REPRODUCTION

Reproduction and reproductive tract morphology of male and female pigs whose mothers were heat stressed during the second month of gestation

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Abstract

The primary objective was to assess the development of fetal gonads and measure the subsequent reproductive capacity of boars and gilts whose mother was either subjected to gestational heat stress (GHS) or thermoneutral (GTN; control) conditions during pregnancy. Gilts were subjected to either GHS (28 to 38 °C; 65% to 88% relative humidity [RH]; $n = 30$) or GTN (17 to 22 °C; 56% to 65% RH; $n = 29$) for the second month of gestation (a period that coincides with a critical window of gonadal development). A subset of GHS ($n = 12$) and GTN ($n = 11$) gilts was sacrificed immediately following treatment for the collection of pregnancy data. The remaining gilts ($n = 18$ GHS and $n = 18$ GTN) were allowed to farrow. Female offspring from the farrowed gilts were studied through puberty, first insemination, and early pregnancy when fetal tissues were again collected. During the treatment period, GHS gilts had greater ($P < 0.001$) rectal temperature and respiration rate at both measurement time points (morning and afternoon) compared with GTN gilts. When assessed at the end of the second month of gestation, the total number of viable fetuses did not differ ($P > 0.10$) for GHS vs. GTN. Likewise, the weight of the fetus, placenta, fetal testes, and fetal ovaries were similar ($P > 0.10$) for GHS and GTN pregnancies. There was a tendency for an effect of treatment (63.3 ± 2.3 vs. 70.1 ± 2.6 ; GHS vs. GTN; $P < 0.073$) on the number of oogonia per histological section in the fetal ovaries. There was no effect of treatment on the number of prespermatogonia per histological section in the fetal testis. For gilts farrowing after treatment, litter size, piglet birth weight, and weaning weight were similar ($P > 0.10$) for the GHS and GTN gilts. Testes collected from castrated GHS boars had fewer prespermatogonia per seminiferous tubule cross section ($P < 0.049$). Female offspring from the GHS ($n = 30$) or GTN ($n = 37$) sows reached puberty at a similar age, and their pregnancies (ninth week of gestation) had fewer corpora lutea (15.6 ± 0.5 vs. 17.1 ± 0.4 ; GHS vs. GTN; $P < 0.038$) but the number of fetuses was similar for GHS and GTN. In summary, compared with GTN, GHS during a critical window of gonadal development tended to reduce the number of oogonia in the fetal ovary, reduced the number of prespermatogonia in the neonatal testes, and reduced ovulation rate at first pregnancy in gilts.

Key words: gestation, heat stress, pig, reproduction

Introduction

High ambient temperature and humidity during the summer create heat stress that can have detrimental effects on reproduction in swine (Johnson et al., 2020; Mayorga et al.,

2020). Most of the previous research on heat stress has focused on the reproductive performance of the sow. Compared with thermoneutral conditions, heat stress delays the return to estrus in sows after weaning and increases the number of

Abbreviations

AI	artificial insemination
bpm	breaths per min
BW	body weight
CL	corpora lutea
MOF	modified open front
RH	relative humidity
ST	seminiferous tubule(s)
THI	temperature humidity index
wt	weight

nonpregnant sows after breeding (Nardone et al., 2006). Heat stress during gestation will increase embryonic mortality and increase the number of stillborn piglets (Edwards et al., 1968; Omtvedt et al., 1971; Wildt et al., 1975). The combined effects of heat stress on conception, early embryonic development, and neonatal viability contribute to a decrease in the number of piglets born alive at farrowing. There are additional effects of heat stress on the growing pig that include a reduction in feed intake that reduces the daily gain in body weight (BW; Quiniou et al., 2000; Kerr et al., 2003). The economic impact of heat stress in pigs through its effects on reproduction and growth is large (St-Pierre et al., 2003).

The true effects of heat stress on farm productivity may be much greater if the developmental program of offspring that is initiated in utero is irreversibly changed by heat stress in the pregnant sow (Lucy and Safranski, 2017). In their recent reviews, Johnson et al. (2020) and Mayorga et al. (2020) outlined a diverse array of biological responses that affect postnatal performance that are impacted in the offspring of heat-stressed sows. These responses include altered growth, body composition, reproduction, immune function, thermoregulation, and stress response. In nearly every case, the effects of gestational heat stress (GHS) are negative with respect to the future performance of the offspring. Despite the accumulation of salient studies, we know relatively little about the effects of GHS on the functional morphology of the ovary and testes or their capacity for gamete production later in life.

Black and Erickson (1968) determined that there is a large increase in the number of germ cells (oogonia) within the porcine fetal ovary from day 20 to 50 of gestation. During this period, they estimated that the germ cell population increased from approximately 5,000 on day 20 to greater than 1 million by 50 d of gestation. This increase was explained by a high level of mitotic activity within the germ cell population. Afterward, they found that mitotic activity decreased so that there were approximately 500,000 germ cells in the ovary at birth. In subsequent work, Erickson and Martin (1984) irradiated pregnant pigs for the first 108 d of gestation and studied the subsequent reproduction of boars and gilts born to the irradiated mothers (Erickson and Martin, 1984). The treatment caused a large decrease in sperm production in boars and a decrease in oocyte numbers in gilts. Fertility through first parity was normal in gilts but appeared to be compromised thereafter. Earlier work in the boar demonstrated that the period of fetal gonad sensitivity began at day 35 with maximum sensitivity beginning at day 50 of gestation (Erickson et al., 1963).

Based on the work of Erickson and Martin (1984) the pool of germ cells for both gilts and boars is established early during pregnancy. Toxic agents can permanently affect this pool to possibly affect the reproductive potential of the offspring long term. In mammals, it is generally accepted that the oocyte pool cannot be regenerated at any other time during the lifetime

of the female (Grive, 2020). In the male, spermatogonial stem cells within the fetal testis form the basis for lifelong sperm production (Mäkelä et al., 2019). Our hypothesis was that GHS during a critical period of germ cell development (second month of gestation) would have long-term negative consequences for the reproduction of the boar and gilt through its effects on fetal gonad development. To test this hypothesis, we heat stressed gilts during the second month of gestation and examined the development of the fetus, placenta, and fetal gonads at the end of treatment compared with a thermoneutral control. We allowed additional sows from the GHS and control treatments to complete gestation and farrow and measured litter size and piglet weight and collected and analyzed testes from the neonatal boars. A subset of intact boars from the treated and control sows was used to assess the effects of GHS on sperm production and morphology (results published by Lugar et al., 2018). Finally, gilts coming from heat-stressed or control sows were tested for age at puberty and reproductive capacity through first pregnancy.

