Heat stress alters ovarian insulin mediated phosphatidylinositol-3 kinase and steroidogenic signaling in gilt ovaries

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Running Title: Endocrine disrupting effects of hyperthermia
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Keywords:
Hyperthermia, Hyperinsulinemia, ovary, infertility

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Abstract:

Heat Stress (HS) compromises a variety of reproductive functions in several mammalian species. Inexplicably, HS animals are frequently hyperinsulinemic despite marked hyperthermia-induced hypophagia. Our objectives were to determine the effects of HS on insulin signaling and components essential to steroid biosynthesis in the pig ovary. Female pigs (35±4 kg) were exposed to constant thermal neutral (TN; 20°C; 35-50% humidity; n = 6) or HS conditions (35°C; 20-35% humidity; n = 6) for either 7 (n = 10) or 35 d (n = 12). After 7d, HS increased ($P < 0.05$) ovarian mRNA abundance of the insulin receptor (*INSR)*, insulin receptor substrate 1 (*IRS1*), protein kinase B subunit 1 (*AKT1*), low density lipoprotein receptor (*LDLR*), luteinizing hormone receptor (*LHCGR*), and aromatase (*CYP19a*). After 35d, HS increased *INSR*, *IRS1*, *AKT1*, *LDLR*, *LHCGR*, *CYP19a*, and steroidogenic acute regulatory protein (*STAR*) ovarian mRNA abundance. In addition, after 35d, HS increased ovarian phosphorylated IRS1 (pIRS1), phosphorylated AKT (pAKT), STAR and CYP19a protein abundance. Immunostaining analysis revealed similar localization of INSR and pAKT1 in the cytoplasmic membrane and oocyte cytoplasm, respectively, of all stage follicles, and in theca and granulosa cells. Collectively, these results demonstrate that HS alters ovarian insulin mediated-PI3K signaling pathway members which likely impacts follicle activation and viability. In summary, environmentally-induced HS is an endocrine disrupting exposure that modifies ovarian physiology and potentially compromises production of ovarian hormones essential for fertility and pregnancy maintenance.

Introduction:

Ambient temperatures that exceed an animals' thermoneutral zone can cause heat stress (HS) and result in detrimental effects on animal welfare and productivity [1]. In a number of livestock species HS is also associated with a variety of female reproductive issues including irregular estrous cyclicity [2-4], reduced conception rate [5, 6], reduced embryonic survival [7], and higher frequency of stillbirths [6, 7]. From an agricultural perspective, HS-induced sub-optimal productivity and reproductive performance limits the production of high quality protein for human consumption. Consequently, HS jeopardizes global food security, and this is especially true for developing countries [8].

Several studies have demonstrated that heat-stressed animals have elevated circulating insulin concentrations [8-11] and an increased insulin response to a glucose tolerance test [8]. Energetically, this is paradoxical as HS causes a well-conserved decrease in nutrient intake and is generally considered a hypercatabolic condition [8]. The biological explanation for elevated blood insulin (a potent anabolic signal) during HS have not yet fully been determined, but likely include insulin's key role in activating and up-regulating heat shock proteins [12]. Though traditionally known for regulating blood glucose homeostasis, insulin is a pleiotropic hormone, and it also plays a pivotal role in normal follicle development [13, 14] potentially through its ability to stimulate ovarian steroidogenesis [15]. In insulin responsive cells, insulin binds to its extracellular receptor (IR) resulting in auto phosphorylation and recruitment of the insulin receptor substrate (IRS) proteins [13, 16, 17], which in turn regulate numerous downstream insulin-mediated signaling events, including the phosphatidylinositol-3 kinase (PI3K) pathway [18, 19]. In humans, both obesity and polycystic ovary syndrome (PCOS) are associated with

impaired reproduction, and hyperinsulinemia, hyperandrogenism along with aberrant secretion of luteinizing hormone (LH) [20-23] are associated metabolic perturbations. Elevated circulating LH is thought to increase 17β-estradiol (E2), decrease oocyte maturation and fertilization, impair embryo quality, increase embryo loss and decrease overall fecundity [21, 24]. In addition to follicle stimulating hormone (FSH; [25-27]) and LH, insulin contributes to regulation of ovarian E2 synthesis, thus altered insulin action could profoundly affect the biological impact of E2 and consequently reduce follicle recruitment from the primordial pool through negative feedback mechanisms [28]. Therefore, we hypothesize that there may be causality between aberrant ovarian insulin signaling and HS-induced seasonal infertility.

Proper insulin-mediated PI3K signaling is not only critical for regulating primordial follicle activation (PFA) and recruitment [29-32], but is also necessary for follicle maturation, oocyte viability and ovarian steroidogenesis [25, 27, 33-35]. Most downstream PI3K signaling events are governed through serine-threonine protein kinase B (AKT), a subfamily comprised of three mammalian isoforms (Akt1, Akt2 and Akt3; [36]). Upon PI3K activation, AKT is phosphorylated and translocated to the cell nucleus where it can regulate several targets including forkhead box O (FOXO 1-4), a transcription factor family critical for follicle activation and maturation [37] as well as apoptosis [38-41]. In rodent models, *Akt*$^{-/-}$ mice have impaired fertility due to reduced primordial follicle viability [33], whereas *Foxo3*$^{-/-}$ mice experience secondary infertility as a result of global PFA and their subsequent depletion [37]. Interestingly, in FOXO3^{OE} mice, unlike *Foxo3*$^{-/-}$ mice, PFA is prevented [42]. Therefore, a proper balance in the insulin mediated PI3K/AKT/FOXO3 signaling pathway is crucial for female reproduction.

