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REPRODUCTION

Heat stress during the luteal phase decreases luteal size but does not affect circulating progesterone in gilts[1]

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Abstract

Heat stress (**HS**) occurs when heat dissipation mechanisms are insufficient to maintain euthermia, and it is associated with seasonal infertility (**SI**), which manifests as smaller litters, longer wean-to-estrus interval, increased abortions, and reduced conception rates. To understand HS-induced mechanisms underlying SI, crossbred post-pubertal gilts (167 ± 10 kg; $n = 14$) experienced either thermal neutral (**TN**, 20 ± 1 °C, $n = 7$) or cyclical HS (35 ± 1 °C for 12 h and 31.6 °C for 12 h, $n = 7$) conditions from 2 to 12 d post-estrus (**dpe**). Estrous cycles were synchronized via altrenogest administration for 14 d, phenotypic manifestation of estrus was observed and gilts were assigned to experimental treatment. Gilts were limit fed 2.7 kg daily with ad libitum water access. Blood was collected at 0, 4, 8, and 12 dpe via jugular venipuncture and animals were humanely euthanized at 12 dpe. The corpora lutea (**CL**) width were measured via digital calipers on both ovaries, and CL from one ovary were excised, weighed, and protein and steroid abundance analyzed via western blotting and ELISA, respectively. Relative to TN, HS increased ($P < 0.01$) rectal temperature and respiration rates and reduced ($P < 0.01$) feed intake. The CL from HS ovaries were reduced in diameter ($P < 0.05$) and weight ($P < 0.01$) relative to those from TN animals. No difference ($P = 0.38$) in CL or serum progesterone concentrations between groups was observed at any time point, though at 12 dpe the serum progesterone:CL weight was increased ($P < 0.10$) by HS. No treatment differences ($P = 0.84$) in circulating insulin were observed. Luteal protein abundance of steroid acute regulatory protein, 3 beta-hydroxysteroid, or prostaglandin F2α receptor were not different between treatments ($P = 0.73$). Taken together, these data demonstrate that the CL mass is HS sensitive, but this phenotype does not appear to be explained by the metrics evaluated herein. Regardless, HS-induced decreased CL size may have important implications to pig SI and warrants additional attention.

Key words: heat stress, ovary, progesterone

Introduction

Heat stress (**HS**) occurs when environmental conditions disable euthermia maintenance. Lack of functional sweat glands (Ingram, 1967) and a thick subcutaneous adipose layer that compromises radiant heat dissipation sensitizes pigs to HS. Genetic selection for fecundity and lean tissue accretion has also increased metabolic heat production (Brown-Brandl et al., 2001; Ross et al., 2015). Seasonal infertility (**SI**), associated with HS, manifests phenotypically as smaller litters (Omtvedt et al., 1971), increased abortions (Love, 1978; Bertoldo et al., 2009), delayed puberty onset (Paterson et al., 1991), reduced conception

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rate (Omtvedt et al., 1971), and longer wean-to-estrus interval (Prunier et al., 1996). Annually, HS costs the U.S. swine industry ~$900 million (Ross et al., 2015).

Heat-stressed pigs reduce heat production by decreasing feed intake (Baumgard and Rhoads, 2013) and dissipating heat by skin vasodilation (Lambert et al., 2002). Blood flow reprioritization compromises intestinal barrier integrity leading to luminal contents [including lipopolysaccharide (**LPS**)] infiltrating portal and systemic circulation (Hall et al., 2001; Pearce et al., 2013a). Despite hypophagia, HS increases basal and stimulated circulating insulin, suggesting that LPS acts as an insulin secretagogue (Baumgard and Rhoads, 2013). Prostaglandin F2α (**$PGF_{2α}$**) causes corpus luteum (**CL**) regression and LPS induces temporally inappropriate $PGF_{2α}$ production (Moore et al., 1991; Hockett et al., 2000). Time to luteinization, CL formation, and sufficient progesterone (**P_4**) production is lengthened by LPS (Suzuki et al., 2001; Lavon et al., 2011) and reduces CL size (Herzog et al., 2012). The CL is necessary for pregnancy maintenance for the entirety of gestation in sows; thus, LPS could be a key player in reproductive dysfunction during HS. Our discoveries of HS-induced hyperinsulinemia and endotoxemia provided the rationale to investigate the hypothesis that HS negatively affects luteal function in post-pubertal gilts.

Methods

Materials

Triton X-100 (T-6878), HEPES (H3537), glycerol (G5516), NaF (S7920), EDTA (E7889), SDS (L3771), beta mercaptoethanol (M3148), Trizma base (T1501), sodium chloride (S3014), trichloroacetic acid (T6399), 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), bovine serum albumin (**BSA**; BP1605-100), dNTP (18427-013) were obtained from ThermoFisher Scientific (Rockford, IL). Laemmli buffer (161-0737), Precision Plus Protein Kaleidoscope (161-0375), and 4% to 20% Mini-PROTEAN TGX Precast Protein Gels (4561096) were obtained from Bio-Rad Laboratories (Hercules, CA). Matrix (altrenogest) was obtained from Merck Animal Health (Madison, NJ). Signal fire-enhanced chemiluminescent (**ECL**) reagent (6883) and antibodies directed against anti-mouse IgG, HRP-linked (7076), and anti-rabbit IgG-HRP (7074) were obtained from Cell Signaling Technology (Danvers, MA). Antibodies directed against steroid acute regulatory protein (**STAR**); NBP1-33485), prostaglandin F2α receptor (**PTGFR**; NLS1049) and 3 beta-hydroxysteroid (**HSD3B**; NB110-78644) were obtained from Novus Biologicals (Littleton, CO). Porcine insulin ELISA (10-1200-01) was obtained from Mercodia (Uppsala, Sweden). Progesterone ELISA (EIA-1561) was obtained from DRG International (Springfield, NJ).