Materials and Methods

Animals, facilities, and treatments

The University of Missouri Animal Care and Use Committee approved all procedures involving pregnant gilts and their piglets. Animal care and use standards were based upon the *Guide for the Care and Use of Agricultural Animals in Research and Teaching* (Federation of Animal Science Societies, 2010). The experiment included three replicates that began between August 2015 and December 2016 and included 10 treated and 10 control pigs per replicate with the exception of replicate 3 that had 10 treated and 9 control pigs. Choice Genetics F1 Landrace × Large White gilts were synchronized using Matrix (Merck Animal Health, De Soto, KS) at the University of Missouri Swine Research Complex (SRC). After estrus was detected, gilts were mated to an unrelated maternal line. Gilts were diagnosed pregnant at the SRC at approximately 24 d after insemination. After pregnancy diagnosis, gilts were blocked by BW and randomly assigned to either the GHS ($n = 30$) or gestational thermoneutral (GTN; $n = 29$) treatment (Figure 1). The BW at the start of treatment was similar ($P > 0.10$) for GHS (147.6 ± 2.7 kg) and GTN (147.8 ± 2.8 kg) but replicates differed for starting BW (160.7 ± 3.3 , 135.3 ± 3.3 , and 147.2 ± 3.4 kg; $P < 0.001$; replicates 1 to 3, respectively).

Two environmental chambers were used for this experiment. Each chamber measured 9.3×5.2 m. One chamber housed GHS gilts and the other chamber housed GTN gilts with 10 pigs per chamber. Gilts were housed in stalls (2.4×0.6 m) during gestation. The front of each stall had a solid floor and the back of each stall had a metal grate that allowed for fecal material and urine to fall into the gutter below. Each stall contained an individual nipple waterer.

Gilts were moved into the environmental chambers as a single group. The GHS temperature cycle was initiated at 0800 hours on the day after they moved into the environmental chambers. The day that the GHS was initiated was defined as treatment day = 0. The synchronization protocol (Matrix treatment) did not result in all gilts in estrus and artificial insemination (AI) on the same day. The actual day of pregnancy on treatment day 0, therefore, was not identical. Across all replicates, the GHS or GTN treatment was started on days 25, 26, 27, 28, and 29 of gestation for $n = 2$, 18, 20, 14, and 5 gilts, respectively. The mean gestation day at the start of treatment (27.0 ± 1.0 ; mean $\pm$ SD) did not differ ($P > 0.10$) for GHS or GTN

Figure 1. Timeline and experimental design. Gilts were subjected to GHS or GTN conditions during the second month of gestation (G0). Data were collected from pregnancies immediately after treatment (day 57) or at farrowing. Female progeny (G1) was maintained through puberty, first AI, and first pregnancy when the tissue was collected (day 59).

or for replicates 1 to 3. The ambient temperature of the GHS chamber was increased so that the maximum temperature, relative humidity (RH), and temperature humidity index (THI; Mellado et al., 2018) cycle (28 to 38 °C; 65% to 88% RH; THI = 81 to 92) was reached on treatment day 6. The ambient temperature, RH, and THI of the GTN chamber remained the same for the entire treatment period (17 to 22 °C; RH 56% to 65%; THI = 62 to 68). The ambient temperature cycle for both GHS and GTN was the minimum temperature from 0 to 0800 hours, a steady increase to the maximum temperature from 0800 to 1200 hours, a steady maximum temperature from 1200 to 1600 hours, and a steady decrease in temperature from 1600 to 2400 hours. The RH cycle for both GHS and GTN was the maximum RH from 0 to 0800 hours, a steady decrease in RH from 0800 to 1200 hours, a steady minimum RH from 1200 to 1600 hours, and a steady increase in RH from 1600 to 2400 hours. The temperature and humidity within the chambers were verified every 15 min using Onset HOBO data loggers (Bourne, MA; data not shown).

Data collected during the treatment period (GHS or GTN)

Gilts were fed 2.2 kg of a standard corn–soybean meal gestation diet that was balanced to meet or exceed the NRC (2012) recommendations and identical to that fed in a previous study from our laboratory (Williams et al., 2013). Feeding was at 0615 hours. Feed was removed and any refused feed was measured and recorded at 0645 hours. Thermal measurements were made at 0700 and 1600 hours daily. Rectal temperatures were measured using a rectal thermometer with a thermistor probe (Cole Parmer North America, Vernon Hills, IL). The thermistor probe was lubricated, inserted approximately 15 cm into the rectum, and remained in the gilt until the temperature stabilized (approximately 1 min). Respiration rate was measured by observing the flank of the gilt and counting breaths for 1 min (breaths per min [bpm]).

Data collection at slaughter immediately following GHS or GTN (mid-gestation)

Gilts were removed from the environmental chambers when they were on days 55, 56, 57, or 58 of gestation for $n = 9, 22, 28$, or 10 gilts. The mean gestation day at the end of treatment (56.5 ± 1.0 ; mean $\pm$ SD) did not differ ($P > 0.10$) for GHS or GTN or for replicates 1 to 3. The total treatment time averaged 29.5 ± 0.6 d (mean $\pm$ SD). Gilts were weighed and randomly selected to be either transported to the University of Missouri Swine Teaching Farm to complete their gestation and farrow ($n = 18$ GHS and $n = 18$ GTN; Figure 1) or sent to the University of Missouri

abattoir for slaughter and collection of the reproductive tract ($n = 12$ GHS and $n = 11$ GTN; Figure 1). BW of pigs selected for slaughter (152.0 ± 4.1 and 151.0 ± 4.3 kg) or farrowing (164.7 ± 3.3 and 157.3 ± 3.3 kg) did not differ ($P > 0.10$) for GHS and GTN (respectively).

Gilts selected for slaughter were killed by electrocution and exsanguination. Reproductive tracts were removed and placed in plastic bags on ice and transported to a laboratory for further dissection and data collection. Dissection was within 1 h of slaughter. Ovaries were then removed and weighed and corpora lutea (CL) were counted. The broad ligament was dissected from the uterine horns. Each uterine horn was opened, and the contents were exposed. The entire conceptus (fetus, placenta, and fluid) was removed and weighed. The fluid was then drained from the placenta, and the fetus and placenta were weighed separately. The number of viable, nonviable, and mummified fetuses was counted. Percentage embryonic survival was calculated by using the equation $100 \times (\text{number of fetuses} / \text{number of CL})$. Placental efficiency was defined by the equation $100 \times (\text{fetal weight} / \text{placental weight})$.

