RESEARCH REPORT
ANIMAL SCIENCE

Title: Investigating Biological Reasons for Seasonal Infertility **– NPB #12-115** revised

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Industry summary:

The US swine industry suffers major economic losses to heat stress during the summer months. Reasons for this fiscal hardship include seasonal infertility and poor sow performance during and immediately following the hot parts of the year. It is likely the negative effects of heat stress will become more severe if climate change continues as some predict and most models forecast more extreme summer conditions in US pig-producing areas. In addition, genetic selection based upon rapid muscle growth is thought to increase pig sensitivity to heat stress. A thorough understanding of the biological reasons contributing to seasonal infertility is necessary in order to develop future mitigating strategies. Heat-stressed pigs have unexplainably increased circulating insulin levels and we believe this detrimentally influences ovarian components necessary for successful reproduction. **Our central hypothesis for this project was that heat-induced elevated insulin impairs ovulation, steroidogenesis, oocyte recruitment and oocyte health and is a contributing mechanism to seasonal infertility**. Our objectives were to 1) measure LH protein levels in the pituitary gland on gilts that had been exposed to thermal-neutral (20ºC) or thermal stress (35ºC) conditions for a period of 35 days; 2) Determine differences in follicle number in the ovaries from gilts exposed to thermal-neutral (20ºC) or thermal stress (35ºC) conditions for a period of 35 days; 3) Quantify mRNA and protein of those genes that are involved in LH-regulated steroid production in the ovary, specifically estradiol.

We have discovered that HS alters a pathway that controls the dynamics of follicular growth activation, and oocyte viability, both of which could result in the ovary essentially becoming barren for an extended period of time – as seen in the lagging effects of HS on reproduction in swine. Also, we have found that the pathway that regulates how ovarian steroid hormones (Estrogen (17β-estradiol; E2) and Progesterone) are produced is altered by HS, and we believe that this could result in decreased E2 synthesis, which would alter ovulation and estrus behavior. Altered progesterone levels could result in lack of pregnancy maintenance, a phenotype of HS swine. In addition, we have generated data to indicate that the ovary may alter the mechanisms by which energy is utilized during HS, both as a consequence of reduced feed intake as well as due to thermal stress directly. Identification of these HS-induced ovarian changes has set the stage for further investigations to lead to mitigation interventions to minimize seasonal infertility in swine.

Producer Take Home messages:

- HS alters a pathway in the ovary that regulates when oocyte (egg)-containing follicles are activated to grow and ovulate.

- HS affects the mechanisms by which the ovary produces key female hormones required for ovulation and maintenance of pregnancy.

- The ovary may switch its energy substrate utilization during HS.

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Keywords:

Ovary, hyperthermia, estrogen, follicle, oocyte

Scientific Abstract:

Effect of heat stress on phosphatidylinositol-3 kinase and steroidogenic signaling in gilt ovaries

Heat stress (HS) negatively affects reproductive performance in swine, but the biological reasons responsible for this impaired fecundity are poorly understood. Paradoxically HS decreases feed intake but unexplainably increases plasma insulin in a variety of animal models including pigs. Insulin can influence ovarian phosphatidylinositol-3 kinase (PI3K) signaling, which is important for follicle viability and regulating follicle activation and steroidogenesis. Two downstream mediators of PI3K action are Protein kinase B subunit 1 (*AKT1*), and the forkhead transcription factor subunit 3a (*FOXO3*). This study investigated the effects of HS on PI3K and steroidogenic signaling in the porcine ovary. In addition, pilot data on the impact of HS on ovarian glucose metabolism were collected through use of a PCR array. Crossbred gilts (35±4 kg) housed in constant climate controlled rooms in individual pens with *ad libitum* feed intake were exposed to thermal neutral (TN) conditions (20°C; 35-50% humidity; n = 3-6) or HS conditions (35°C; 20-35% humidity; n = 3-6) for 7 or 35 d to simulate acute and chronic HS, respectively. Additionally, a pair fed (PF) group were included in the 7d treatment. Gilts were euthanized, one ovary was stored at -80°C and the other ovary was fixed in 4% paraformaldehyde. Total RNA was isolated and levels of *IR*, *IRS1*, *AKT1*, *FOXO3, LDLR*, *LHR*, *STAR*, and *CYP19a* mRNA were quantified by RT-PCR. Also, a PCR array to measure glucose metabolism was performed on the 7d TN, PF and HS samples. The IR and phosphorylated AKT (pAKT) proteins were localized by immunofluorescence staining. Western blotting was used to quantify the impact of HS on pIRS1, pAKT, STAR, and CYP19a protein levels. After 7 d of HS, increased ($P < 0.05$) levels of *IR*, *IRS1*, *AKT1*, *LDLR*, *LHR*, and *CYP19a* mRNA relative to TN and PF gilt ovaries. After 35d, all genes measured were increased ($P < 0.05$) by HS. Oocyte cytoplasm and cytoplasmic membrane of all stage follicles stained positive for the IR protein, while pAKT1 protein was located in the oocyte cytoplasm of all stage follicles, with apparent greater expression in larger stage follicles. Additionally, theca and granulosa cells of pre-ovulatory follicles were positive for pAKT protein. Western blotting revealed that IR, pIRS1, pAKT1, STAR and CYP19a were increased ($P < 0.05$) by HS after 7 or 35d. These data suggest HS leads to altered expression of PI3K signaling pathway members, which could alter dynamics of follicle activation and affect follicle viability. Additionally, thermal stress acts as an endocrine disrupting environmental exposure. These findings have identified altered ovarian signaling that could be at least partially responsible for negative impacts of HS on fertility in swine.