Animals

Fourteen crossbred post-pubertal gilts (167 ± 10 kg) were utilized for this experiment. Pigs were fed a diet that meets NRC requirements and were limit fed 2.7 kg at 0600 h daily throughout the trial. Animals were individually housed at the Iowa State University Swine Nutrition Farm. All animal procedures were reviewed and approved by the Iowa State University Institutional Animal Care and Use Committee (approved IACUC no. 12-16-8400-S).

Gilts were synchronized in estrus using altrenogest (Matrix) for 14 d and animals acclimated during this period to their surroundings with individual access to feed and water in thermal neutral (**TN**) conditions (20 ± 1 °C, 36% to 57% relative humidity). Beginning 4 d post-altrenogest withdrawal, they were checked for behavioral signs of standing estrus twice daily using boar exposure. Characteristics of estrus such as a swollen vulva, discharge, and reduced feed intake were noted; however, an animal was classified as in estrus when she would stand for back pressure. At first detection of estrus, animals were assigned to 1 of 2 treatments: TN (n = 7) or HS (n = 7). The time of estrus detection was assigned as 0 d post-estrus (**dpe**). TN and HS treatments began at 2 dpe at 1900 h, and the HS exposure occurred in a different room. To induce HS, pigs were exposed to cyclical HS over a 24-h period with 12 h at 35 ± 1 °C, 21% to 31% relative humidity and 31.6 ± 1 °C, 21% to 31% relative humidity for 12 h in a diurnal pattern. The control pigs remained in TN conditions (20 ± 1 °C, 36% to 57% relative humidity). Rectal temperatures were measured at 3 morning (700, 800, and 900 h) and 3 afternoon (1700, 1800, 1900 h) time intervals (6 measurements per pig per day) via a digital thermometer (Welch Allyn, Skaneateles Falls, NY). Respiration rates were collected at these identical times. Feed disappearance was measured at 0600 and 1500 h daily. Body weights were obtained at 2 and 12 dpe. Pigs were individually housed but were in the same room depending on the temperature to which they were being exposed (n = 7 in TN room; n = 7 in HS room).

Serum Collection

Blood samples were collected via jugular venipuncture at 0, 4, 8, and 12 dpe. The 10-mL blood sample was collected into a vacuum tube, placed on ice, transported to the laboratory, and allowed to clot at room temperature for 15 min before processing. Serum samples were harvested via centrifugation at 4 °C for 15 min at 1,500 × g and stored at −20 °C until further analysis.

Tissue Collection

Animals were humanely euthanized on 12 dpe utilizing captive bolt penetration followed by exsanguination. Ovaries were removed and the number of CL on each ovary was counted and the diameter of each CL measured via digital calipers. Corpora lutea were excised, individually weighed, and snap frozen in liquid nitrogen.

Table 1. Antibody dilutions used for protein detection

Protein[1]	Primary antibody	Primary dilution	Secondary antibody	Secondary dilution
STAR	Rabbit anti-STAR	1:1,000	Goat anti-rabbit IgG-HRP linked	1:1,000
HSD3B	Mouse anti-HSD3B	1:1,000	Horse anti-mouse IgG-HRP linked	1:1,000
PTGFR	Rabbit anti-PTGFR	1:500	Goat anti-rabbit IgG-HRP linked	1:500

[1]STAR = steroidogenic acute regulatory protein; HSD3B = 3 beta-hydroxysteroid dehydrogenase; PTGFR = prostaglandin F2 alpha receptor.

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Corpus Luteum Progesterone Extraction

From each animal, 2 to 4 CL, to represent 25% of luteal tissue per animal, were powdered with a mortar and pestle. Approximately 100 mg of powdered tissue per animal was weighed into a new tube, 1 mL of 5% trichloroacetic acid added and homogenized (Blitek et al., 2016). These tubes were centrifuged at 10,621 × *g* at 4 °C for 5 min to clarify the supernatant, which was then removed and placed into a new tube. The extractions were stored at −80 °C until further analysis.

Progesterone Quantification

Concentrations of serum and CL P_4 were obtained using a colorimetric competitive binding ELISA specific to P_4, according to manufacturer's protocol. Serum collected at 0, 4, and 8 dpe was run undiluted. Serum collected at 12 dpe was diluted 1:3, and CL extractions were diluted 1:100 to bring P4 concentrations into the detectible range of the assay as per manufacturer's protocol. This approach has been used previously in quantification of porcine P_4 (Rak-Mardyla et al., 2014; Rak et al., 2015), and the concentrations were at an estrous stage-appropriate level (Anderson, 2009).