Male and female fetuses were dissected, and the fetal testes and ovaries were removed and weighed. The fetal testes and ovaries were then placed in 10% buffered formalin phosphate (Fisher Scientific, Fair Lawn, NJ) for fixation and taken to the University of Missouri College of Veterinary Medicine Diagnostic Laboratory for tissue embedding, sectioning, and hematoxylin and eosin staining. A single 4- μ m section was collected from the sagittal plane at the largest cross-sectional area of the fetal ovary and testes. Electronic images were taken at 400 $\times$ magnification using a Leica DM400 B microscope (Leica Microsystems, Wetzlar, Germany). The fetal ovary was comprised of nests of oogonia and surrounded by ovarian stroma (Figure 2A and B). The oogonia have round nuclei, whereas stromal cells have cigar-shaped nuclei (Black and Erickson, 1968; Bielańska-Osuchowska, 2006). The number of oogonia was counted objectively by using the public domain NIH ImageJ program (<http://rsb.info.nih.gov/ni-image/>). Image files were opened in ImageJ, and the background was subtracted using a rolling ball radius of 1 pixel and the light background. The image was then converted to a binary image. Particles (oogonia) were counted by using the analyze particles function within ImageJ with the following settings: size = 0.015 to 0.1, circularity = 0.55 to 1.00, exclude edges, and include holes. Summary files were created that included the count (total number of oogonia per section) and the average size of oogonia per section. The ImageJ settings were derived empirically by manually counting a subset of slides and comparing these data with the data from the automated analysis in ImageJ. The final method was validated by using 30 individual sections. The correlation (r) between manual counting and automated (ImageJ) counting was 0.73. The relationship was explained by the regression equation: manual count of oogonia = $1.0 \times$ ImageJ count of oogonia + 20.1 ($r^2 = 0.53$; $P < 0.001$).

Morphological characteristics of fetal testes were analyzed by using manual counting, manual measurements, and ImageJ analysis. Both the fetal and neonatal testes are comprised of seminiferous tubules (STs) lined with Sertoli cells and prespermatogonia (Figure 2C and D). The prespermatogonia are more centrally located than the Sertoli cells and have round nuclei (Erickson et al., 1963; Mutembei et al., 2005). The number of Sertoli cells was counted for each of two tubules, and the tracing function within ImageJ was used to measure the area. The average number of Sertoli cells per tubule and the average area of the tubules were calculated. The total

Figure 2. Histological section of the fetal ovary (A and B), fetal testis (C), and newborn testis (D). Regions of the same image (A and B) are annotated to show developing oocytes (oogonia) in egg nests and ovarian stroma. Oogonia have round nuclei and develop in nests (regions encircled by white dashed lines in B). Ovarian stroma cells have cigar-shaped nuclei (regions encircled by black dashed lines in B). The fetal testis (C) consists of STs lined with prespermatogonia and Sertoli cells. The STs in newborn testis (D) are larger and also possess prespermatogonia and Sertoli cells. Bar = 50 µm in all images.

number of STs across the entire section was then counted manually. The number of prespermatogonia was counted using a method similar to that used for the fetal ovary. The image was converted to a binary image (background subtraction was not necessary). Particles (prespermatogonia) were counted by using the analyze particles function with the following settings: size = 0.02 to 0.1, circularity = 0.55 to 1.00, exclude edges, and include holes. The final method was validated by using 30 individual sections. The correlation (r) between manual counting and automated (ImageJ) counting was 0.77. The relationship was explained by the regression equation: manual count of prespermatogonia = $0.65 \times \text{ImageJ count of spermatogonia} + 15.7$ ($r^2 = 0.59$; $P < 0.001$). The number of prespermatogonia per ST was calculated by dividing the total number of prespermatogonia per section by the total number of tubules per section.

Data collection at farrowing following GHS or GTN treatment

Gilts that were selected for farrowing were moved from the environmental chambers to an environmentally controlled gestation facility (MU Swine Teaching Farm) and then moved into farrowing crates within 1 wk before expected farrowing. Gestation and lactation diets were balanced to meet or exceed NRC (2012) recommendations and identical to those fed in a previous study from our laboratory (Williams et al., 2013). Farrowings were attended. At birth, each piglet was caught, dried, weighed, and identified with an individual ear tag. Gestation length, the number of live born, and the number of stillborn pigs were recorded. Offspring were designated as gestational heat stress-generation 1 (GHS-G1) or gestational thermoneutral-generation 1 (GTN-G1). At 3 d of age, piglets had their tails docked, teeth clipped, and

ears notched for identification. Iron was administered to prevent anemia. A subset of males remained intact for a collaborative study on the reproductive capacity of boars from treated and control dams (Lugar et al., 2018). The remaining males were castrated by 3 d of age. The neonatal testes were weighed, fixed in 10% buffered formalin phosphate, and processed for histological analysis as described for the fetal testis (preceding paragraph). Piglets were weighed at processing, at 1 wk of age, at 2 wk of age, and at weaning. After weaning, boars, barrows, and gilts were moved to a nursery for 5 to 7 wk. After leaving the nursery, barrows were sold and gilts were moved to an environmentally controlled finishing room at the Swine Teaching Farm.

Morphology of neonatal testes was analyzed by using both manual counting and ImageJ analysis in a manner similar to that used for the fetal testes. The number of Sertoli cells was counted for two tubules, and the tracing function within ImageJ was used to measure the area of each tubule. The average number of Sertoli cells per tubule and the average area of the tubules were analyzed. A method to use ImageJ to count prespermatogonia could not be validated. The total number of STs and the total number of prespermatogonia across the entire section, therefore, were counted manually. The number of prespermatogonia per ST was calculated by dividing the total number of prespermatogonia per section by the total number of tubules per section.

Data collection for GHS-G1 and GTN-G1 gilts (puberty, breeding, and slaughter)

The GHS-G1 and GTN-G1 gilts were moved from the finishing room to a naturally ventilated modified open front (MOF) building at the University of Missouri Teaching Farm for the purpose of estrus detection and AI. Gilts were exposed to boars for 10 min daily beginning at 160 d of age. Gilt behavior was observed and vulva scores were recorded daily. Gilts were scored on a 4-point scale (0 = no signs of estrus; 1 = some signs of estrus [i.e., swollen vulva, increased interest in the boar, and vulvar discharge]; 2 = close to standing estrus; 3 = standing estrus). Gilts in replicates 1, 2, and 3 were exposed to the boar for 58, 86, and 60 d, respectively. Two blood samples were collected at a 1-wk interval for any gilt that was not observed in estrus beginning on days 58, 86, or 30 for replicates 1 to 3, respectively, and analyzed for plasma progesterone using radioimmunoassay (Pohler et al., 2016). Gilts with progesterone concentrations of 0.5 ng/mL or greater for one or both samples were defined as pubertal (Magness and Ford, 1983).

Gilts that had reached puberty were inseminated on their first postpubertal estrus after 210 d of age. The gilts were inseminated on the first day of standing estrus and 24 h later using commercial Duroc semen. Pregnancy was diagnosed by ultrasound approximately 24 d after AI.

Pregnant gilts at the end of the second month of gestation (56 to 63 d of pregnancy) were transported to the University of Missouri abattoir where they were killed by electrocution and exsanguination (GHS-G1 $n = 30$; GTN-G1 $n = 37$). The mean gestation day at slaughter (59.4 ± 2.1 ; mean $\pm$ SD) did not differ ($P > 0.10$) for GHS and GTN or for replicates 1 to 3. Processing of the reproductive tracts was identical to that described for the treatment gestation with the exception that fetal ovaries and testis were not collected.