Understanding the molecular mechanism(s) by which HS compromises reproduction is a prerequisite for developing strategies and therapeutics to mitigate HS-induced suboptimal fertility. Our working hypothesis is that heat-induced hyperinsulinemia and aberrant ovarian insulin signaling may be responsible for seasonal infertility during the warm summer months. Our objective in this project was to determine the ovarian molecular response to HS.

Materials & Methods:

Animals and Tissue Collection: Tissues analyzed in this manuscript were obtained as a subset from previous experiments with all procedures approved by the Iowa State University Institutional Animal Care and Use Committee. Details regarding experimental design, animal handling and environmental conditions have been previously described [9, 11, 43]. In brief, crossbred pre-pubertal gilts (35±4 kg) were housed in constant climate controlled rooms in individual pens with *ad libitum* feed intake. Gilts were exposed to thermoneutral (TN) conditions (20°C; 35-50% humidity; n = 5-6) or HS conditions (35°C; 20-35% humidity; n = 5-6) for 7 (n = 5 per treatment) or 35 (n = 6 per treatment) days. To eliminate the effects of dissimilar feed intake, an additional group of thermoneutral gilts (n = 3) were pair-fed to the HS treatment for 7d (PFTN). Gilts were euthanized by captive bolt penetration, one ovary was snap frozen in liquid nitrogen and stored at -80^{o}C and the other ovary was fixed in 4% paraformaldehyde.

RNA isolation and quantitative reverse transcription polymerase chain reaction (qRT-PCR): Three ovaries per treatment were chosen randomly at each time point. Ovaries were ground in liquid nitrogen using a hand held pestle. The resulting powder was divided into two halves; one

half was used for RNA isolation while the other half for protein purification. Total RNA was isolated using an RNeasy Mini kit and concentrated using an RNeasy MinElute kit according to the manufacturers' protocols (Qiagen, Germantown, MD). RNA was eluted using 14 μL of RNase-free water and RNA concentration determined using an ND-1000 Spectrophotometer (λ = 260/280nm; NanoDrop technologies, Inc., Wilmington, DE). Prior to RT-PCR, total RNA was reverse transcribed into cDNA utilizing a Superscript III system (Life Technologies, Grand Island, NY). Two microliters of diluted cDNA (1:50) was amplified using gene-specific primers (see Table 1) on an Eppendorf Mastercycler using a Quantitect™ SYBR Green PCR kit. The 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 was acquired. A melting curve analysis was conducted using a temperature gradient from 72°C to 99°C with a 1°C increase at each step. Since there was no difference in ovarian glyceraldehyde-3-phosphate dehydrogenase (*GAPDH)* mRNA level across treatments, the relative mRNA expression of each target gene measured in the current study was normalized using *GAPDH* as a housekeeping gene. The relative fold change was calculated using the $2^{-\Delta\Delta CT}$ method and the results are presented as mean fold difference ± standard error relative to the TN control group.

Protein Isolation and Western Blot Analysis: Three ovaries per treatment were chosen randomly at each time point. Using tissue lysis buffer, samples were homogenized and the ovarian protein homogenates were placed on ice for 30 min, followed by two rounds of centrifugation at 10,000 rpm for 15 min at 4°C. Supernatant was collected and protein concentration determined using a standard BCA protocol on a 96-well assay plate during which 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). Samples were then stored at -80^{o}C until further use. Using 10% SDS-PAGE, 50 µg of protein homogenates were separated and 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. Equal protein loading and transfer efficiency was confirmed by Ponceau S staining of nitrocellulose membranes prior to antibody incubation. 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 sized bands was measured using Carestream molecular imaging software version 5.0 (Carestream Health Inc., Rochester, NY) which eliminates background noise. Proteins of interest were normalized to Ponceau S measurement.

Immunofluorescence staining and microscopy analysis: Immunofluorescence staining was performed as previously described [44]. Briefly, following euthanasia, ovaries were fixed in 4% paraformaldehyde for 24 h, transferred to 70% ethanol, embedded in paraffin, serially sectioned (5 micron thickness), and every 6th section mounted. Three slides per ovary were deparaffinized and incubated with primary antibody specific to either IRα or pAKT^{Ser473} (1:100) at 4°C overnight. Secondary antibodies conjugated to fluorescein were applied for 1 h, followed by counterstaining with DAPI nuclear stain for 7 min. Immunofluorescence was visualized on a Leica DMI300B fluorescent microscope at λ = 461 and 665 nm for DAPI (blue) and fluorescein conjugated (green), respectively.

Statistical Analysis: Chi-square/Goodness of fit was used to test for normality under the null hypothesis (the data follows a normal distribution) against the alternative hypothesis (the data does not follow a normal distribution). To perform the test, the log transformed expression data for 4 genes (*IRS1*, *FOXO3*, *LHCGR*, and *CYP11A1*) were randomly chosen. The sample mean, standard deviation and size of the data were calculated using the descriptive statistics tool in Excel. Using the mean, standard deviation and sample size, the expected values under the conditions of the null hypothesis (which assumes mean = 0 and STDEV =1) using the NORM.DIST function of Excel were computed. Further, the Chi-Square Statistic, degrees of freedom and the P - value at alpha level 5% was determined. For all the genes tested, the Chi-Square P – values were greater than 0.05, confirming that our data followed normal distribution. All data were statistically analyzed using GraphPad Prism software. Comparison of two treatments was performed using two-tailed t-test; comparison of more than two treatments was performed by ANOVA. A *P*-value < 0.05 was considered significantly different, with *P* < 0.1 considered a trend for a difference.