Protein Isolation and Western Blotting

Powdered CL 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), 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 5 µg/µL in sterile water with 1× Laemmli buffer. Protein samples were incubated at 95 °C for 5 min and were separated on a Mini-PROTEAN TGX 4% to 20% precast gel followed by transfer to a nitrocellulose membrane utilizing the iBlot 2 Dry Transfer System (Protocol 0: 20 V for 1 min, 23 V for 4 min, and 25 V for 2 min). 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 to 3 h at room temperature with gentle rocking action. Membranes were then incubated with primary antibodies (Table 1) overnight at 4 °C with rocking, then were washed 3 times in PBST for 10 min each. Membranes were incubated with the appropriate secondary antibody (Table 1) for 1 h at room temperature with rocking, followed by 3 washes of 10 min each in PBST. Membranes were incubated with ECL reagent for 3 min before imaging in a ChemiImager 5500 (Alpha Innotech, San Leandro, CA) with AlphaEaseFC software (v3.03 Alpha Innotech). Appropriate-sized bands were identified and densitometric analysis of the appropriate band performed using ImageJ

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Table 2. Impact of heat stress during luteal phase on body weight, circulating insulin, and CL number[1]

		TN	HS	SEM	P-value Trt	P-value Day	P-value Trt × day
Body weight, kg	d2	167.20	166.77	3.12	0.71	<0.001	0.14
	d12	173.54	170.77	3.12			
Serum insulin, µg/L	d0	0.094	0.091	0.023	0.84	0.78	0.84
	d12	0.096	0.106	0.023			
CL number, avg		17.71	17.71	0.99	0.99		

[1]TN = thermal neutral; HS = heat stress, Trt = treatment, CL = corpus luteum, avg = average.

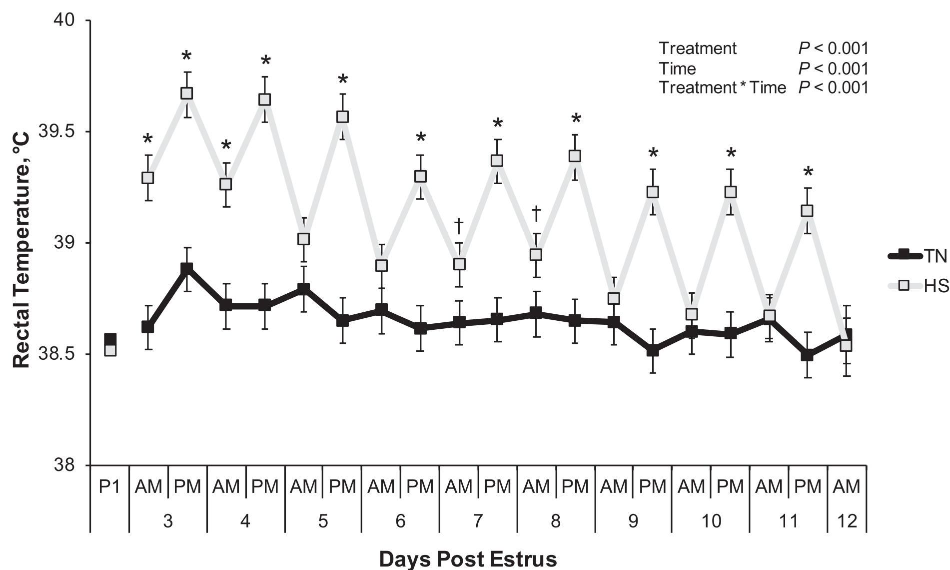

Figure 1. Impact of chronic heat stress on rectal temperature. Post-pubertal gilts were exposed to diurnal heat stress (35 ± 1 °C for 12 h/31.6 °C for 12 h, 21% to 31% relative humidity; HS) or thermal neutral (20 ± 1 °C, 36% to 57% relative humidity; TN) conditions from 3 to 12 dpe. Rectal temperature was recorded 3 times each morning and evening. Line graph represents average morning (AM) and evening (PM) rectal temperature per treatment ± SEM. There was an increase ($P < 0.001$) in average rectal temperature in the HS treatment compared to TN. *Difference between treatments at individual time points ($P < 0.05$); †$P < 0.1$.

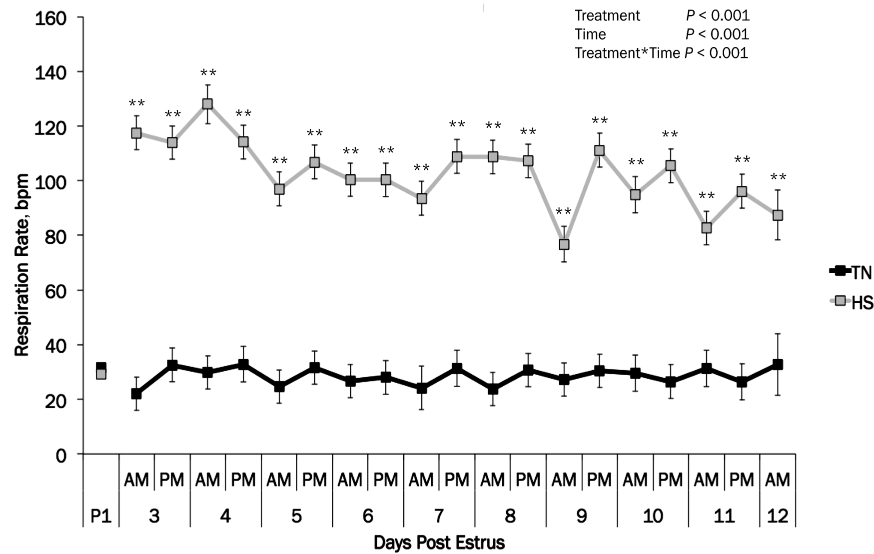

Figure 2. Impact of chronic heat stress on respiration rate. Post-pubertal gilts were exposed to diurnal heat stress (35 ± 1 °C for 12 h/31.6 °C for 12 h, 21% to 31% relative humidity; HS) or thermal neutral (20 ± 1 °C, 36% to 57% relative humidity; TN) conditions from 3 to 12 dpe. Respiration rate was recorded 3 times each morning and evening. Line graph represents average morning (AM) and evening (PM) respiration rate per treatment ± SEM. There was an increase ($P < 0.001$) in average respiration rate in the HS treatment compared to TN. **Difference between treatments at individual time points ($P < 0.001$).