Statistical Analyses

Data collected during the treatment period (GHS or GTN)

Data with one measurement per gilt (BW) were analyzed using the Mixed Models procedure (PROC MIXED) of SAS 9.4 (SAS Inst. Inc., Cary, NC). The model included the main effects of treatment,

replicate, and treatment by replicate interaction. The replicate was defined as random. The day of gestation at slaughter was included as a covariate. Data with multiple measurements per gilt (morning rectal temperature, afternoon rectal temperature, etc.) were analyzed using PROC MIXED of SAS 9.4. The model included the main effects of treatment, replicate, treatment by replicate, day, treatment by day, and replicate by day. Day was defined as the repeated variable. Sow nested within treatment and replicate was defined as the subject variable.

Data collection at slaughter immediately following GHS or GTN treatment (mid-gestation)

Data with one measurement per gilt (total number of viable fetuses per pregnancy, etc.) were analyzed using PROC MIXED. The model included the main effects of treatment, replicate, and treatment by replicate interaction. Replicate was defined as random. Data with multiple measurements per gilt (fetal weight, placental weight, etc.) were analyzed using PROC MIXED. The model included the main effects of treatment, replicate, treatment by replicate, fetal sex, and treatment by sex interaction. The day of pregnancy and the total number of fetuses in the litter were included as covariates in the analyses. Sow nested within treatment and replicate was defined as random. Fetus was defined as a repeated variable. For the sex-specific analyses (fetal testes weight, fetal ovarian weight, etc.), the mixed model was used but sex or interactions were not included in the model statement.

Data collection at farrowing following GHS or GTN treatment

Data with one measurement per gilt (gestation length, total born, born alive, etc.) were analyzed using PROC MIXED. The model included the main effects of treatment, replicate, and treatment by replicate interaction. Replicate was defined as random. Data with multiple measurements per gilt (piglet birth weight, weaning weight, etc.) were analyzed using PROC MIXED. The model included the main effects of treatment, replicate, treatment by replicate, sex, treatment by sex, and replicate by sex. Sow nested within treatment and replicate was defined as random. For the sex-specific analyses (testes weight, gilt age at puberty, etc.), the mixed model was used but sex was not included in the model statement. Binomial data (survival to weaning, survival in the nursery, etc.) were analyzed for the effects of treatment, replicate, treatment by replicate, sex, treatment by sex, and replicate by sex by using PROC GLIMMIX of SAS 9.4. The distribution was defined as binary, and sow nested within treatment and replicate was defined as random.

Data collection for GHS-G1 and GTN-G1 at slaughter (mid-gestation)

Data with one measurement per gilt (litter size, ovarian weight, number of CL, etc.) were analyzed using PROC MIXED. The model included the main effects of treatment, replicate, and treatment by replicate interaction. Gilt was defined as a repeated variable. The original dam nested within treatment and replicate was defined as the subject variable. Data with multiple measurements per gilt (fetal weight, placental weight, etc.) were analyzed by using PROC MIXED. The model included the main effects of treatment, replicate, treatment by replicate, fetal sex, and treatment by sex interaction. The day of pregnancy was included as a covariate in the analyses. Gilt was defined as a repeated variable. The original dam nested within treatment and replicate was defined as the subject variable.

Data are presented as least squares means $\pm$ SE. Means were considered significant at $P < 0.05$. A statistical tendency was defined as $0.05 < P < 0.10$. Mean separation procedures (PDIF in SAS 9.4) were used where appropriate. Both compound symmetry and heterogeneous compound symmetry covariance structures were tested for the analyses of repeated measures using PROC MIXED. The covariance structure that successfully solved and met the convergence criteria and with the lowest Akaike's Information Criteria and Bayesian Information Criteria was used. Given these criteria, the compound symmetry covariance structure was used for the majority of the analyses.

Results

Data collected during the treatment period (GHS or GTN)

The GHS gilts had greater rectal temperature in the morning (38.4 ± 0.02 vs. 38.0 ± 0.02 °C; $P < 0.001$) and afternoon (38.5 ± 0.02 vs. 38.1 ± 0.02 °C; $P < 0.001$) and greater respiration rate in the morning (35.2 ± 0.9 vs. 19.3 ± 0.9 bpm; $P < 0.001$) and afternoon (49.4 ± 1.5 vs. 19.9 ± 1.5 bpm; $P < 0.001$) compared with GTN gilts (Figure 3). There was a treatment by replicate interaction ($P < 0.015$) for morning rectal temperature but treatment by replicate was not significant ($P > 0.10$) for afternoon rectal temperature, morning respiration rate, or afternoon respiration rate. There was an effect of day ($P < 0.001$), treatment by day ($P < 0.001$), and replicate by day ($P < 0.001$) for each of morning rectal temperature, afternoon rectal temperature, morning respiration rate, and afternoon respiration rate (Figure 3).

Regardless of treatment, nearly 99% of all morning feed rations were fully consumed. Across all replicates and feedings ($n = 1,961$), there were 16 instances (1.9% of feedings) where a GHS gilt did not consume her entire morning ration and 4 instances (0.5% of feedings) where a GTN sow did not consume her entire ration ($X^2 = 6.9$; $P < 0.01$ for treatment comparison).

There was an effect of treatment ($P < 0.014$) and replicate ($P < 0.001$) for BW gain during treatment because the GHS sows gained more weight than GTN (12.0 ± 1.3 and 7.2 ± 1.3 kg, respectively) and replicate 1 gilts gained less weight than replicate 2 or 3 gilts during the treatment period (3.2 ± 1.6 , 11.4 ± 1.6 and 14.3 ± 1.6 kg, respectively).

Slaughter immediately following GHS or GTN treatment (mid-gestation)

There was no effect ($P > 0.10$) of treatment or replicate on the total ovarian weight, the number of CL, the total number of fetuses (viable + nonviable), the total number of viable fetuses, the number of mummies, or the percent survival of fetuses (number of fetuses/number of CL; Table 1). There was a treatment by replicate interaction ($P < 0.05$) for the total number of fetuses, the number of viable fetuses, and the percent survival of fetuses that was explained by numerically smaller litters for GHS vs. GTN in replicates 1 and 3 but larger litters for GHS vs. GTN in replicate 2.

There was no effect ($P > 0.10$) of treatment or replicate on fetal weight, placental weight, or placental efficiency (Table 1). There was an effect of sex of fetus ($P < 0.001$) on fetal weight because male fetuses weighed more than female fetuses (85.2 ± 2.4 and 81.5 ± 2.4 g, respectively). Weight of fetus increased by 7.4 ± 1.0 g/d (effect of day; $P < 0.01$) during the period that gilts were slaughtered (day 55 to 58 of gestation).