Data from this project were presented at the American Society of Animal Science annual meeting in Phoenix. Nteeba, J., Ullerich, E.E., Pearce, S.C., Boddicker, R., Ross, J.W., Baumgard, L.H., and Keating, A.F. 2012. Effect of heat stress on phosphatidylinositol-3 kinase signaling in gilt ovaries.

Introduction:

Annual economic losses to global animal agriculture due to thermal (heat) stress surpass billions of dollars. In the United States, summer-induced decreased production is well-documented in every aspect of animal agriculture. Nationally, the swine industry is estimated to lose at least $113 million for sows and $202 million for growing-finishing swine, resulting in reduced income of over $300 million annually due to heat stress (1). This heat-induced economic burden is due to poor sow performance, increased morbidity, mortality, suboptimal growth, inefficient nutrient utilization, decreased carcass value and carcass processing problems (1). The fiscal losses occur despite recent advances in cooling systems, barn management, and 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 heat stress 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 heat stress (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 (Figure 1), 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 heat stress (9). We hypothesized that heat-induced elevated insulin may be responsible for seasonal infertility and poor sow performance during the summer months.

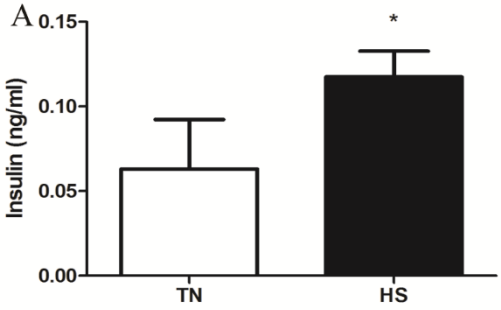


Figure 1 (left). Effect of heat stress (HS) on circulating insulin levels in gilts. Gilts (~90 days of age) were maintained in thermal neutral conditions (control; 20°C) or heat stressed (HS; 35°C) for 7 days. * indicates difference between treatments; $P < 0.05$.

A physiological link between abnormally high insulin levels and impaired reproduction is observed in polycystic ovarian syndrome (PCOS) in humans. PCOS females demonstrate high levels of circulating insulin and hyper-secretion of luteinizing hormone (LH) from the pituitary gland (10-12). High LH levels increase 17β-estradiol (E2), decrease oocyte maturation and fertilization, impair embryo quality, increase embryo loss and decrease fecundity (13). Additionally, both insulin and LH regulate ovarian E2 synthesis, thus altered insulin and/or LH levels will have profound effects on E2 concentrations. Dominant follicles produce E2 (14), which negatively feeds back to reduce follicle recruitment from the primordial pool. Therefore, we hypothesized that increased E2 (in response to elevated LH and/or insulin levels) reduces the supply of growing follicles (the follicles that will be ovulated during future cycles) in the heat-stressed pig ovary.

Another critically important pathway for both maintaining the primordial follicle pool (the ovarian reserve) and ovarian E2 production is the phosphatidyinositol-3 kinase (PI3K) pathway. Proper PI3K signaling maintains the ovarian reserve of primordial follicles and regulates the rate at which these resting follicles become activated/recruited into the growing pool (15-17) (so they can be ovulated and fertilized in the future). The major downstream signaling component of PI3K is the serine-threonine protein kinase AKT1. AKT1 knock-out mice have impaired fertility, which is primarily explained by the destruction of the ovarian primordial follicle reserve (18).

Project objective:

Investigation of heat stress-induced elevated insulin levels on pituitary gland luteinizing hormone (LH) secretion and consequences for LH-regulated ovarian function.

Materials & Methods:

Animals: Crossbred gilts (35±4 kg) housed in constant climate controlled rooms in individual pens with *ad libitum* feed intake were exposed to thermal neutral (TN) conditions (20°C; 35-50% humidity; n = 3-6) or HS conditions (35°C; 20-35% humidity; n = 3-6) for 7 or 35 d to simulate acute and chronic HS, respectively. In addition, in the day 7 time point, a pair fed (PF) group was included that received the same amount of food as the HS gilts. Gilts were euthanized, one ovary was stored at -80°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°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°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°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.

Immunofluorescence staining and microscopy analysis: Ovaries were fixed in 4% buffered formalin for 2 h, transferred to 70% ethanol, embedded in paraffin, serially sectioned, and every 6th section mounted. Sections were deparaffinized (approximately 10 sections/ovary) and incubated with primary antibody at 4°C overnight. Secondary biotinylated antibody will be applied for 1 h, followed by CY-5-streptavidin (1 h; 1:100 dilution). Sections were treated with Ribonuclease A (100μg/ml) for 1 h, followed by staining with Hoechst. Slides were repeatedly rinsed with phosphate buffer saline (PBS), cover-slipped, and stored in the dark (4°C) until visualization. Immunofluorescence was visualized on a Leica DMI300B fluorescent microscope at λ = 461 and 633 nm for Hoechst (green) and CY-5 (red), respectively. All images were captured using a 40 X objective lens. Multiple readings were taken throughout the sections. Analysis was performed using ImageJ software to measure staining intensity for the parameter of interest in 10 follicles of each stage, comparing control to treated ovaries (n = 3-5 ovaries per treatment).