Results:

The mRNA of ovarian *INSR* and *IRS1* genes are upregulated during HS

After 7d, HS markedly increased (*P* < 0.001**)** ovarian *INSR* compared to TN controls (Figure 1A). Although not to the same extent, *INSR* mRNA was also increased in the PF pigs compared to the TN controls (Figure 1A). The HS-induced increased ovarian *INSR* response was maintained (*P* < 0.01) after 35d (Figure 1B). In contrast to *INSR* mRNA, the *IRS1* mRNA abundance on d7 was only increased in the HS ovaries as the PF and TN controls had similar

levels of transcript abundance (Figure 1A). Although the magnitude of increase was less, *IRS1* mRNA level remained increased after 35d in the HS compared to TN controls (Figure 1B).

HS increases ovarian phosphorylated IRS1

Concomitant with HS-induced ovarian *INSR* and *IRS1* mRNA levels, d7 ovarian phosphorylation of the tyrosine 632 residue on the IRS1 protein was increased ($P < 0.05$; 1.3-fold increase) in HS relative to the TN pig ovaries (Figure 2A) while PF pIRS1 abundance was intermediate and not statistically different from either TN or HS groups. The increase in ovarian pIRS1 was maintained for 35d of HS compared to the TN controls (Figure 2B; 1.8-fold increase). There was no impact of HS on total IRS1 protein abundance (Supplemental Figure 1).

***AKT1* and *FOXO3* mRNA is increased during HS**

Following increased activation of insulin signaling pathway members during HS, whether the downstream insulin mediated PI3K members, *AKT1* and *FOXO3*, were also impacted by HS was determined. Ovarian *AKT1* mRNA was increased ($P < 0.05$) after 7 d of HS (Figure 3A), compared to PF and TN controls and remained greater after 35 d of HS relative to TN controls (Figure 3B). Interestingly, it is noteworthy that although there was no difference ($P > 0.05$) in *FOXO3* mRNA abundance between ovaries obtained from TN and HS pigs, reduced ($P < 0.05$) ovarian *FOXO3* mRNA levels were observed after 7 d of PF (Figure 3A). However, after 35 d and in a similar pattern to *AKT1*, there was a trend for increased ovarian *FOXO3* mRNA expression ($P = 0.06$) in HS pigs relative to their TN counterparts (Figure 3B).

HS increases ovarian AKT1 phosphorylation

Immunofluorescence staining revealed that pAKT1 protein was highly expressed in the porcine oocyte, in addition to being localized to a lesser extent to the theca and granulosa cells (Figure 4A, B). Western blotting to quantify the impact of HS on phosphorylated, activated AKT1 (pAKT1) protein demonstrated that ovaries from HS pigs had increased ($P < 0.05$; 1.4-fold increase) pAKT1 protein levels compared to TN controls after 35 d of thermal treatment (Figure 4C). No statistical effect of HS on total AKT protein level was observed (Supplemental Figure 2).

HS affects ovarian expression of genes involved in steroid hormone production

We determined the impact of HS on mRNA expression of the genes involved in ovarian steroid production (Figure 5). Our data indicate that compared to TN and PF controls, mRNA levels of *LDLR*, *LHCGR* and *CYP19a* were elevated ($P < 0.05$) after 7 d (Figure 5A) and this HS-induction of increased mRNA abundance of these genes was maintained for 35 d (Figure 5B). Interestingly, *STAR* gene expression was not impacted by 7 d of HS (Figure 5A), but was increased after 35d of HS.

Ovarian STAR and CYP19a are elevated during HS

Since the increase in *STAR* and *CYP19a* mRNA were unexpected, protein levels of these two critically important enzymes were quantified during HS. Relative to the TN controls, ovaries from pigs following 35 d of HS had greater than a 40-fold increased abundance ($P < 0.05$) of STAR protein (Figure 6). It is important to note that the seeming lack of STAR in the TN pigs was not surprising, since they are pre-pubertal. Compared to TN and PF females, ovarian CYP19a protein was elevated ($P < 0.05$) by HS after 7 d (Figure 7A; 2.4-fold increase relative to

TN) and this HS-induced increase was maintained for 35 d (Figure 7B; 1.6-fold increase). There was no difference ($P > 0.05$) in CYP19a protein concentrations between PF and TN pig ovaries (Figure 7A).

Discussion:

Insulin plays a pivotal role in controlling carbohydrate, protein and lipid metabolism as well as influencing normal reproductive function [13, 14, 45]. Physiological conditions in which hyperinsulinemia is one of the alterations to central metabolism, including obesity and PCOS, are often associated with reduced fecundity. Hyperinsulinemia has been reported in a number of environmentally-induced HS models, including pigs [9, 10], therefore the HS female pig was utilized as a model of hyperinsulinemia to explore impacts of increased circulating insulin on ovarian function. Since pigs in this experiment were in a pre-pubertal physiological state, this model presented an opportunity to determine ovarian impacts of environmentally-induced hyperthermia while avoiding the confounding influence of an active hypothalamic-pituitary-ovarian axis.

Following insulin binding, the INSR undergoes conformation changes resulting in auto-phosphorylation of INSRβ subunits which in turn activate kinase cascades leading to phosphorylation of IRS proteins at various tyrosine/serine sites. Phosphorylated IRSs provide binding sites for the activation of various insulin mediated downstream effectors including PI3K. Plasma insulin was increased (49%; $P < 0.05$) in d 7 HS pigs compared with PFTN controls [9]. Additionally, HS-induced hyperinsulinemia appears to be caused by increased pancreatic insulin