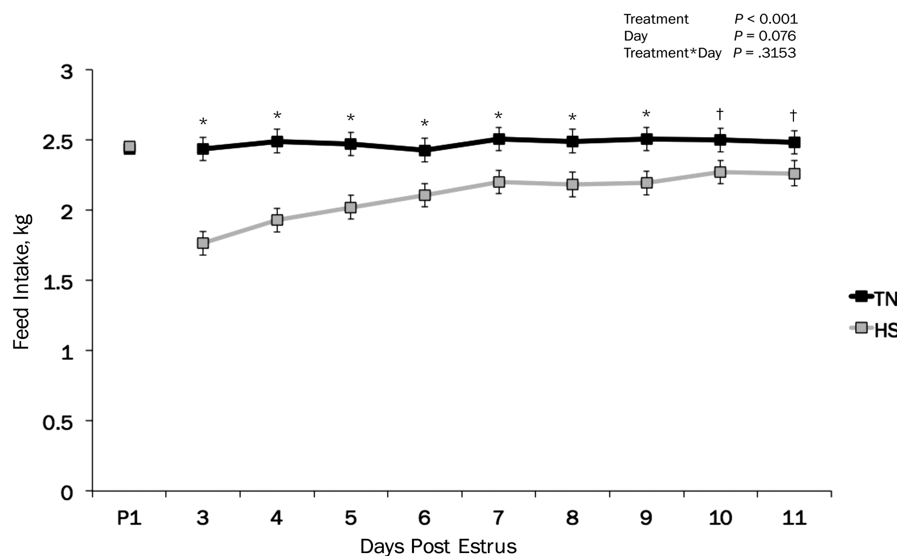

Figure 3. Impact of chronic heat stress on feed intake. Post-pubertal gilts were exposed to diurnal heat stress (35 ± 1 °C for 12 h/31.6 °C for 12 h, 21% to 31% relative humidity; HS) or thermal neutral (20 ± 1 °C, 36% to 57% relative humidity; TN) conditions from 3 to 12 dpe. Animals were limit fed 2.7 kg daily at 0600 h. Feed disappearance was recorded daily. Line graph represents feed disappearance each day in each group ± SEM. *Difference between treatments at individual time points ($P < 0.05$); †$P < 0.1$.

software (NCBI). Densitometric analysis of Ponceau S staining was performed measuring the entire protein stain in each lane to confirm equal protein loading and for normalization of specific proteins of interest. Technical controls included primary antibody only, secondary antibody only, and primary antibody species-specific IgG with secondary antibody, to demonstrate antibody specificity, for all antibodies.

Statistics

Rectal temperature, feed intake, respiration rate, serum P_4, and serum insulin were analyzed in SAS (Cary, NC) utilizing a PROC MIXED analysis procedure. Fixed effects were treatment, day, treatment × day, and replicate. Day was utilized as a repeated measure. The synchronization/acclimation period values were used as a covariate for rectal temperature, feed intake, and respiration rate. Corpora lutea number was analyzed using the PROC GLIMMIX of SAS with a Poisson adjustment.

Corpora lutea weight, CL diameter, and protein abundance were analyzed using unpaired *t*-tests in GraphPad Prism. Statistical significance was set at $P < 0.05$, with a tendency for difference set at $P < 0.10$. †$P < 0.10$, *$P < 0.05$, **$P < 0.001$, and ***$P < 0.0001$.

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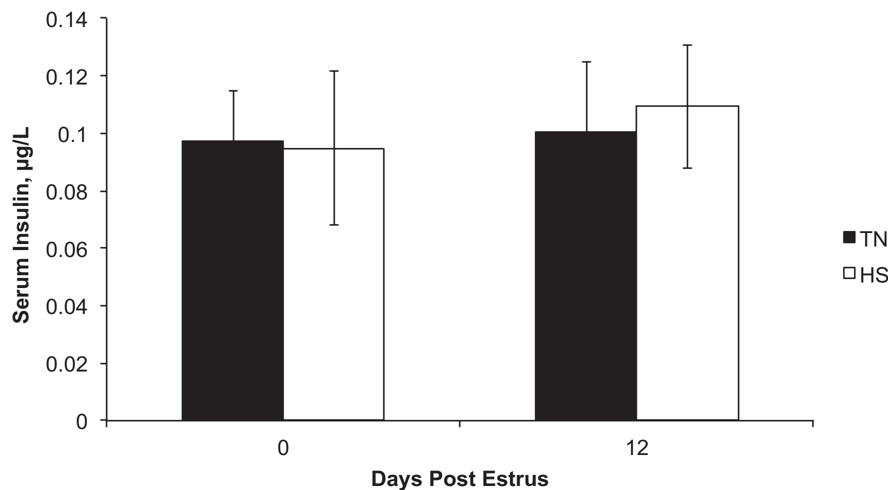

Figure 4. Impact of chronic heat stress on serum insulin concentrations. Post-pubertal gilts were exposed to diurnal heat stress (35 ± 1 °C for 12 h/31.6 °C for 12 h, 21% to 31% relative humidity; HS) or thermal neutral (20 ± 1 °C, 36% to 57% relative humidity; TN) conditions from 3 to 12 dpe. A fasting blood sample was collected on 0 and 12 dpe and analyzed for insulin concentrations via colorimetric ELISA. Bar graph represents the average serum insulin concentration in each group ± SEM. $P > 0.05$ between groups at each time point.