Figure 3. Least square means $\pm$ SE (bar) for rectal temperature (A) and respiration rate in bpm (B) for gestational heat stress (GHS) or gestational thermoneutral (GTN) gilts during the treatment period.

There was no effect ($P > 0.10$) of treatment or treatment by replicate interaction for ovarian weight, ovarian weight as a percentage of fetal weight, testicular weight, or testicular weight as a percentage of fetal weight (Table 1). There was a tendency for an effect of replicate on ovarian weight ($P < 0.051$) and ovarian weight as a percentage of fetal weight ($P < 0.080$) because fetuses had progressively lighter ovaries for replicates 1 to 3. For the female fetuses, there was a tendency ($P < 0.073$) for an effect of treatment on the number of oogonia per section (fewer oogonia in GHS compared with GTN; Table 1). There was an effect of replicate on the number of oogonia per section (79.2 ± 3.0 , 56.2 ± 3.2 and 64.6 ± 3.2 ; replicates 1 to 3; $P < 0.001$). For male fetuses, there was no effect ($P > 0.10$) of treatment or treatment by replicate on the number of STs per section, the average area of STs, the number of prespermatogonia per section, the number of prespermatogonia per ST, or the number of Sertoli cells per tubule (Table 1). There was a tendency for an effect of replicate ($P < 0.056$) on the number of prespermatogonia per tubule (3.0 ± 0.1 , 2.4 ± 0.2 , and 2.8 ± 0.2 ; replicates 1 to 3).

Farrowing after the GHS or GTN treatment

There was no effect ($P > 0.10$) of treatment, replicate, or treatment by replicate on gestation length, total born, or born alive (Table 2). There was a tendency ($P < 0.070$) for an effect of sex on piglet birth weight (males heavier than females; 1.32 ± 0.03 vs.

Table 1. Least squares means $\pm$ SE for ovarian, litter, fetal, and placental measurements at the end of the second month of gestation in gilts subjected to GHS or GTN during the second month of gestation

Item	Treatment ¹		P-value
	GHS	GTN	
N	12	11	—
Maternal ovarian data			
Total ovarian wt, g	17.6 $\pm$ 1.5	16.8 $\pm$ 1.6	0.724
Number of CL	15.8 $\pm$ 0.9	16.9 $\pm$ 0.9	0.422
Maternal litter data			
Total number of fetuses	14.1 $\pm$ 0.8	15.4 $\pm$ 0.9	0.346
Number of viable fetuses	13.8 $\pm$ 0.8	15.3 $\pm$ 0.9	0.253
Number of mummies	0.2 $\pm$ 0.1	0.3 $\pm$ 0.1	0.576
Survival (number of fetuses/number of CL), %	88.6 $\pm$ 4.1	90.4 $\pm$ 4.3	0.758
Female fetal data			
N	83	67	—
Fetal wt, g	79.1 $\pm$ 3.4	83.7 $\pm$ 3.4	0.355
Placental wt, g	152.0 $\pm$ 17.5	167.7 $\pm$ 17.3	0.534
Efficiency (fetal wt/placental wt), %	59.4 $\pm$ 4.5	55.1 $\pm$ 4.5	0.512
Ovarian wt, mg	25.7 $\pm$ 1.0	25.9 $\pm$ 1.0	0.886
Ovarian wt as a percentage of fetal wt, %	0.032 $\pm$ 0.001	0.031 $\pm$ 0.001	0.702
No. of oogonia per ovarian section	63.3 $\pm$ 2.3	70.1 $\pm$ 2.6	0.073
Male fetal data			
N	87	101	—
Fetal wt, g	83.8 $\pm$ 3.4	87.0 $\pm$ 3.3	0.513
Placental wt, g	154.0 $\pm$ 15.1	164.6 $\pm$ 14.9	0.626
Efficiency fetal wt/placental wt), %	60.1 $\pm$ 4.6	58.7 $\pm$ 4.5	0.836
Fetal testes wt, mg	34.0 $\pm$ 1.5	33.1 $\pm$ 1.5	0.666
Testes wt as a percentage of fetal wt, %	0.040 $\pm$ 0.001	0.038 $\pm$ 0.001	0.222
Number of ST per section	18.8 $\pm$ 0.5	18.8 $\pm$ 0.5	0.977
ST area, μm^2	11,205 $\pm$ 373	11,141 $\pm$ 353	0.904
Number of prespermatogonia per section	50.4 $\pm$ 2.2	48.7 $\pm$ 2.0	0.589
Number of prespermatogonia per ST cross section	2.8 $\pm$ 0.1	2.7 $\pm$ 0.1	0.566
Number of Sertoli cells per ST cross section	18.3 $\pm$ 0.5	18.4 $\pm$ 0.5	0.934

¹GHS, gestational heat stress; GTN, gestational thermoneutral.

1.28 $\pm$ 0.03 kg, respectively) but there was no effect ($P > 0.10$) of treatment, replicate, treatment by replicate, or interactions with sex on piglet birth weight.

Growth and survival from birth to weaning for GHS-G1 and GTN-G1 piglets

Weights at processing (3 d of age), week 1 of age, week 2 of age, and weaning (Table 2) were not affected ($P > 0.10$) by treatment, replicate, treatment by replicate, sex of piglet, or interaction with sex of piglet. The percentage of G1 gilts that survived from birth to weaning was similar ($P > 0.10$) for GHS-G1 and GTN-G1 (Table 2). The effect of sex was not significant for survival from birth to weaning ($P > 0.10$). There was an effect of replicate ($P < 0.035$) because fewer piglets from replicate 1 survived to weaning (117/156 [75.0%], 143/157 [91.1%], and 144/167 [86.2%]; replicates 1 to 3, respectively). The lower survival in replicate 1 was associated with an outbreak of “greasy pig” (*Staphylococcus hyicus*) in the farrowing room during replicate 1 (diagnosis based on consultation with the attending veterinarian; University of Missouri College of Veterinary Medicine).

Histological analyses of testes collected from GHS-G1 and GTN-G1 boar piglets

For boar piglets castrated on day 3 of age, there was no effect ($P > 0.10$) of treatment, replicate, or treatment by replicate on testes weight or testes weight as a percentage of BW (Table 2).

There was no effect ($P > 0.10$) of treatment, replicate, or treatment by replicate on the number of STs per histological cross section or the number of Sertoli cells per tubule (Table 2). The area of the cross section of the STs was not affected by treatment or treatment by replicate but there was an effect of replicate ($P < 0.001$) because the area of the tubules was greater for replicate 3 (15,394 $\pm$ 365, 15,014 $\pm$ 353, 17,648 $\pm$ 312 μm^2 ; replicates 1 to 3, respectively). There was a tendency ($P < 0.100$) for an effect of treatment on the total number of prespermatogonia per section and an effect of treatment ($P < 0.049$) on the number of prespermatogonia per ST because the number of prespermatogonia was reduced for male piglets born to GHS compared with GTN gilts (Table 2). There was an effect of replicate on the number of prespermatogonia per section (21.6 $\pm$ 1.2, 19.3 $\pm$ 1.2, 15.5 $\pm$ 1.1; replicates 1 to 3, respectively; $P < 0.004$) and an effect of replicate on the number of prespermatogonia per tubule (3.0 $\pm$ 0.2, 2.8 $\pm$ 0.2, 2.3 $\pm$ 0.2; replicates 1 to 3, respectively; $P < 0.014$) because the number of prespermatogonia per section and the number of prespermatogonia per tubule decreased from replicates 1 to 3.