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:

Three experiments were proposed in this project and the results from each are described below:

Experiment 1.1: Measure LH protein levels in the pituitary gland on gilts that had been exposed to thermal-neutral (20ºC) or thermal stress (35ºC) conditions for a period of 35 days.

Tissues for this project were donated to us from a larger HS project at ISU. We experienced considerable problems with the pituitary gland – in some cases pituitary samples were obtained and in others not. This was likely due to the method of euthanization (captive bolt). Since we did not have a complete set of samples to work with, we decided to determine the ovarian capability to respond to LH (Experiment 1.2). In addition, we utilized the resources allocated to this work to study the effect of HS on porcine energy utilization in the ovary, since this is an area in which some therapeutic manipulation may be possible. These data are presented in Experiment 1.4 (below).

Experiment 1.2: Determine differences in follicle number in the ovaries from gilts exposed to thermal-neutral (20ºC) or thermal stress (35ºC) conditions for a period of 35 days.

Our original approach was to count follicles in ovaries obtained from TN and HS gilts. We discovered that there was considerable variation in the proximity to puberty entry within the gilts from which we had tissue, thus we took a mechanistic approach to determine alterations to the pathways that govern primordial follicle activation.

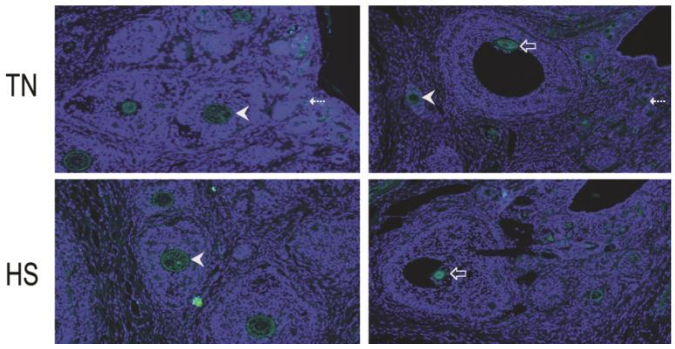


Figure 2 (above). Effects of 7 days of HS (35°C) on IR protein levels in ovaries from pre-pubertal gilts relative to gilts in thermo-neutral conditions receiving pair-feeding to the HS conditions (TN; 20°C). Thin arrow indicates small primary or primordial follicles; arrowhead indicates oocyte in secondary follicle, block arrow indicates oocyte in antral follicle. Green staining indicates IR protein; Blue staining represents DNA.

The ovary possesses both insulin and insulin-like growth factor 1 (IGF-1) receptors. Insulin not only controls central bioenergetic functions associated with carbohydrate and lipid metabolism but also plays a crucial role in reproductive function. Insulin receptor (IR) is a heterodimer comprised of two alpha and two beta subunits (19, 20). Insulin binds to the alpha subunits which activates the IR tyrosine kinase in the beta subunits, with subsequent auto-phosphorylation and recruitment of different substrate adaptors such as the IR substrate (IRS 1-4) family of proteins. Tyrosine phosphorylated IRS then display binding sites for numerous signal transduction partners including the PI3K/Protein kinase B (PKB/AKT) signaling pathway. Defects in IRS, more specifically IRS1 and IRS2, have been implicated in female infertility and rodent models with dysfunctional IRS1 and IRS2 causes both ovarian and hypothalamic dysfunction (21-23).

In order to confirm that the IR is present in the porcine ovary, we performed immunofluorescence staining to detect the IR (green). It is evident that the oocyte is highly enriched in IR protein (Figure 2), and thus is likely to be acutely responsive to changing blood insulin levels.

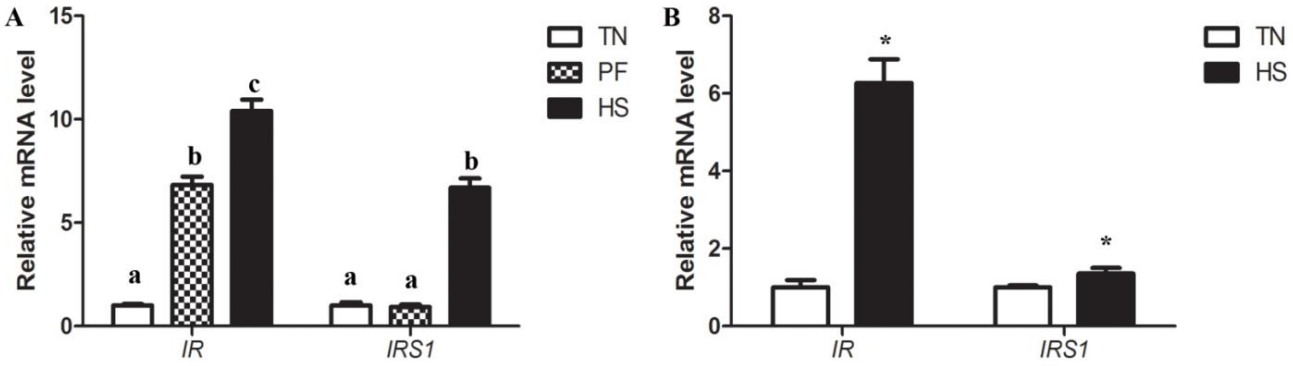


Figure 3 (above). Effects of HS (35°C) after (A) 7 days, or (B) 35 days on mRNA encoding the *IR* and *IRS1* in ovaries from pre-pubertal gilts relative to gilts in thermo-neutral conditions receiving pair-feeding to the HS conditions (TN; 20°C). (A) different letters indicate statistical difference; $P < 0.05$. (B) * indicates $P < 0.05$, different from TN.