Results

Impact of Chronic Heat Stress on Rectal Temperature and Respiration Rate

Gilts were successfully heat stressed as evidenced by increased ($P < 0.01$) rectal temperatures in HS compared with TN animals (Fig. 1). Rectal temperatures were reduced in a diurnal pattern and did not differ at night from the TN gilts from day 9 of experimental treatment onwards, indicating that gilts were employing physiological mechanisms in response to HS with time (Fig. 1). Heat-stressed gilts also had elevated ($P < 0.01$) respiration rates (101 bpm) compared with TN counterparts (29 bpm) which, in contrast to rectal temperature, did not normalize to TN status at any point in the experimental time frame (Fig. 2).

Effect of Heat Stress on Feed Intake and Circulating Insulin Concentration

Overall feed disappearance was reduced (15%, $P < 0.01$) in HS gilts compared with TN controls, but the decrease was most pronounced immediately following HS and it progressively increased with time (Fig. 3). There were no treatment differences in serum insulin concentrations between groups ($P > 0.05$; Table 2 and Fig. 4).

Influence of Heat Stress on Corpus Luteum Diameter and Weight

There was no difference in the number of CL between treatments (Table 2). No treatment differences were observed in CL diameter between treatment groups ($P = 0.45$, Fig. 5A); however, the average CL weight was decreased (15%; $P = 0.07$, Fig. 5B) in HS gilts.

Effect of Heat Stress on Circulating or CL Progesterone Levels

Serum P_4 levels did not differ ($P = 0.16$) on 0, 4, 8, or 12 dpe between HS and TN animals (Fig. 6A). In addition, CL progesterone content was unchanged ($P = 0.48$) in HS gilts compared with TN gilts (Fig. 6B). However, when total circulating serum P_4 was calculated on a total CL weight basis, there was a tendency for increased ($P = 0.07$) P_4 in the CLs from the HS gilts, implying that the smaller CL are producing a greater relative amount of P_4 in HS animals (Fig. 7).

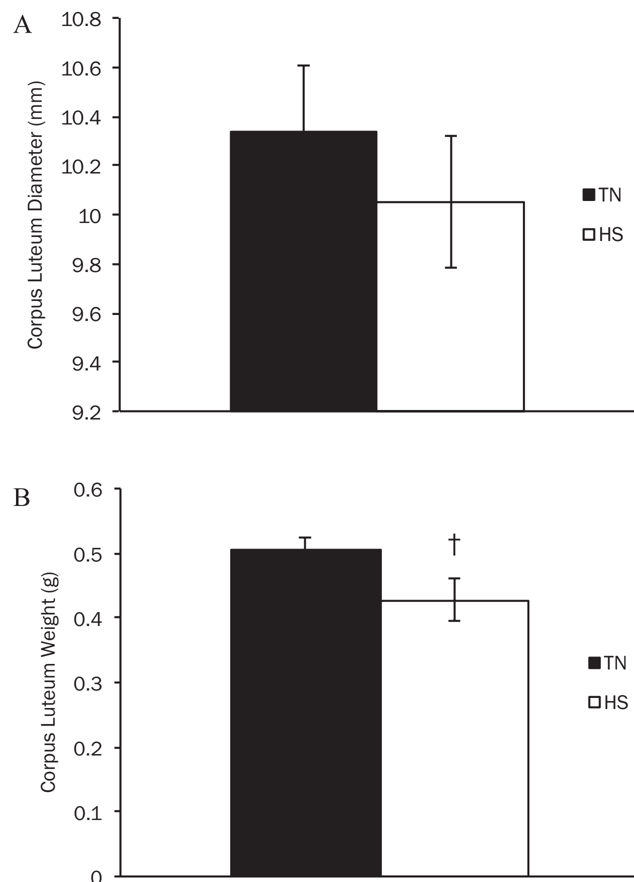

Figure 5. Impact of chronic heat stress on corpus luteum diameter and weight. Post-pubertal gilts were exposed to diurnal heat stress conditions from 3 to 12 dpe. After euthanasia, ovaries were removed and the diameter of each corpus luteum measured using digital calipers. Bar charts represent the average (A) diameter and (B) weight in each group ± SEM. †$P < 0.1$.