secretion rather than changes in insulin clearance, as evidenced by increased C-peptide during HS [11]. We report increased ovarian *INSR* mRNA abundance during acute (7 d) and chronic (35 d) HS. Interestingly, ovarian *INSR* mRNA levels were also elevated in ovaries from PF gilts suggesting that reduced feed intake and presumably decreased nutrient delivery to the ovary increases the initial capacity for insulin signaling. Concomitant with increased *INSR* mRNA expression, ovaries from HS pigs had increased *IRS1* mRNA and pIRS1 protein levels during acute and chronic HS, confirming that the insulin-mediated signaling pathway activity was elevated during HS. These results suggest that the ovary not only is responsive to insulin but that insulin signaling is upregulated during HS. This is also in agreement with previous work in rat models of diet-induced obesity, where in the face of elevated circulating insulin, the ovary maintained insulin sensitivity [19], and in our previous work using both a mouse high fat diet-induced obesity model [44] and a model of progressive obesity due to lack of satiety [46, 47]. In rodent models, defects in IRS1 and IRS2 cause ovarian and hypothalamic dysfunction leading to impaired female fertility [13, 17, 48]. Since the described pig model is independent of hypothalamic input, it is likely that the observed HS effects on IRS1 are ovarian specific. Despite the up-regulation of the INSR, the PF pigs had decreased pIRS1 protein compared to the HS pigs suggesting a reduced capacity of intracellular insulin signaling. This differential effect between the HS and PF pIRS1 also demonstrates that HS directly (independent of reduced nutrient intake) alters insulin signaling. The increased ovarian insulin action observed is similar to studies demonstrating that HS ameliorates systemic proxy measurements of insulin insensitivity in diabetic rodents [49, 50] or in rodents fed high fat diets [51]. Further, these observations are also similar to reports indicating thermal therapy (saunas and hot baths) improves insulin sensitivity in humans [52]. One potential mechanism(s) by which heat offers

protection from insulin resistance is by upregulating HSP72; which inhibits the activation of stress kinases c-Jun N-terminal kinase (JNK) and inhibitor of kappa B kinase β (IKKβ) [53, 54], both enzymes involved in insulin resistance.

To determine if the downstream pathways mediated by insulin's binding to the INSR are altered during HS, we investigated mRNA encoding genes involved in PI3K signaling. Both acute and chronic HS increased the expression of both *AKT1* and *FOXO3*. Interestingly, it is noteworthy that during reduced feed intake in the PF group, *FOXO3* is reduced, thus the impact of inadequate nutrient intake during HS did not impact *FOXO3* as would be energetically expected. Previous studies have demonstrated that FOXO3 plays a pivotal role in maintaining the resting primordial follicular pool [37] in addition to influencing cell apoptosis [38-41]. Therefore, increased *FOXO3* during HS could be associated with impaired PFA similar to that observed in $FOXO3^{OE}$ mice [42]. Such a scenario could negatively impact reproduction and result in a "barren" ovary since a lack of growing follicles progressing towards ovulation would effectively reduce the number of dominant follicles required for E2 production. In addition, increased *FOXO3* could indicate increased apoptosis, which is important in the ovary since approximately 99% of oocytes are eliminated through pro-apoptotic pathways, potentially as a mechanism to ensure ovulation of oocytes having the greatest developmental potential. Thus, apoptosis prevention is not necessarily beneficial for the ovary and alterations to this process may result in imperfect oocyte ovulation. pAKT is highly expressed in the porcine oocyte and is increased in response to HS. We surmise that increased AKT activation is an ovarian attempt to sustain oocyte viability and also could increase steroid production by the dominant follicles. These data indicate that HS could directly (not associated with decreased feed intake) alter the ovarian

mechanisms that regulate oocyte quality, viability and growth activation - all of which are potentially negative events for reproductive success.

Similar to humans, porcine reproductive cycles are tightly regulated by a system of positive and negative feedback of reproductive hormones synthesized and secreted from the hypothalamus (gonadotropin-releasing hormone), the pituitary (FSH, LH, oxytocin), the ovaries (progesterone, 17β-estradiol, inhibins and relaxin) and, depending on species, from the ovary or the uterus (prostaglandin F2α) [55]. While the major physiological functions of E2 in ovulation and regulation of follicular recruitment are well-documented, it is also important to recognize that E2 is necessary for behavioral estrus display and maternal recognition of pregnancy in the pig, thus altered insulin signaling negatively impacting E2 production could contribute to compromised fertility and increase the potential for early embryonic death [24, 28]. Unlike humans, pigs are typically multiple ovulators (i.e. >15 oocytes during estrus), however, like humans, the ovulation rate and follicle quality is impacted by the preceding folliculogenesis period [55] by the action of multiple signaling pathways including PI3K signaling. Thus, any disruption in folliculogenesis could compromise ovulation and subsequent conception, while impaired steroidogenesis could reduce conception rate and cause embryonic death. Additionally, unlike cyclic follicle recruitment which is controlled by gonadotropins, initial PFA is gonadotropin independent and it is thought to be tightly regulated by the PI3K signaling pathway [29-32, 37, 56-59]. Any external stress or exposure which alters the PI3K and PFA can therefore detrimentally alter fertility.