Additionally, ovarian mRNA encoding the *IR* is increased during heat stress (Figure 3) after 7 days and this increase is maintained until day 35 of heat stress. Also, we have demonstrated that *IRS1* is increased due to heat stress, and is at a greater level after 7 days of heat stress than 35 days. Coupling our increased circulating insulin levels with our mechanistic data we demonstrate increased IR signaling capabilities, through IRS1 mRNA increase and increased phosphorylation of the Tyrosine 632 residue on the IRS1 protein (Figure 4) supporting that the ovary not only maintains insulin sensitivity but that the signaling pathway activated by insulin binding to the IR is also increased during HS.

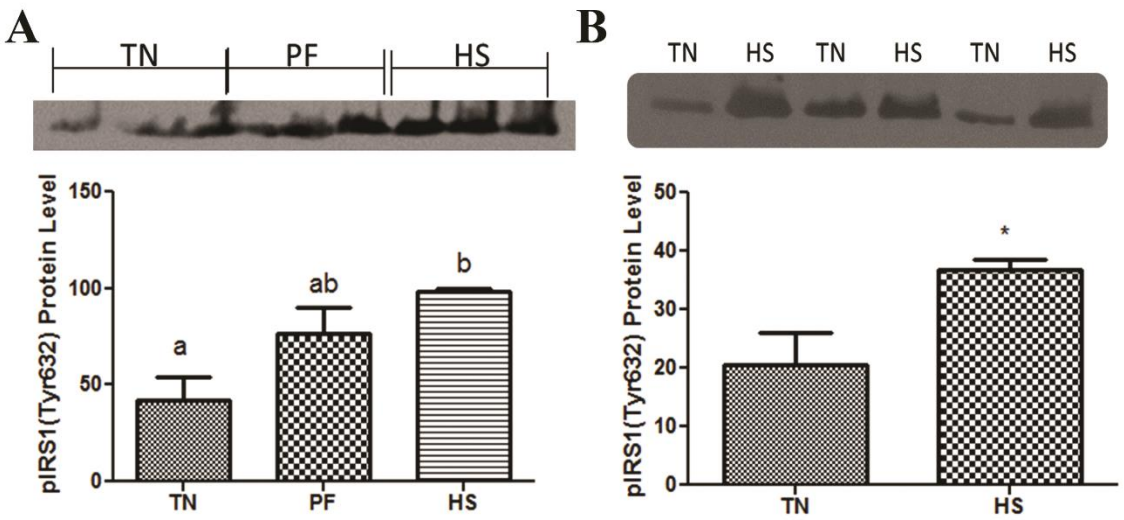


Figure 4 . Impact of HS (35°C) on pIRS^{Tyr632} after (A) 7 days, or (B) 35 days in ovaries from pre-pubertal gilts relative to gilts in thermo-neutral conditions receiving pair-feeding to the HS conditions (TN; 20°C). (A) different letters indicate statistical difference; $P < 0.05$. (B) * indicates $P < 0.05$, different from TN.

To determine if the downstream pathways mediated by insulin's binding to the IR are altered during heat stress, we investigated mRNA encoding genes involved in PI3K signaling (Figure 5). Heat stress increased the expression of *AKT1* and *FOXO3*. Interestingly, it is noteworthy that during PF, *FOXO3* is reduced, thus the impact of less energy intake during HS did not impact *FOXO3* as expected. After 35d of HS, (Figure 4B) both AKT and FOXO3 were increased.

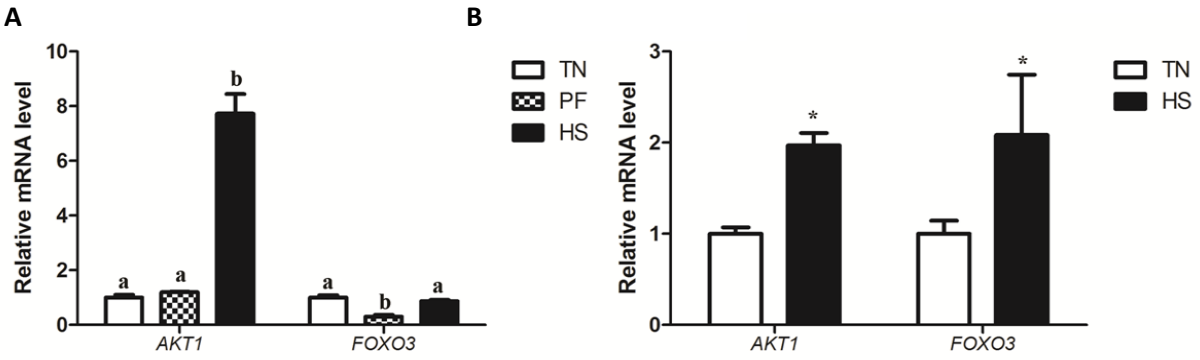


Figure 5 . Effects of HS (35°C) after (A) 7 days, or (B) 35 days on mRNA encoding genes in the PI3K pathway (*AKT1*, *FOXO3*) in ovaries from pre-pubertal gilts relative to gilts in thermo-neutral conditions receiving pair-feeding to the HS conditions (TN; 20°C). (A) different letters indicate statistical difference; $P < 0.05$. (B) * indicates $P < 0.05$, different from TN.

