher if increased ovarian LPS-induced activity is present during HS, we determined the level of the TLR4 protein during HS and discovered that HS increases ovarian TLR4 in a dramatic manner (Figure 10).

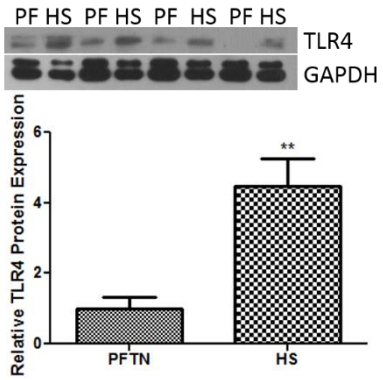

Figure 10. HS increases ovarian TLR4 protein. Total ovarian protein from thermal neutral (PF) or HS gilts was separated via SDS-PAGE and western blotting to detect TLR4 protein abundance performed. Quantification revealed HS increases the ovarian receptor for LPS, TLR4. ** indicates $P < 0.05$.

Discussion:

In totality, HS represents a bigger economic threat to the sustainability of the US pork industry that infections disease, with seasonal infertility in females representing a component of the negative biological impacts of HS. Unfortunately, climate change models predict that hotter, humid summers will become the norm rather than the exception. For these reasons, understanding biological changes that could contribute to poor reproductive performance in heat-stressed swine is a critical need. Until this understanding is gained, development of mitigation strategies to improve fertility during HS will be limited.

Our previous published work, funded by the IPPA, provided us a framework from which to interrogate the hypothesis in this study, that heat-induced elevated insulin and lipopolysaccharide (LPS) are causative factors in seasonal infertility. We previously demonstrated that ovarian pathways involved in regulation of follicle growth and maturation towards ovulation as well as steroid hormone synthesis (17β-estradiol and progesterone) were impacted in ways that could negatively alter ovarian function. We had

demonstrated that the mRNA encoding the *INSR, IRS1*, AKT1 and FOXO3 were increased by HS (23), however, the current study discovered that during HS in the presence of hyperinsulinemia, that these HS affected genes were unaltered. These data are interesting and raise questions about what role hyperinsulinemia is playing in this experimental paradigm. It may be that insulin has a greater impact on some genes than others, therefore, this is mechanistically informative in terms of porcine ovarian function regulation. In contrast to these genes, we did demonstrate in this study that genes responsible for producing proteins that convert cholesterol to 17β-estradiol were increased by HS with accompanying hyperinsulinemia. These data are in agreement with our previous findings with HS alone and provide mechanistic data since exogenous insulin did not abrogate the impact of HS on steroidogenic gene expression. It is important to note that this pathway produces 17β-estradiol during the follicular phase, but synthesizes progesterone during the luteal phase. We also determined some very novel findings with respect to 17β-estradiol metabolism. During the normal estrous cycle, steroid hormone degradation and removal is required so that 17β-estradiol wanes, while progesterone subsequently increases and then both are reduced to continue the cyclical nature of the estrous cycle. We investigated if HS altered 17β-estradiol metabolism and discovered that the enzyme that sulfonates 17β-estradiol, SULT1E1, to make it recognizable for transport from the ovary is increased by HS. In addition, we determined that the sulfonated-17β-estradiol transporter, ABCC1, is also increased by HS. These are innovative findings may offer new targets for mitigation strategies in swine. Some future questions are which phase of the estrous cycle has greater sensitivity to HS. If the follicular phase is affected, then ovulation and estrus display would be the phenotypic endpoint. If the luteal phase is sensitive to HS, then pregnancy maintenance effects would be an observed phenotype. It also may be a combination of both phases, thereby HS would have an additive effect on the estrous cycle, and understanding the changes during each phase will be critical to developing different strategies based upon the phenotypic observations to mitigate season infertility.

The novel previous findings from our group (10, 11) that LPS is increased in heat-stressed swine led us to investigate the impacts of LPS on ovarian function. There is a body of information on the effects of LPS in rodent and bovine species (18, 25, 29) and the phenotypic observations of LPS exposure include spontaneous abortion, primordial follicle loss and reduced progesterone synthesis during the luteal phase of the estrous cycle. These are similar phenotypes observed during seasonal infertility in swine. It should also be noted that any infection or stress that alters intestinal integrity in swine (porcine epidemic diarrhea, off feed events as examples) would result in increased LPS leaking from the intestine into systemic circulation, thus these investigations are not limited to understanding the effects of HS on fertility in female pigs. We found that the ovary was sensitive to a bolus of LPS administered 8 h prior. The classic LPS receptor, TLR4, was increased in LPS exposed gilt ovaries. pAKT, the proxy marker of phosphatidyinositol-3 kinase signaling, a pathway with critical functions in reproduction was increased by LPS exposure, indicating that LPS could be altering ovarian physiology in a variety of manners that would negatively impact fertility We discovered that LPS increased mRNA encoding *FOXO3*, *LDLR*, *STAR* and *CYP11A1* but decreased *LHR*, *CYP17A* and *CYP19A1* mRNA. The data of LPS-induced increased *LDLR* and *STAR* mRNA and pAKT protein, are in agreement with our findings in the pre-pubertal heat-stressed gilt ovary, however the decrease in *LHR*, *CYP17A* and *CYP19A1* oppose our

previous findings in HS gilts. We also did not note any change to IRS1 protein, meaning that insulin was unlikely to be driving the ovarian changes observed in these gilts, and increased TLR4 indicates that LPS signaling within the ovary is predominating in this experimental system. It may be that LPS can activate expression of a subset of genes within the steroidogenic pathway (*FOXO3*, *LDLR*, *STAR* and *CYP11A1*) while insulin may positively influence another subset (*LHR*, *CYP17A* and *CYP19A1*). It is important to note that this LPS paradigm was an acute high level exposure, thus future studies to mimic chronic low level LPS exposure during HS are warranted.

Taken together, these data add mechanistic information to our understanding of the biological impacts of HS that could contribute to seasonal infertility. The data gleaned from completion of both objectives add significantly to our biological understanding of the causes of seasonal infertility in swine and have opened new area(s) of research by our group to decipher these causes with the ultimate goal to develop mitigation strategies to prevent seasonal infertility due to HS.

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