Since both insulin [60-62] and PI3K [25] signaling pathways influence ovarian steroidogenesis, we next examined the impact of HS on expression of genes encoding proteins essential for E2 production. Our data revealed that mRNA levels of *LDLR*, *LHR*, *STAR* and *CYP19a* were elevated following HS for either 7 or 35 d, though the increase in *STAR* was not fully evident until d 35. HS also increased STAR and CYP19a protein abundance, relative to PF and TN conditions indicating the effects of HS are direct and not mediated by reduced nutrient intake. Normally, STAR is upregulated in steroid producing cells in response to conditions that induce acute steroid biosynthesis. Under such circumstances, STAR increases cholesterol translocation from the outer to the inner mitochondrial membranes; the rate-limiting step in steroid production [63]. Therefore increased STAR expression during HS could be locally interpreted as an increased steroid production capacity, however, we suspect that HS compromises steroid biosynthesis and the observed increase in ovarian STAR abundance is a compensatory mechanism in response to the unfulfilled steroid production required of ovarian cells. Additionally, increased *LHCGR* during HS could indicate that the ovary is attempting to upregulate the amount of LH receptors necessary to ensure ovulation. Increased CYP19a could also locally imply that HS potentially alters the amount of circulating E2 since the pathway that produces E2 is increased, resulting in compromised fertility. The impact of HS on reproductive plasma hormone concentrations have been previously noted [6, 64-67]. In dairy cows, blood LH levels have been reported to either increase [68], decrease [69, 70] or stay unchanged [65, 71] during HS. Similarly, plasma P4 concentrations have been reported to increase [65, 72, 73], decrease [65, 74] or be unaltered [3, 75] in response to HS. Our finding that the LHCGR was increased by HS may indicate an ovarian effort to increase the capacity to respond to LH, and this warrants further investigation. Furthermore, HS has been associated with reduced blood E2

concentrations [65, 75, 76] in dairy cows. Reasons for the inconstancies within the literature are not clear but likely include differences in 1) species, 2) length and magnitude of the heat load, and 3) the incorporation of a pair-feeding model. Regardless, these data suggest that HS alters gonadotropin and ovarian steroid metabolism, in ways that are likely detrimental to reproduction and our future work is aimed at quantifying E2 and androgen production, as well as mRNA and protein abundance of steroidogenic genes that were not characterized in this study, including *CYP17* and *HSD3B*, within the HS ovary.

Taken together, our data indicates that the utilization of a hyperinsulinemic pre-pubertal porcine model has benefits for understanding ovarian physiological alterations in the absence of hypothalamic-pituitary input and that HS could be considered an endocrine disrupting environmental exposure that can negatively affect female reproduction. Further, our results clearly demonstrate that the effects of HS on ovarian insulin signaling and steroid synthesis are the direct effects of environmental hyperthermia and not mediated by reduced nutrient intake.

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Figure Legends:

Figure 1. Effects of HS (35°C) after (A) 7 days, or (B) 35 days on mRNA encoding the *INSR* and *IRS1* in ovaries from pre-pubertal pigs relative to pair-fed pigs in thermoneutral conditions receiving(TN; 20°C). (A) different letters indicate statistical difference; $P < 0.05$. (B) * indicates $P < 0.05$, different from TN.

Figure 2. Impact of HS (35°C) on $pIRS^{Tyr632}$ after (A) 7 days, or (B) 35 days in ovaries from pre-pubertal pigs relative to pair-fed pigs in thermoneutral conditions (TN; 20°C). (A) different letters indicate statistical difference; $P < 0.05$. (B) * indicates $P < 0.05$, different from TN.

Figure 3. 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 pigs relative to pair-fed pigs in thermoneutral conditions (TN; 20°C). (A) different letters indicate statistical difference; $P < 0.05$. (B) * indicates $P < 0.05$, † indicates $P < 0.1$; different from TN.

Figure 4. Effect of HS on pAKT1 protein localization (after 7 days) and level (after 35 days) in ovaries from pre-pubertal pigs relative to pair-fed pigs thermoneutral conditions (A = TN; 20°C; B = HS; 35°C). Thin arrow indicates small primary or primordial follicles; arrowhead indicates oocyte in secondary follicle. Green staining indicates pAKT1 protein; Blue staining represents DNA. (C) Western blot to detect pAKT1 in ovaries from TN or HS gilts after 35 days.

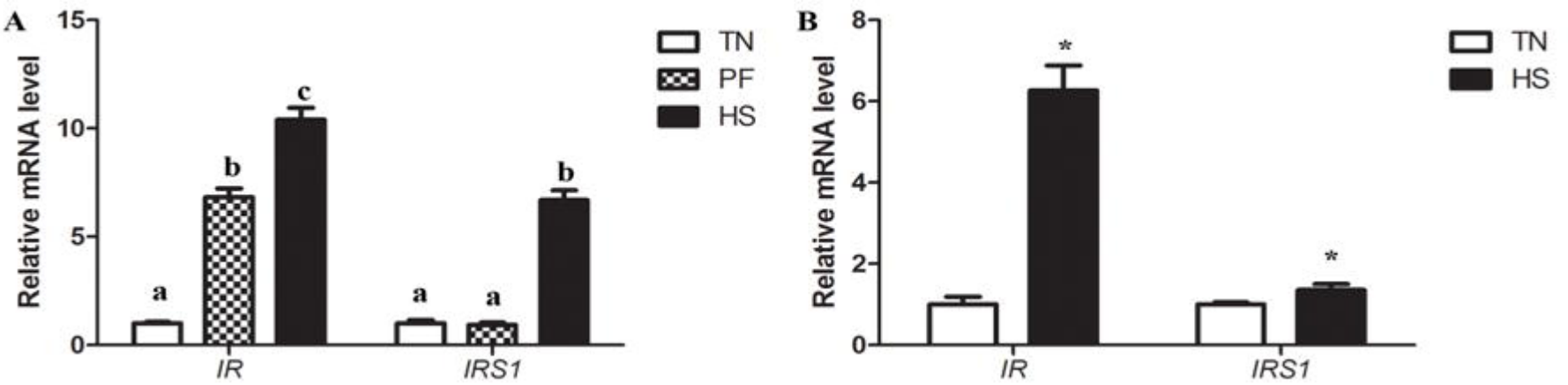
Figure 5. Effects of HS (35°C) after (A) 7 days, or (B) 35 days on mRNA encoding genes in the steroidogenic pathway (*LDLR*, *LHR*, *STAR*, *CYP19A*) in ovaries from pre-pubertal pigs relative to pair-fed pigs in thermoneutral conditions(TN; 20°C). (A) different letters indicate statistical difference; $P < 0.05$. (B) * indicates $P < 0.05$, different from TN.