Impact of Chronic Heat Stress on Luteal STAR, HSD3B, and PTGFR protein

There was no observable effect ($P = 0.73$) of HS on luteal STAR protein abundance in gilts relative to TN controls (Fig. 8A). Luteal

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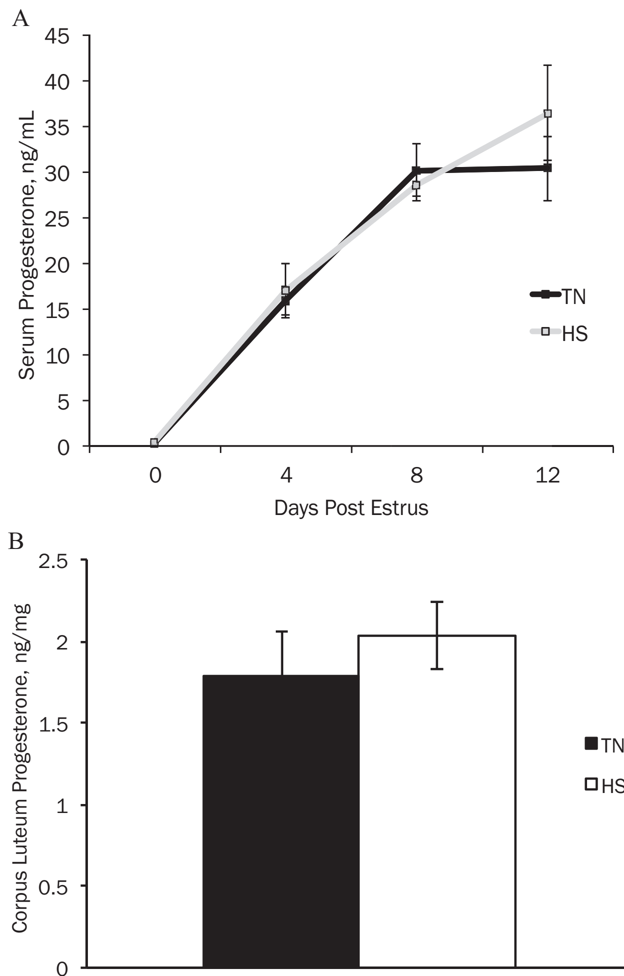

Figure 6. Impact of chronic heat stress on serum and luteal progesterone concentrations. Post-pubertal gilts were exposed to diurnal heat stress (35 ± 1 °C for 12 h/31.6 °C for 12 h, 21% to 31% relative humidity; HS) or thermal neutral (20 ± 1 °C, 36% to 57% relative humidity; TN) conditions from 3 to 12 dpe. (A) Blood was collected on 0, 4, 8, and 12 dpe and analyzed for progesterone concentrations via colorimetric ELISA. Line graph represents the average serum progesterone concentration in each group ± SEM. P_4 levels were different between time points, but within each time point no differences were noted, $P > 0.05$. (B) Corpora lutea from each animal were powdered and homogenized in 5% trichloroacetic acid and diluted samples run on a colorimetric ELISA to quantify P_4 content. Bar graph represents the average luteal P_4 concentration per milligram of tissue in each group ± SEM; $P = 0.48$.

HSD3B protein abundance was comparable ($P = 0.90$) between HS and TN gilts (Fig. 8B), and there was no difference ($P = 0.94$) in PTGFR protein between TN and HS pigs (Fig. 9).

Discussion

Swine SI is characterized by increased abortions (Love, 1978), smaller litters (Omtvedt et al., 1971), delayed puberty (Paterson et al., 1991), reduced conception rates (Omtvedt et al., 1971), and longer wean-to-estrus intervals (Prunier et al., 1996). Heat stress is a major cause of SI and given that pigs lack functional sweat glands and the increased thermal load from genetic selection for valuable production traits (Ingram, 1967; Brown-Brandl et al., 2001; Bloemhof et al., 2008; Renaudeau et al., 2011) and SI is a major economic and animal welfare issue affecting pork production in the United States. To dissipate heat, pigs increase dermal vasodilation, leading to intestinal hypoxia and increased intestinal permeability (Hall et al., 1999; Lambert et al., 2002; Pearce et al., 2013b). This, in turn, allows for passage of LPS into circulation, which stimulates insulin secretion and induces inflammation (Hijiya et al., 2002; Pearce et al., 2013b). Reproduction is detrimentally affected by LPS (Bidne et al., 2018) and LPS triggers pre-term labor in many species (Koga and Mor, 2010). Indeed, intraperitoneal LPS injection is an established experimental tool for pre-term labor induction (Deb et al., 2004; Elovitz and Mrinalini, 2004; Agrawal et al., 2013). Considering the importance of P_4 for pregnancy maintenance, we hypothesized that HS could alter luteal function in exposed gilts, this contributing to SI. To interrogate the posit, gilts were subjected to TN or HS conditions for a total of 10 d beginning at 2 d post-estrus. Gilts were successfully heat stressed as evidenced by increased rectal temperature and respiration rate and decreased feed intake. Though there was decreased feed intake in HS animals, serum insulin concentrations were not different between the groups, implying that the HS gilts were hyperinsulinemic relative to the TN gilts since their insulin:feed intake was elevated, as has been documented in other porcine HS studies (Pearce et al., 2013b; Sanz-Fernandez et al., 2015). Whether this observation is dependent on pancreatic insulin production or hepatic insulin metabolism remains to be determined.