We sought to determine the localization of AKT protein in the porcine ovary and have found that the major PI3K mediator, AKT1, (27-31) shows a similar localization pattern to the IR, and is highly expressed in the porcine oocyte, in addition to being localized to the theca and granulosa cells (Figure 6A,B). Western blotting to quantify the impact of heat stress on phosphorylated and activated AKT1 (pAKT1) protein demonstrates that ovarian pAKT1 is increased during heat stress (Figure 6C).

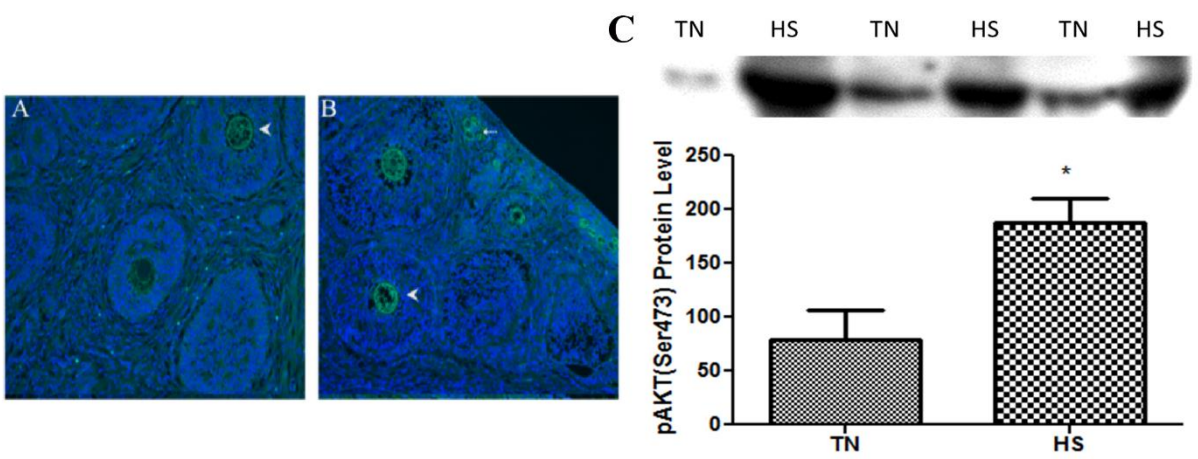


Figure 6 . Effect of HS (35°C) on pAKT1 protein (A,B) localization (after 7 days) and (C) level (after 35 days) in ovaries from pre-pubertal gilts relative to gilts in thermo-neutral conditions receiving pair-feeding to the HS conditions (TN; 20°C). Thin arrow indicates small primary or primordial follicles; arrowhead indicates oocyte in secondary follicle. Green staining indicates pAKT1 protein; Blue staining represents DNA.

Increased FOXO3 is associated with primordial follicle activation being halted, which could negatively impact reproduction. In addition, FOXO3 increases cell death by apoptosis, which is important in the ovary since 99% of oocytes are eliminated through pro-apoptotic pathways, likely as a mechanism to ensure ovulation of oocytes that have the greatest reproductive potential. Thus, apoptosis prevention is not necessarily good for the ovary and alterations to this process may result in imperfect oocytes being ovulated, further contributing to negative reproductive outcomes. These data also indicate that heat stress alters the ovarian mechanisms that regulate oocyte quality and viability - both of which are negative events for reproductive success.

Experiment 1.3: Quantify mRNA and protein of those genes that are involved in LH-regulated steroid production in the ovary, specifically E2.

Circulating E2 is required for ovulation and uterine growth. In the pig, E2 is also necessary for estrus display and maternal recognition of pregnancy, thus, any alteration to E2 production could severely compromise fertility. Progesterone is required for implantation along with establishment and maintenance of pregnancy. In the pig, progesterone production by the corpus luteum is required for the entire pregnancy, rather than by the placenta for the final two-thirds of pregnancy as in the human.

Since PI3K signaling was impacted by HS, and it is known that PI3K regulates steroidogenic pathways in the ovary, to produce E2, our first approach was to measure mRNA of the genes encoding the LDL receptor (*LDLR*), the LH receptor (*LHR*), *STAR* (the enzyme involved in cholesterol transport into the steroidogenic pathway) and *CYP19a* (aromatase) the enzyme involved in the final stage of E2 synthesis. LDLR, LHR, and CYP19A were increased after 7d of HS (Figure 7A). Interestingly, STAR was not impacted by 7d of HS at

the mRNA level. However, after 35d (Figure 7B), all members of the steroidogenic pathway measured were increased, pointing towards an endocrine disrupting effect of HS in the ovary.