Survival from weaning to 160 d of age for GHS-G1 and GTN-G1 gilts

The percentage of G1 piglets that survived from weaning to the end of the nursery phase (5 to 7 wk after weaning) was similar ($P > 0.10$) for GHS-G1 and GTN-G1 (Table 2). There was an effect

Table 2. Least squares means $\pm$ SE for neonatal litter and offspring (G1) data in gilts subjected to GHS or GTN during the second month of gestation and subsequently allowed to farrow

Item	Treatment ¹		P-value
	GHS	GTN	
Number of gilts	18	18	—
Number of piglets ²	238	249	—
Litter data			
Total born	13.1 $\pm$ 0.7	13.6 $\pm$ 0.7	0.598
Born alive	12.7 $\pm$ 0.6	13.4 $\pm$ 0.6	0.419
Gestation length, d	114.7 $\pm$ 0.4	114.6 $\pm$ 0.4	0.768
Female piglet data (G1)			
N	109	118	—
Birth wt, kg	1.27 $\pm$ 0.04	1.28 $\pm$ 0.04	0.859
Day 3 wt, kg	1.61 $\pm$ 0.06	1.58 $\pm$ 0.06	0.662
Weaning wt, kg	5.54 $\pm$ 0.23	5.50 $\pm$ 0.23	0.907
Survival birth to weaning	92/109 (84%)	101/118 (86%)	0.822
Survival in nursery	65/92 (71%)	76/101 (75%)	0.766
Pubertal by 225 d, %	42/64 (66%)	58/76 (76%)	0.200
Age at puberty, d	207 $\pm$ 3	206 $\pm$ 2	0.800
Male piglet data (G1)			
N	127	126	
Birth wt, kg	1.32 $\pm$ 0.05	1.33 $\pm$ 0.05	0.875
Day 3 wt, kg	1.62 $\pm$ 0.06	1.65 $\pm$ 0.06	0.795
Weaning wt, kg	5.48 $\pm$ 0.21	5.75 $\pm$ 0.21	0.365
Survival birth to weaning	105/127 (83%)	106/126 (84%)	0.632
Survival in nursery	87/105 (83%)	96/106 (91%)	0.488
Testes wt, g	1.33 $\pm$ 0.06	1.44 $\pm$ 0.06	0.213
Testes wt as a percentage of BW, %	0.084 $\pm$ 0.003	0.088 $\pm$ 0.003	0.294
Number of ST per section	7.1 $\pm$ 0.1	6.9 $\pm$ 0.1	0.259
ST area, μm^2	15,700 $\pm$ 281	16,337 $\pm$ 281	0.120
Number of prespermatogonia per section	17.6 $\pm$ 1.0	20.0 $\pm$ 1.0	0.100
Number of prespermatogonia per ST cross section	2.5 $\pm$ 0.1	2.9 $\pm$ 0.1	0.049
Number of Sertoli cells per ST cross section	21.9 $\pm$ 0.3	21.6 $\pm$ 0.3	0.575

¹GHS, gestational heat stress; GTN, gestational thermoneutral.²There were 6 mummies and 1 piglet with unidentified sex across all farrowings.

of sex ($P < 0.001$) because more male piglets survived in the nursery than female piglets (183/211 [86.7%] vs. 141/193 [73.1%], male vs. female, respectively). There was an effect of replicate ($P < 0.001$) because fewer piglets from replicate 1 survived in the nursery (71/117 [60.7%], 129/143 [90.0%], and 124/144 [86.1%]; replicates 1 to 3, respectively). The lower survival in the nursery for replicate 1 was associated with an outbreak of hemolytic *Escherichia Coli* during replicate 1 (diagnosis from the attending veterinarian and confirmed by the University of Missouri College of Veterinary Medicine Diagnostic Laboratory).

Male piglets were sold after leaving the nursery. Gilts that left the nursery were moved to the MOF. All gilts leaving the nursery survived in the MOF until 160 d of age when estrus detection for puberty was initiated. There was no effect of treatment or treatment by replicate on the percentage that reached puberty or the age at puberty (Table 2). There was an effect of replicate for the percentage pubertal by 225 d and age because a lesser percentage of pigs from replicate 3 were detected in estrus by 225 d (24/28 [85.7%], 49/59 [83.1%], and 27/53 [50.9%]; replicates 1 to 3, respectively; $P < 0.018$). The average age at puberty was lesser ($P < 0.007$) for replicate 1 gilts (198 $\pm$ 3, 212 $\pm$ 2, and 210 $\pm$ 3 d; replicates 1 to 3, respectively).

G1 mid-gestation slaughter

There was no effect ($P > 0.10$) of treatment, replicate, or treatment by replicate on the weight of the ovary at slaughter (Table 3). There were fewer CL ($P < 0.038$) on the ovaries of GHS-G1

compared with GTN-G1 gilts (Table 3). The effects of replicate or treatment by replicate were not significant for the number of CL. There was no effect ($P > 0.10$) of treatment or treatment by replicate on the total number of fetuses, the number of viable fetuses, or the percent survival (number of fetuses/number of CL; Table 3). There was an effect of replicate because the total number of fetuses (10.9 $\pm$ 0.8, 11.6 $\pm$ 0.6, and 14.3 $\pm$ 0.9; replicates 1 to 3; $P < 0.021$) and the number of viable fetuses (10.9 $\pm$ 0.8, 11.5 and 0.6, and 14.1 $\pm$ 0.9; replicates 1 to 3; $P < 0.029$) were greater for replicate 3. The percent survival increased from replicates 1 to 3 (64.9 $\pm$ 5.5, 72.9 $\pm$ 4.4, and 88.9 $\pm$ 5.7%; replicates 1 to 3; $P < 0.019$).