Figure 6. Effects of HS (35°C) after 35 days on STAR protein in ovaries from pre-pubertal pigs relative to pair-fed pigs in thermoneutral conditions (TN; 20°C); * indicates $P < 0.05$, different from TN.

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

Gene	Forward Primer Sequence (5' – 3')	Reverse Primer Sequence (5' – 3')
GAPDH	ACCCAGAAGACTGTGGATGG	AAGCAGGGATGATGTTCTGG
LDLR	GAGTTGGCTTTTGCTCTGCT	GGGTTTTGGTGAATGAATGG
LHR	CATGGCACCGATCTCTTTCT	CGGAATGCCTTTGTGAAAAT
STAR	TTGGAAGAGACGGATGGAAG	CCCACATTCCTGCTATTGCT
CYP11a	AGGCCAATGTTACCGAGATG	ATTGCAGCATCTTGCTTGTG
3β-HSD	GACACACCTCCCCAAAGCTA	TGTAGGAGACGGTGAACACG
CYP19a	GGAGCTTGGGGTTAATGGAT	GGGAAGGATGCTCTTTGATG
IRβ	AGAGATTGCTGATGGGATGG	AAAGGACCACATGTCGGAAG
IRS1	GTGCGACAGAGCATCATTGT	GCCTTGTTTTAAGCCTCTGC
AKT1	ATCGTGTGGCAGGATGTGTA	CTGGCCGAGTAGGAGAACTG
FOXO3	TCAGCCAGTCTATGCAAACC	CCATGTCACATTCCAAGCTC