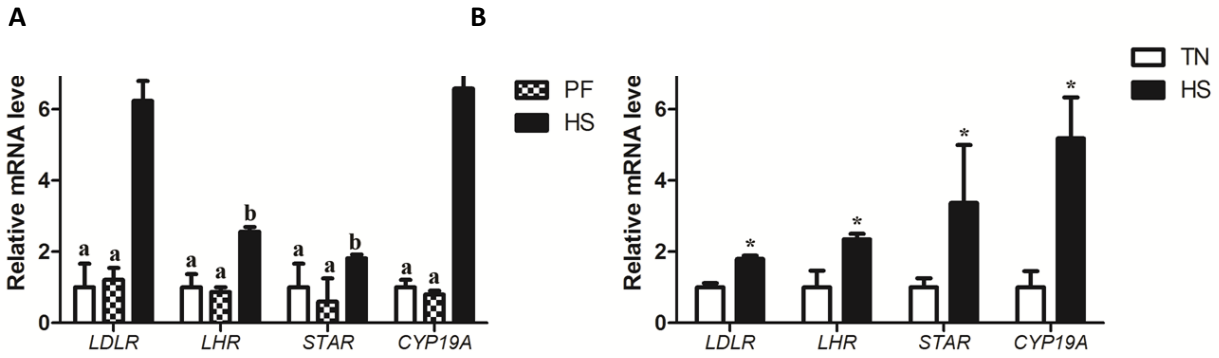
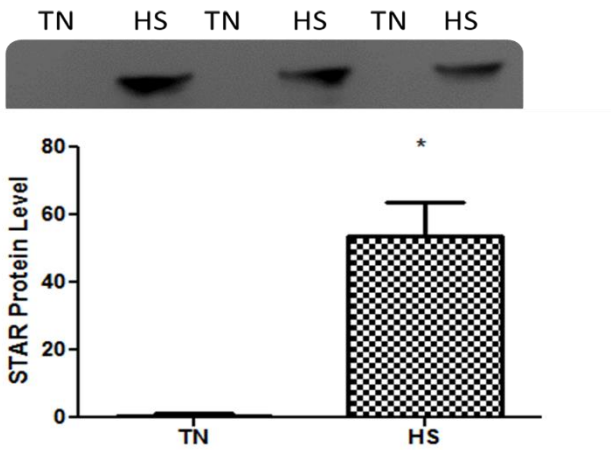


Figure 7 (above). Effects of HS (35°C) after (A) 7 days, or (B) 35 days on mRNA encoding genes in the PI3K pathway (*AKT1*, *FOXO3*) and steroidogenesis (*LDLR*, *LHR*, *STAR*, *CYP19A*) in ovaries from pre-pubertal gilts relative to gilts in thermo-neutral conditions receiving pair-feeding to the HS conditions (TN; 20°C). (A) different letters indicate statistical difference; $P < 0.05$. (B) * indicates $P < 0.05$, different from TN.



Since the increase in *STAR* and *CYP19a* mRNA were unexpected, we quantified protein levels of these two critically important enzymes during HS. STAR protein was increased compared to the TN gilts. The seeming lack of STAR was not surprising in the TN gilts, since they are pre-pubertal, however HS increased STAR tremendously (Figure 8).

Figure 8 (left). Effects of HS (35°C) after 35 days on STAR protein in ovaries from pre-pubertal gilts relative to gilts in thermo-neutral conditions receiving pair-feeding to the HS conditions (TN; 20°C); * indicates $P < 0.05$, different from TN.

Our Western blotting data also confirmed that porcine ovarian CYP19a protein was elevated by heat stress after 7d (Figure 9A) and 35 d (Figure 9B) of HS. Since CYP19a catalyzes the conversion of testosterone to estradiol, heat stress potentially alters the amount of circulating E2, which would compromise fertility.

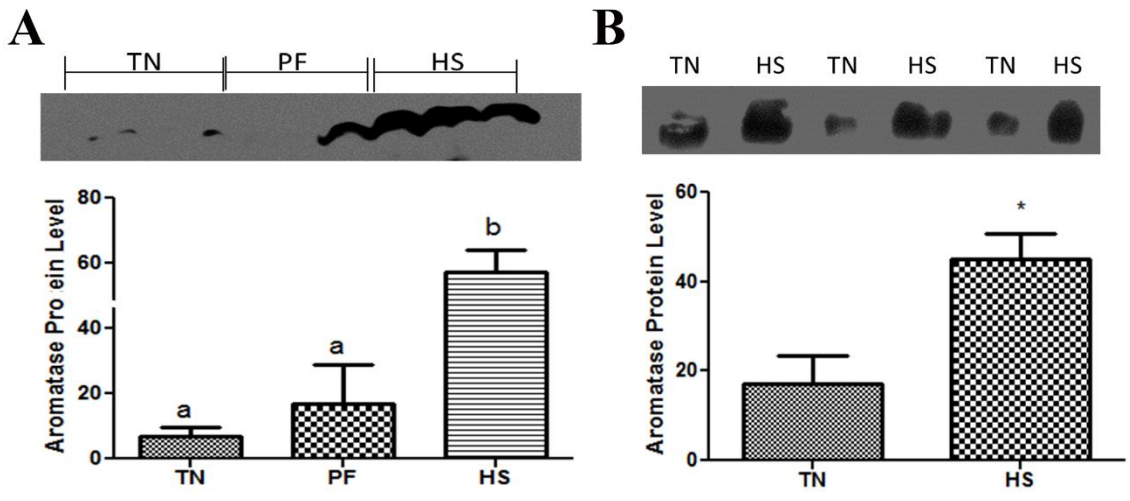


Figure 9 . Impact of HS (35°C) on CYP19a after (A) 7 days, or (B) 35 days in ovaries from pre-pubertal gilts relative to gilts in thermo-neutral conditions receiving pair-feeding to the HS conditions (TN; 20°C). (A) different letters indicate statistical difference; $P < 0.05$. (B) * indicates $P < 0.05$, different from TN.