There was no effect ($P > 0.10$) of treatment or replicate on fetal weight, placental weight, or placental efficiency (Table 3). There was an effect of sex of fetus on fetal weight and placental weight because male fetuses weighed more than female fetuses (123.1 $\pm$ 1.7 and 114.0 $\pm$ 1.8 g, respectively; $P < 0.001$) and had heavier placenta (173.6 $\pm$ 5.6 and 154.8 $\pm$ 5.6 g, respectively; $P < 0.001$) than female fetuses. The weights of fetus and placenta increased by 10.7 $\pm$ 0.3 g/d ($P < 0.001$) and 8.4 $\pm$ 0.9 g/d ($P < 0.001$) during the period that gilts were slaughtered (day 56 to 63 of gestation). The effect of replicate was significant for fetal weight (114.9 $\pm$ 2.9, 116.0 $\pm$ 2.5, and 124.9 $\pm$ 2.9 g; replicates 1 to 3; $P < 0.042$), placental weight (159.2 $\pm$ 9.3, 140.0 $\pm$ 8.4, and 193.5 $\pm$ 9.6 g; replicates 1 to 3; $P < 0.001$), and placental efficiency (78.1 $\pm$ 4.0, 89.3 $\pm$ 3.6, and 67.8 $\pm$ 4.1%; replicates 1 to 3; $P < 0.002$). There was a tendency for a treatment by sex

Table 3. Least squares means $\pm$ SE for uterine, ovarian, litter, and placental measurements in G1 gilts whose mothers were subjected to GHS or GTN ambient temperature during the second month of gestation

Item	Treatment ¹		P-value
	GHS	GTN	
Number of gilts	30	37	—
Maternal ovarian data			
Total ovarian wt, g	16.4 $\pm$ 0.6	16.7 $\pm$ 0.6	0.712
Number of CL	15.6 $\pm$ 0.5	17.1 $\pm$ 0.4	0.038
Maternal litter data			
Total number of fetuses	11.9 $\pm$ 0.7	12.7 $\pm$ 0.6	0.333
Number of viable fetuses	11.8 $\pm$ 0.7	12.5 $\pm$ 0.6	0.434
Survival (number of fetuses/number of CL), %	77.7 $\pm$ 4.5	73.4 $\pm$ 4.1	0.492
Female fetal data			
N	176	213	
Fetal wt, g	113.1 $\pm$ 2.6	116.6 $\pm$ 2.5	0.334
Placental wt, g	158.9 $\pm$ 7.7	151.0 $\pm$ 7.5	0.470
Efficiency (fetal wt/placental wt), %	77.7 $\pm$ 3.7	83.0 $\pm$ 3.7	0.316
Male fetal data			
N	164	255	
Fetal wt, g	123.5 $\pm$ 2.4	121.5 $\pm$ 2.1	0.526
Placental wt, g	182.3 $\pm$ 7.6	163.9 $\pm$ 6.8	0.085
Efficiency (fetal wt/placental wt), %	73.2 $\pm$ 3.0	80.2 $\pm$ 2.6	0.096

¹GHS, gestational heat stress; GTN, gestational thermoneutral.

interaction ($P < 0.077$) because male GHS-G1 fetuses had heavier placentas than male GTN-G1 fetuses but this effect was not observed for females (Table 3). Placental efficiency tended to be lesser for GHS-G1 males fetuses compared with GTN-G1 male fetuses (Table 3).

Discussion

We tested the hypothesis that GHS during the second month of gestation would have long-term negative consequences for the reproduction of boars and gilts through its effects on fetal gonad development. The temperature and humidity cycle that we imposed approximated a summer heat wave in Missouri and created an afternoon THI in excess of 90. Compared with GTN, the GHS gilts had a 0.4 °C increase in the afternoon rectal temperature and approximately 2.5-fold increase in the afternoon respiration rate. We have found similar responses in gestating sows in our previous work (reviewed by Lucy and Safranski, 2017). Others have reported increases in rectal temperature in excess of 1 °C in gilts exposed to a similar heat stress (Wildt et al., 1975). It is possible that the housing that we used in this study afforded a greater opportunity for convective heat loss (through the concrete floor) or evaporative heat loss through the wetting of the floor with nipple waterers. In either case, the extent of the thermal response to the stress that we applied was quantified with respect to body temperature and respiration rate. A greater thermal response may have had a greater effect on the endpoints that we measured.

There was no effect of treatment on the size of the litter or weights of the fetuses or placenta (Table 1). The heat stress was initiated at the end of the fourth week of pregnancy after gilts had been diagnosed pregnant and moved into the environmental chambers. Heat stress reduces litter size when applied early in gestation (Edwards et al., 1968; Omtvedt et al., 1971; Wildt et al., 1975) but our treatment application was after this early window of development. There was a numeric decrease in the total number of fetuses (approximately 1.5 fewer for the GHS treatment; Table 1) but the experiment was not designed

to detect relatively small differences in litter size. We applied the total number of fetuses in the litter as a covariate in the remaining statistical analyses of the fetal data that we collected (Table 1). This was done to account for possible differences in the litter size that were established before the heat stress was applied. We found that GHS did not affect the weight of the fetus or placenta. Regardless of sex, the GHS and GTN fetuses and placenta were nearly identical in weight (Table 1).

The earliest developmental measurements that were related specifically to our hypothesis were the counts of oogonia and prespermatogonia in the fetal gonad. We used the ImageJ program to count the number of prespermatogonia and oogonia. Every nucleus was evaluated across all sections in an objective manner using predefined criteria for size and circularity to define the specific cell type. We selected ImageJ over manual counting because it was an objective method and also faster than manual counting. The correlation coefficients that we obtained for manual vs. automated counting (approximately 0.75) would be classified as on the lower end of “high positive” (greater than 0.7) but not “very high positive” (above 0.9; Mukaka, 2012). Our correlation coefficients with manual counting were comparable to other published studies that used automated procedures to count the number of cells in tissue sections (Meruvia-Pastor et al., 2011; Dordea et al., 2016). Compared with GTN, we found no effect of the GHS on the weight or the morphology of the fetal testes (Table 1). The GHS did not affect the weight of the fetal ovary but we detected a tendency for a reduced number of oogonia per section in the GHS vs. GTN gilts (Table 1). Numerically, the GHS fetuses had approximately 10% fewer oogonia per section.

The next series of developmental endpoints was measured on the offspring of the GHS and GTN gilts. These analyses involved litters from 18 GHS gilts and 18 GTN gilts and nearly 500 G1 piglets (Table 2). We found no effect of GHS on litter size, gestation length, or piglet weights from birth to weaning. We castrated G1 boars at 3 d of age and studied the development of the testes in boars that came from the GHS compared with GTN gilts. As with the fetal testes, the GHS did not affect testis weight, the number of STs,

the area of STs, or the number of Sertoli cells in neonatal testes. There was an effect of GHS, however, on the average number of prespermatogonia per ST cross section (Table 2). The magnitude (approximately 15% reduction) and direction of the response corroborated data from Lugar et al. (2018) who showed a 23% reduction in sperm production from a subset of boars from these litters that were left intact and transported to Purdue University for the Lugar study. Erickson and Martin (1984) reported reduced sperm production and increased sperm abnormalities in boars irradiated in utero. Collectively, the data from Erickson and Martin (1984), Lugar et al. (2018), and the present study indicate that the fetal testes are sensitive to environmental insults. Heat stress is one potential environmental insult, and furthermore, a 1-mo period of maternal heat stress during a critical period of testicular development can cause long-term effects on sperm production. We are not aware of any data in any species that has applied a targeted period of heat stress to a specific period of testicular development in the fetus and demonstrated permanent damage to the testes.