Table 1: Primer sequences used for gene amplification.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Relative mRNA level
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10
5
0
a
b
c
a
a
b
IR
IRS1
TN
PF
HS
B
Relative mRNA level
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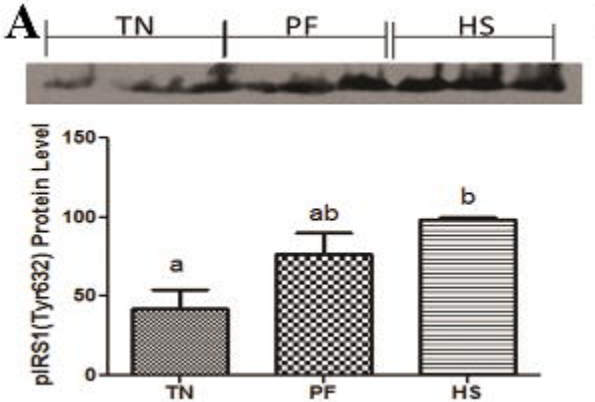
6
4
2
0
*
*
IR
IRS1
TN
HS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PF
HS
pIRS1(Tyr632) Protein Level
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100
50
0
a
ab
b
TN
PF
HS
B
TN
HS
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HS
TN
HS
pIRS1(Tyr632) Protein Level
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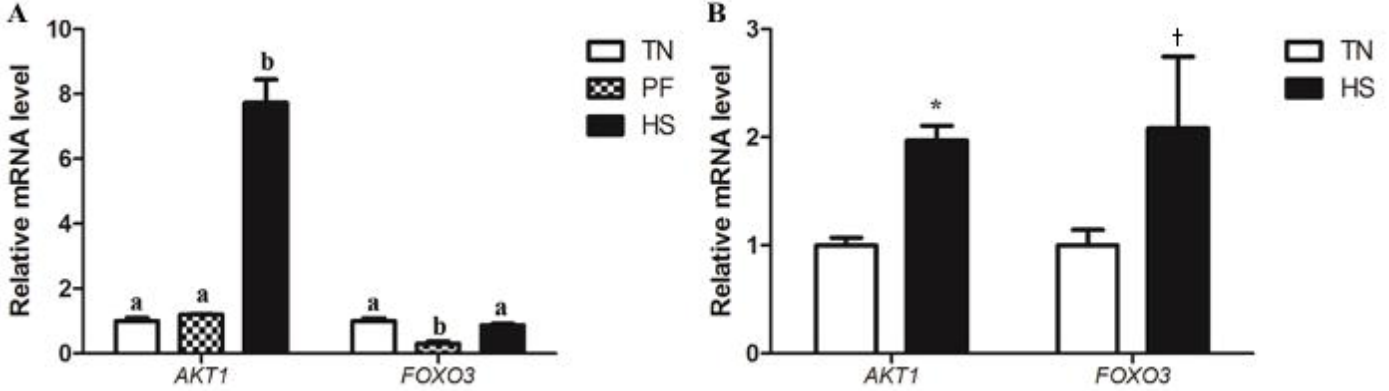
30
20
10
0
*
TN
HS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6
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2
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a
a
b
a
b
a
AKT1
FOXO3
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PF
HS
B
Relative mRNA level
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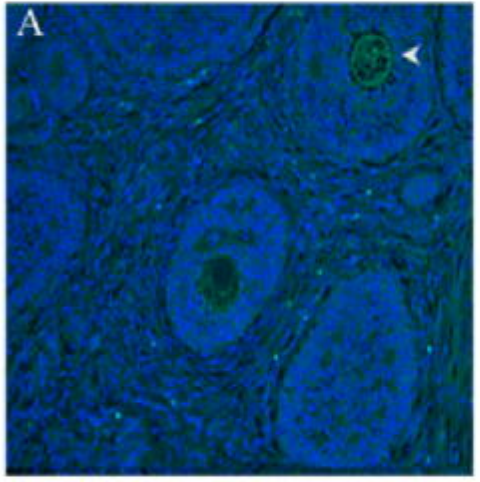
1
0
*
†
AKT1
FOXO3
TN
HS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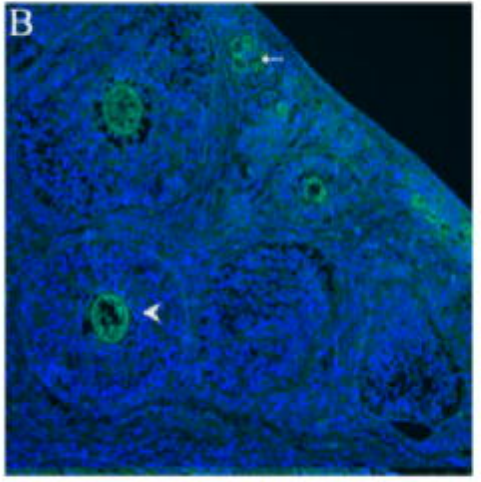