These data could mean that E2 is increased since the pathway that produces E2 is increased. However, we hypothesize that E2 is likely decreased and that the increase in the production pathway is an attempt by the ovary to increase this critical reproductive hormone.

Experiment 1.4. Quantification of HS impact on ovarian energy utilization gene expression.

In light of the reduced feed intake by gilts during HS, we hypothesized that the ovary may undergo internal stress from lack of sufficient energy reserves. We investigated the impact of HS on glucose metabolism using PCR arrays. We used the 7 day time point, since we had the PF animals in this group. We found that both PF and HS increased genes involved in gluconeogenesis (Figure 10A). The genes involved in glycolysis showed a general trend of reduction by PF (ALDOB, ALDOC, HK2, HK3; Figure 10B). HS however, increased ALDOB, ALDOC, and GPI, demonstrating that thermal stress itself may impact energy requirements by the ovary. While glycolysis was increased by HS, the pentose phosphate pathway was reduced (Figure 10C). The genes in the TCA cycle were regulated in the same manner by PF and HS, with the exception of ACO1 (Figure 10D). In the regulation of glucose metabolism gene group, HS increased PDK2 and PDPR compared to TN and PF (Figure 10E). These data are not useful only in the context of HS, but also provide valuable information regarding the ovarian response to nutrient deprivation.

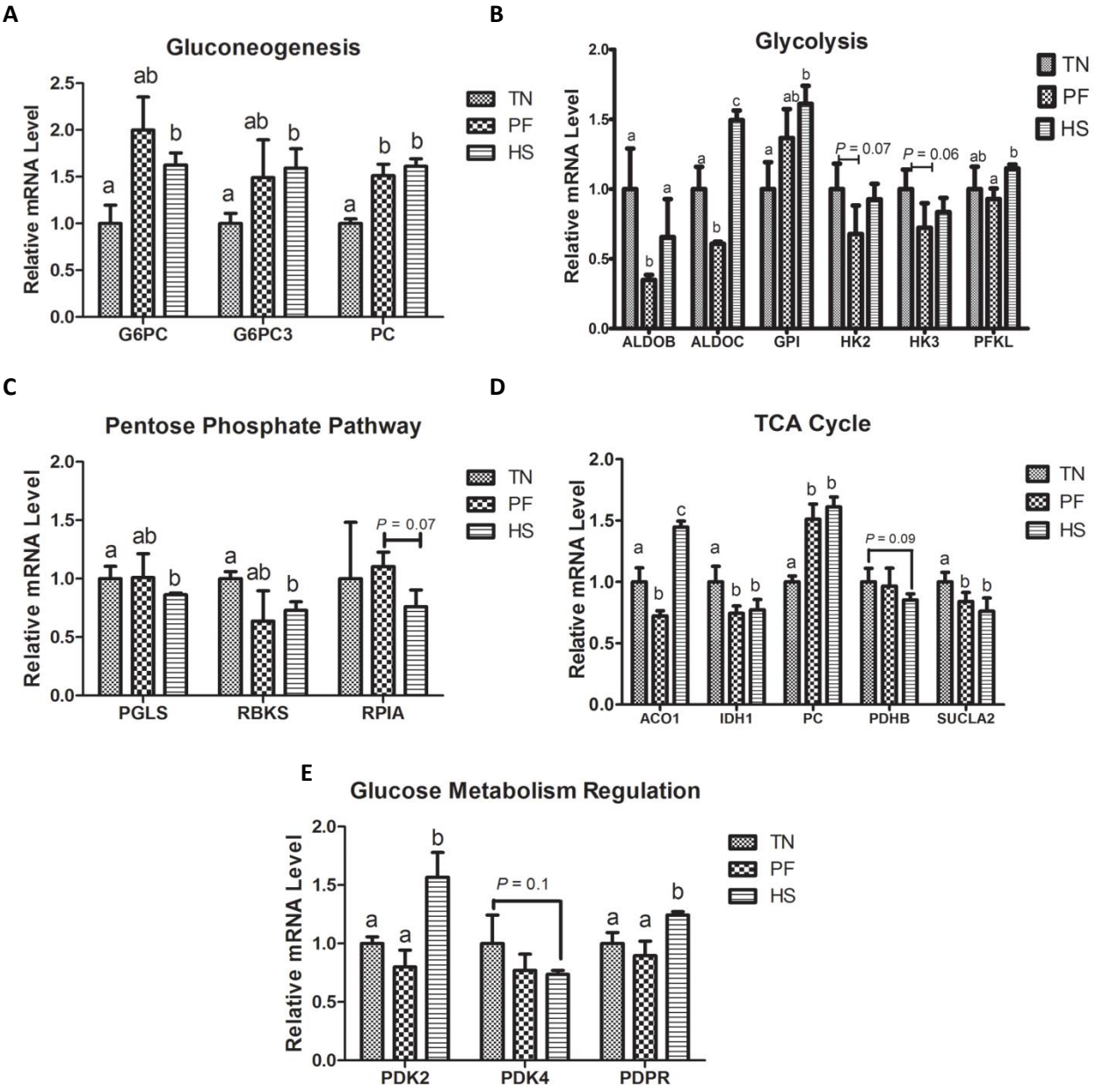


Figure 10 (above). Effects of HS (35°C) after 7 day on mRNA encoding genes involved in glucose metabolism: (A) Gluconeogenesis, (B) Glycolysis, (C) Pentose Phosphate Pathway, (D) TCA cycle and (E) Glucose metabolism regulation in ovaries from pre-pubertal gilts relative to gilts in thermo-neutral conditions receiving pair-feeding to the HS conditions (TN; 20°C). (A) different letters indicate statistical difference; $P < 0.05$.