Gilts from the GHS and GTN litters (G1) were left intact so that their reproductive capacity through first pregnancy could be studied. Neither the percentage of gilts pubertal by 225 d of age nor age at puberty was affected by GHS (Table 2). The G1 gilts were inseminated at their first postpubertal estrus after 210 d of age and slaughtered at the end of their second month of pregnancy. At the time of slaughter, we observed the number of CL was reduced for the GHS-G1 compared with GTN-G1 gilts (Table 3). The reduction in the number of CL for GHS was approximately 10% compared with control and of similar magnitude to the numeric decrease in oögonia in the fetal gilts (Table 1). One interpretation for these data is that the heat stress applied during gonadal development reduced the population of ovarian follicles in the G1 gilts and this led to a reduction in ovulation rate and the number of CL formed thereafter. The work of Erickson and Martin (1984) demonstrated that irradiation of the pregnant pig could reduce the number of oocytes in the offspring to cause infertility by second parity. The depletion of the oocyte pool is one explanation for infertility in younger women that experience premature menopause (Vabre et al., 2017). A similar phenomenon may exist in gilts that are exposed to heat stress in utero. As with the testes, we are not aware of any data in any species that has applied a targeted period of heat stress to a specific period of ovarian development in the fetus and demonstrated permanent damage to the ovary.

Ovulation rate was reduced but we did not detect a significant reduction in the litter size for GHS-G1 gilts (Table 3). This perhaps demonstrates that uterine capacity was limiting litter size in the GHS-G1 gilts. The experiment was not designed to test the long-term effects of GHS on the fertility of sows. The fertility of older sows (second or greater parity) may be compromised through oocyte depletion. This should be explored in future studies. A complete histological analysis of the adult ovaries where primordial and developing follicles are counted should also be conducted.

An unexpected finding from the G1 slaughter was that male fetuses from the GHS-G1 gilts were similar in weight but tended to have heavier placenta compared with male fetuses from the GTN-G1 gilts (Table 3). This difference in placental weight was not observed for female fetuses for GHS-G1 vs. GTN-G1. The oocytes that gave rise to the male fetuses in the G1 gilts were forming within a fetal ovary exposed to heat stress. There may be unique imprinting of DNA caused by heat stress that specifically affects male but not female placental development. The autosomes in the male fetuses were derived from both the

G1 oocytes (heat-stressed in utero) and the sperm from the boars used for AI (presumably not heat stressed). In the fetal boars, the X chromosome was derived from the G1 oocytes and the Y chromosome was derived from the boars used for AI. The fetal gilts (not affected by GHS in this study) possessed an X chromosome from the G1 oocyte and the AI boar. In most eutherian mammals, the paternal X chromosome is inactivated in the placenta (Hemberger, 2002). The pig is different because there is random X inactivation of maternal and paternal X chromosomes in the placenta (Zou et al., 2019). Placental development in the pig, therefore, is controlled by genes expressed from both the paternal and maternal X chromosome. In the female fetus, both the paternal and maternal X chromosomes are randomly inactivated in the placenta. In the male fetus, there is a single X chromosome from the mother and it is not inactivated. We speculate that this unique aspect of X chromosome inactivation in the pig may have enabled the unexpected result that we observed—specifically that the placenta of male fetuses but not female fetuses was increased in size. Aberrant expression of genes on the maternally derived X chromosome in the male fetuses may have led to enlarged placenta. In the female fetuses, where there is random inactivation of one of two X chromosomes, the influence of the maternally derived X chromosome may be less. Our line of reasoning depends on the assumption that the imprinting of the X chromosome partially controls placental development, which is true for many mammalian species (Gabor et al., 2013). The possibility that the X chromosome controls placental development in the pig and that random X inactivation in the placenta leads to sexual dimorphism in response to heat stress should be studied further.

Three observations from this study were not specifically related to the original hypothesis but nonetheless should be discussed. First, the heat stress applied during the second month of gestation led to an increase in BW for the GHS gilts. We have observed this phenomenon in other studies and attributed it to reduced activity in pigs that are heat stressed (Lucy and Safranski, 2017). Second, the GHS did not affect birth weight, the growth of piglets from birth to weaning, or survival from birth to weaning or survival in the nursery compared with control. Lugar et al. (2018) measured the growth in the boars taken from this study and also reported no effect on growth (BW through 37 wk of age). Johnson et al. (2020) reviewed studies that demonstrated reduced growth and immune function in offspring exposed to heat stress in utero. Maskal et al. (2020), for example, demonstrated reduced average daily gain for GHS pigs after weaning; a period during which we did not make BW measurements in the present study. The possibility that the focused period of GHS that we applied (second month of gestation) does not affect growth in offspring should be studied further. Second, there was typically an effect of replicate on the data that we collected but the effect of treatment by replicate was generally not significant. The effects of replicate could be explained by a number of factors unique to each replicate, including season, the boar used for AI, or health of the individual animals. In replicate 1, for example, fewer piglets survived to weaning because there was an outbreak of “greasy pig” (*S. hyicus*) in the farrowing room. Fewer pigs survived in the nursery for replicate 1 as well because there was an outbreak of hemolytic *E. Coli*. Although survival was reduced for replicate 1, the treatment by replicate interaction was not significant for survival in the farrowing room or nursery. The capacity of the GHS-G1 pigs to survive these unexpected disease challenges appeared to be equivalent to the GTN-G1 pigs. This conclusion

was inconsistent with studies reviewed by Johnson et al. (2020) showing reduced immune function in GHS pigs and other farm animals. As with the effects on growth, the possibility that the focused period of GHS during the second month of gestation does not affect immune function in offspring should be studied further. It may be necessary to delineate more defined periods of pregnancy to completely understand the effects of GHS on the postnatal performance of offspring.

In summary, gilts were heat stressed during the second month of gestation when the gonads were developing. We found evidence for: 1) a reduced number of prespermatogonia in neonatal boars, 2) reduced sperm production in adult boars (Lugar et al., 2018), 3) a reduced number of oogonia in fetal gilts, and 4) lesser ovulation rate in adult gilts during the first pregnancy. The heat stress did not affect the other aspects of pregnancy development during the treated gestation, growth or survival of offspring from farrowing through the nursery phase, puberty in offspring, or establishment of first pregnancy in offspring. From an applied perspective, heat stress during the second month of gestation in sows whose offspring are destined to be replacement gilts or boars may reduce the performance of the breeding herd. Additional studies should examine the long-term implications of maternal heat stress during gonadal development on the productivity of older boars and sows (parity 2 or greater).

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Conflict of interest statement

No conflict of interest, financial, or otherwise are declared by the author(s).

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