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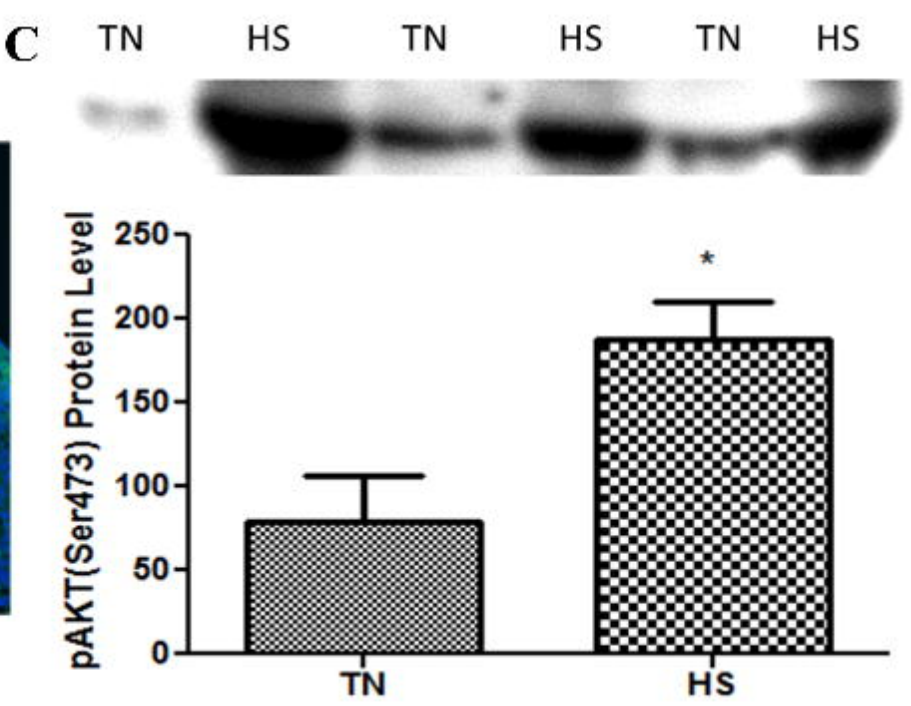
C
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HS
TN
HS
TN
HS
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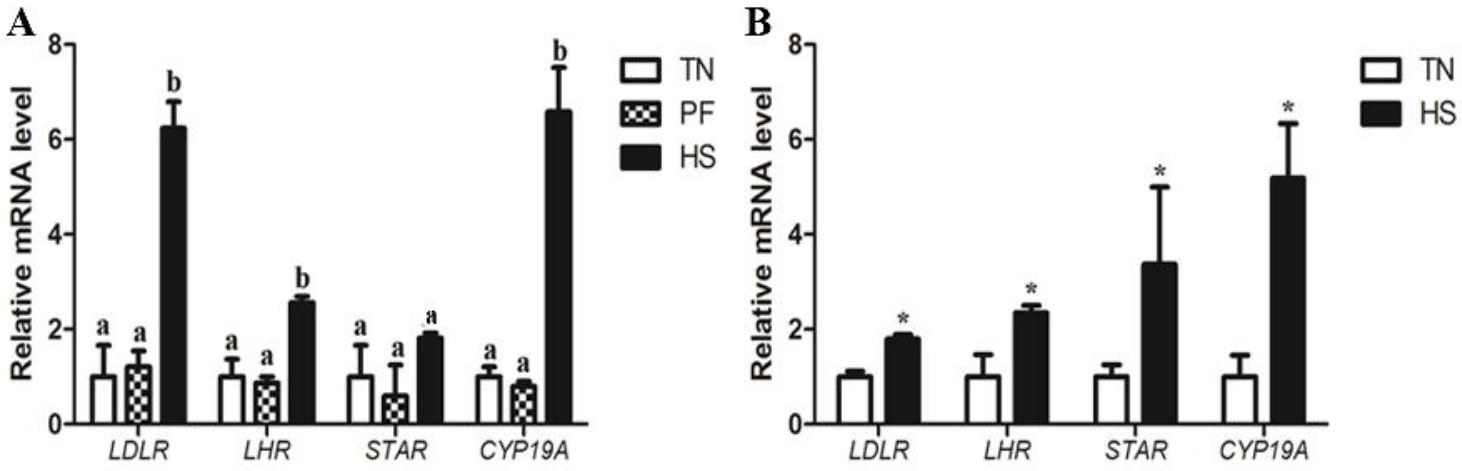
200
150
100
50
0
*
TN
HS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PF
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b
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a
b
a
a
a
a
a
b
LDLR
LHR
STAR
CYP19A
B
Relative mRNA level
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4
2
0
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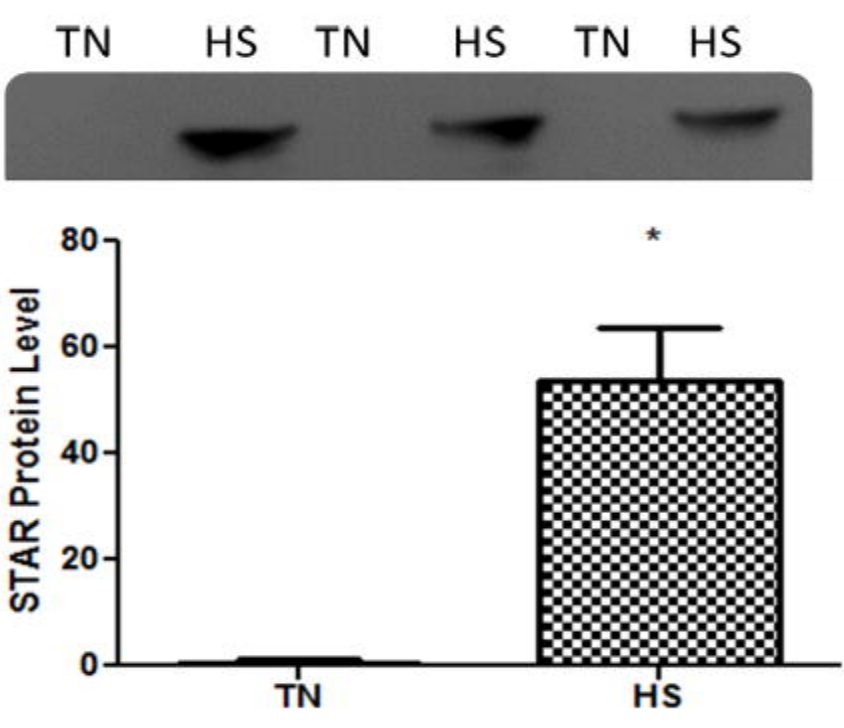
HS
*
*
*
*
LDLR
LHR
STAR
CYP19A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HS
TN
HS
TN
HS
STAR Protein Level
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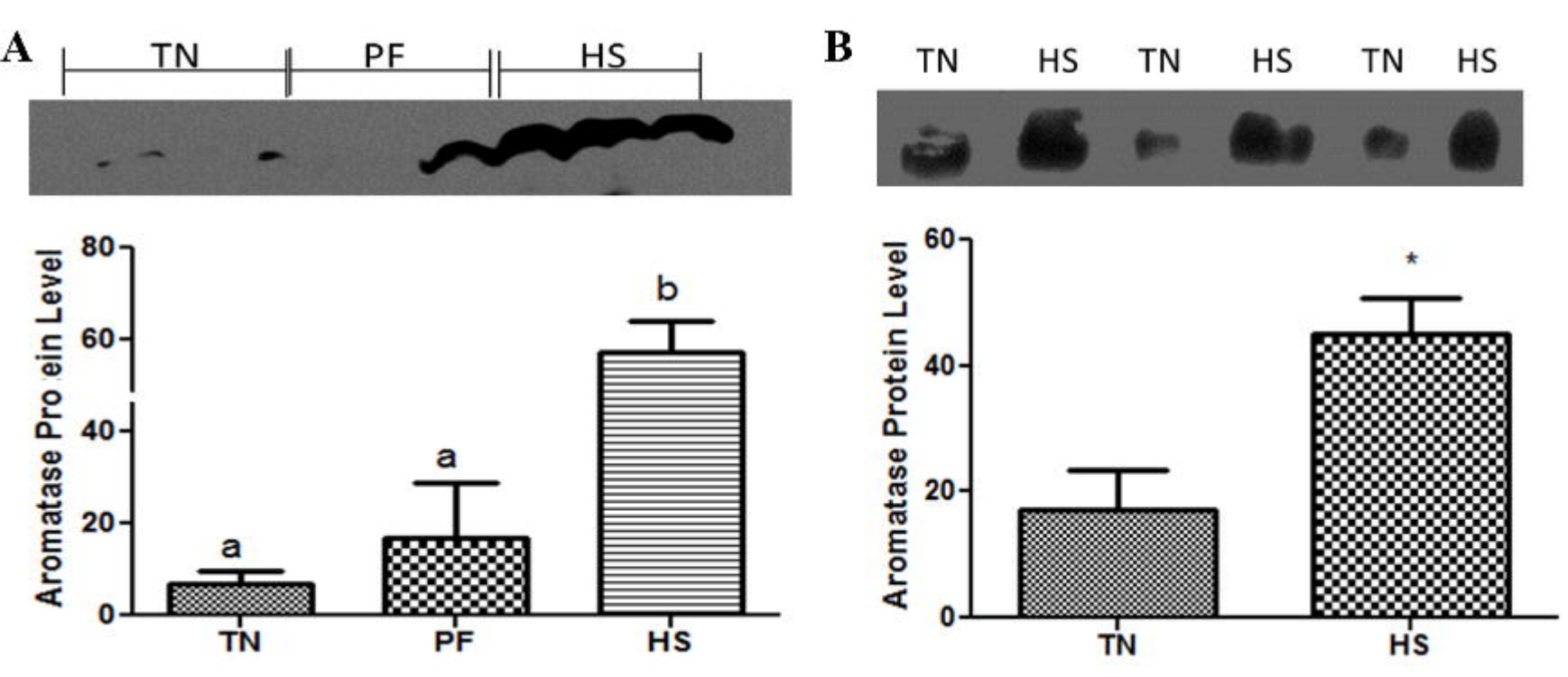
*
TN
HS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PF
HS
Aromatase Pro ein Level
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b
TN
PF
HS
B
TN
HS
TN
HS
TN
HS
Aromatase Protein Level
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20
0
*
TN
HS