Discussion:

In summary, our data funded by the IPPA has discovered the following: 1) the IR is localized to the porcine oocyte; 2) genes encoding the IR and its substrate (IRS1) are increased by HS, relative to PF and TN gilts; 3) HS activates PI3K signaling as evidenced by increased pIrs^{Tyr632} and pAKT1; 4) HS increased mRNA and protein for a number of genes involved in E2 production; and 5) we have data to suggest that ovarian energy utilization is altered by reduced feed intake and during HS.

These data support that the ovary not only maintains insulin sensitivity but that the signaling pathway activated by insulin binding to the IR is also increased during HS. Insulin has a variety of potential actions

within the ovary, and we concentrated on the impact that increased insulin and elevated insulin signaling had on the PI3K pathway and the steroidogenic pathway.

Several lines of evidence have identified the importance of PI3K signaling in ovarian function (24). PI3K regulates proliferation and differentiation of granulosa cells (GC) in response to gonadotropins (25, 26). Once PI3K is activated, AKT is recruited to the cell membrane where it becomes phosphorylated (pAKT) and regulates follicular activation (27, 28), recruitment and survival (27-31). In rodent models, *Akt*$^{-/-}$ mice have reduced primordial follicle viability (18). AKT phosphorylates and inactivates several targets including forkhead transcription factors (FOXO). FOXO3 is critical for early stages of follicular growth (32) and it has been reported to trigger apoptosis through either up-regulation of genes necessary for cell death or down-regulation of anti-apoptotic genes (33-36). *Foxo3*$^{-/-}$ mice had global primordial follicle activation by postnatal day 14, leading to oocyte death, early depletion of functional ovarian follicles and secondary infertility (32) while in FOXO3^{OE} mice, activation of the primordial follicle pool was prevented (29).

We found increase activation of AKT, which would point to an ovarian attempt to increase oocyte viability, and also could increase steroid production by the dominant follicles in the gilt. Increased FOXO3 is associated with primordial follicle activation being halted, which could negatively impact reproduction and result in a "barren" ovary since lack of growing follicles toward ovulation would result. In addition, FOXO3 increases cell death by apoptosis, which is important in the ovary since 99% of oocytes are eliminated through pro-apoptotic pathways, likely as a mechanism to ensure ovulation of oocytes that have the greatest reproductive potential. Thus, apoptosis prevention is not necessarily good for the ovary and alterations to this process may result in imperfect oocytes being ovulated, further contributing to negative reproductive outcomes. These data indicate that HS alters the ovarian mechanisms that regulate oocyte quality, viability and growth activation - all of which are negative events for reproductive success.

When we examined the steroid production pathway in the ovary, we found increased levels of LHR and CYP19a after 7 days and that this was maintained to 35 days of HS. The increased LHR indicates that there is either increased circulating LH, or that the ovary needs to up-regulate the amount of receptors to ensure ovulation. Since CYP19a catalyzes the conversion of testosterone to estradiol, HS potentially alters the amount of circulating E2, which could compromise fertility and estrus demonstration (heat behavior) in gilts.

Surprisingly, STAR mRNA was increased after 35d of HS. STAR is the rate-limiting step in steroid production by the ovary, since it uptakes the steroid hormone precursor, cholesterol, into the cell for conversion to E2. Even more interesting was that in TN gilts, STAR protein was absent, unsurprising since these gilts have not undergone puberty. However, STAR protein was present at a high level in the HS gilts. These results could indicate that HS is driving gilts towards puberty at a quicker rate, or simply be a feedback response to a reduction in E2 synthesis within the ovary. Overall, our data could mean that E2 is increased since the pathway that produces E2 is increased. However, we hypothesize that E2 is likely decreased and that the increase in the production pathway is an attempt by the ovary to increase this critical reproductive hormone. In addition, progesterone production could be impacted by HS, and this would result in spontaneous abortion as is a phenotype in HS pigs. Thus, our results indicate that HS can be thought of as an endocrine disrupting environmental exposure that can negatively affect the reproductive status in the pig in a number of ways.

Our data on the energy utilization/production genes within the ovary open up new avenues of investigation to understand why the reproductive capacity of HS animals is reduced. These results are not

useful only in the context of HS, but also provide valuable information regarding the ovarian response to nutrient deprivation (PF).

To summarize, HS affects pathways involved in maintaining follicle dynamics and viability, increases genes involved in the steroid production pathway, and is an endocrine disrupting exposure to the pig. Identification of these changes provides a first step in understanding seasonal infertility in the U.S. swine herd. Until the mechanisms that govern these negative impacts seen during hot summer months and into the fall season are determined, development of strategies to alter these consequences will remain limited. Of future benefit to producers is that our results identify new areas to pursue to unravel the mechanisms that result in costly economic impacts on the Iowan and U.S. swine herd.

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