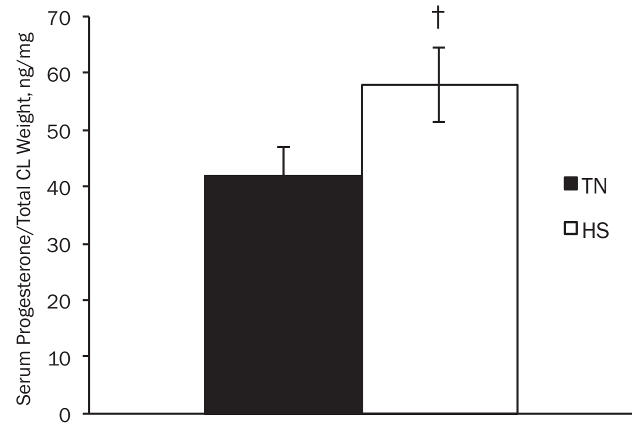

Figure 7. Impact of chronic heat stress on serum progesterone relative to total CL weight. Post-pubertal gilts were exposed to diurnal heat stress (35 ± 1 °C for 12 h/31.6 °C for 12 h, 21% to 31% relative humidity; HS) or thermal neutral (20 ± 1 °C, 36% to 57% relative humidity; TN) conditions from 3 to 12 dpe. Blood was collected on 0, 4, 8, and 12 dpe and analyzed for progesterone concentrations via colorimetric ELISA. After euthanasia, ovaries were removed and the diameter of each corpus luteum measured using digital calipers. Bar graph represents the average total serum progesterone concentration per total CL tissue weight in each group ± SEM; †$P < 0.1$.

Treatment onset was designed to avoid confounding effects of HS during ovulation, an event that typically occurs 38 to 42 h after the onset of estrus, or 60% of the way through sexual receptivity (Anderson, 2009). We do not anticipate that HS affected either ovulation or luteinization because, by experimental design, all gilts were in TN conditions during this phase. Timing of ovarian collection coincided with peak luteal P_4 production and before luteal regression occurs (Noguchi et al., 2010). The CL of HS pigs were reduced in weight compared to their TN counterparts. Although we did not observe differences between environmental treatment groups in either circulating P_4 or P_4 per microgram of luteal tissue, when considering that CL weight was reduced by HS, there was a tendency for the HS CL to produce more P_4 on a per weight basis than the TN animals. Lack of correlation between bovine CL volume and serum P_4 concentrations have been reported (Sartori et al., 2002; Sartori

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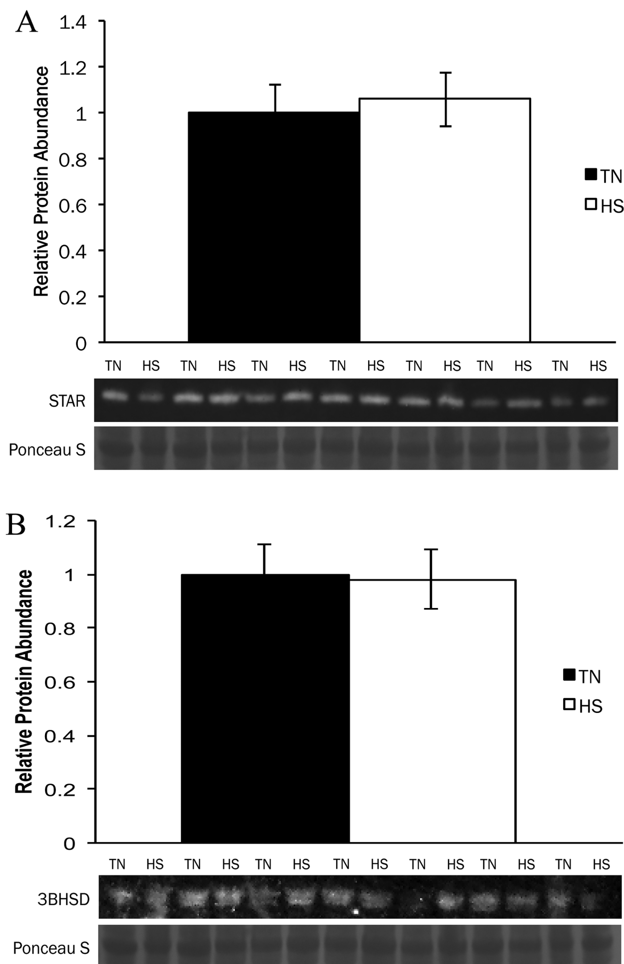

Figure 8. Impact of chronic heat stress on luteal STAR and HSD3B protein abundance. Post-pubertal gilts were exposed to diurnal heat stress (35 ± 1 °C for 12 h/31.6 °C for 12 h, 21% to 31% relative humidity; HS) or thermal neutral (20 ± 1 °C, 36% to 57% relative humidity; TN) conditions from 3 to 12 dpe. Bar charts represent relative luteal (A) STAR and (B) HSD3B protein abundance for TN and HS animals as densitometric mean ± SEM, $P > 0.05$. Representative western blots are presented for STAR and HSD3B proteins. Equal protein loading is confirmed by Ponceau S staining.

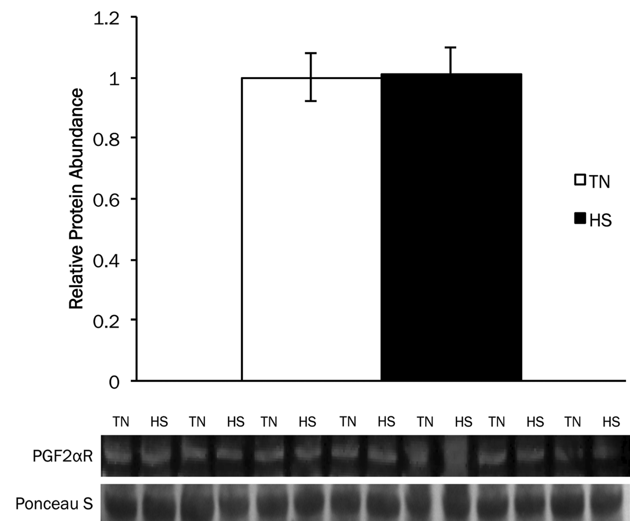

Figure 9. Impact of chronic heat stress on luteal PTGFR abundance. Post-pubertal gilts were exposed to diurnal heat stress (35 ± 1 °C for 12 h/31.6 °C for 12 h, 21% to 31% relative humidity; HS) or thermal neutral (20 ± 1 °C, 36% to 57% relative humidity; TN) conditions from 3 to 12 dpe. Bar chart represent relative luteal PTGFR protein abundance for TN and HS animals as densitometric mean ± SEM, $P = 0.94$. Representative western blots are presented for PTGFR protein. Equal protein loading is confirmed by Ponceau S staining.

et al., 2004; Lopez et al., 2005). Because the CL size and diameter were assessed at a single time point in the study, we cannot assess the rate of growth or the initiation of regression during the experimental period. We also cannot address whether CL P_4 production was increased or P_4 metabolism was reduced either by luteal tissue or hepatic metabolism. Additionally, whether there was a difference in the luteal cell fraction of the HS CL cannot be answered by this study. All of these possibilities present as avenues for further future exploration.

Our previous investigations into the ovarian impacts of HS on pre- and post-pubertal gilts have identified effects on steroidogenesis. In pre-pubertal gilts who experienced HS for 35 d, increased abundance of mRNA encoding LDLR, LHR, STAR, and CYP19A1 were observed (Nteeba et al., 2015). Additionally, both STAR and CYP19A1 protein abundance was increased, supporting potential endocrine disruption due to HS (Nteeba et al., 2015). An additional study in post-pubertal gilts exposed to HS only during the follicular phase of the estrous cycle discovered lack of an impact of HS on STAR or CYP19A1 protein abundance but did demonstrate altered levels of estrogen sulfotransferase 1E1 (**SULT1E1**; Dickson et al., 2018). Because SULT1E1 is involved in estrogen inactivation and metabolism (Polei et al., 2014), increased SULT1E1 due to HS suggested increased E2 turnover within the ovary (Dickson et al., 2018). We, therefore, determined any impact of HS on the steroidogenic enzymes within the CL but did not observe any effect of HS on STAR protein abundance in luteal tissue. This enzyme performs the rate-limiting step of conversion of cholesterol to P_4, namely, the transport of cholesterol into the inner mitochondrial matrix in the luteal cells (Stocco, 2001). Additionally, HS did not affect luteal abundance of HSD3B protein, which performs the final step in P_4 synthesis (Smith et al., 1994). Thus, there was no impact of HS on steroidogenic protein abundance per unit of luteal tissue. There could be altered P_4 metabolism due to HS within the CL that could affect P_4 levels and this is a consideration for our future studies.

Administration of $PGF_{2\alpha}$ to gilts can induce luteolysis, but only after day 12 of the estrous cycle (Guthrie and Polge, 1976), before this, the CL does not possess luteolytic capacity in normal physiological conditions (Guthrie and Polge, 1976; Diaz et al., 2000). However, Estill et al. (1993) have demonstrated that repeated administration of $PGF_{2\alpha}$ to gilts every 12 h from day 5 to day 10 of the estrous cycle reduced serum P_4 beginning on day 8.5 and shortened the cycle length by 6.5 d on average, which suggests that the CL can attain luteolytic capacity with repeated $PGF_{2\alpha}$ exposure. Heat stress increases production of proinflammatory cytokines, including tumor necrosis factor α (Chow et al., 1999). Culture of bovine luteal cells in vitro with TNFα increased media concentrations of $PGF_{2\alpha}$ in a dose-dependent manner (Townson and Pate, 1996), and short-term in vitro isolated perfusion culture of bovine ovaries with constant LPS administration increased $PGF_{2\alpha}$ media concentrations (Lüttgenau et al., 2016). Moreover, in vitro culture of porcine luteal tissue after in vivo administration of a $PGF_{2\alpha}$ analog indicated that luteal tissue itself produced $PGF_{2\alpha}$ (Diaz et al., 2000). There was no difference between TN and HS gilts in luteal PTGFR protein abundance; however, tissue collection occurred at a time point when PTGFR is expected to be present on the CL and the study design does not allow investigation of premature PTGFR expression due to environmental conditions.

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Taken together, these data support that HS during the luteal phase in post-pubertal gilts induces hyperinsulinemia, reduces CL weight, and increases the amount of P_4 in circulation per unit of luteal tissue. No evidence of altered steroidogenic enzyme abundance or elevated PTGFR was observed in the present study; however, we were successful in implementing HS only during the luteal phase, paving the way for further interrogations into HS-induced effects on this phase of the estrous cycle. Though we cannot speak to the temporal growth or regression of the CL in HS animals, we propose a mechanism by which HS may induce premature regression of the CL, potentially contributing to the reduced conception rate and increased spontaneous abortion that are phenotypic manifestations of SI during HS.

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