Characterization of the acute heat stress response in gilts: III. Genome-wide association studies of thermotolerance traits in pigs

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ABSTRACT: Heat stress is one of the limiting factors negatively affecting pig production, health, and fertility. Characterizing genomic regions responsible for variation in HS tolerance would be useful in identifying important genetic factor(s) regulating physiological responses to HS. In the present study, we performed genome-wide association analyses for respiration rate (RR), rectal temperature (T_R), and skin temperature (T_S) during HS in 214 crossbred gilts genotyped for 68,549 single nucleotide polymorphisms (SNP) using the Porcine SNP 70K BeadChip. Considering the top 0.1% smoothed phenotypic variances explained by SNP windows, we detected 26, 26, 21, and 14 genes that reside within SNPs explaining the largest proportion of variance (top 25 SNP windows) and associated with change in RR (ΔRR) from thermoneutral (TN) conditions to HS environment, as well as the change in prepubertal T_R (ΔT_R), change in postpubertal ΔT_R, and change in T_S (ΔT_S), respectively. The region between 28.85 Mb and 29.10 Mb on chromosome 16 explained about 0.05% of the observed variation for ΔRR. The growth hormone receptor (*GHR*) gene resides in this region and is associated with the HS response. The other important candidate genes associated with ΔRR (*PAIP1*, *NNT*, and *TEAD4*), ΔT_R (*LIMS2*, *TTR*, and *TEAD4*), and ΔT_S (*ERBB4*, *FKBP1B*, *NFATC2*, and *ATP9A*) have reported roles in the cellular stress response. The SNP explaining the largest proportion of variance and located within and in the vicinity of genes were related to apoptosis or cellular stress and are potential candidates that underlie the physiological response to HS in pigs.

Key words: genome-wide association, gilt, heat stress, pig

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INTRODUCTION

Heat stress (HS) is a hurdle to efficient animal agriculture productivity (Renaudeau et al., 2012; Baumgard and Rhoads, 2013) and the global changes in temperature are expected to become increasingly erratic (IPCC, 2007). In pigs, HS is an annual limiting factor affecting production, health, and fertility and results in significant economic losses (St-Pierre et al., 2003; Ross et al., 2017). From a traditional production parameter standpoint, HS increases mortality (D'Allaire et al., 1996), reduces milk production (Renaudeau and Noblet, 2001) and litter survival (Wettemann and Bazer, 1985; Renaudeau et al., 2003; St-Pierre et al., 2003), markedly decreases growth rate and feed intake (FI) (Collin et al., 2001; Campos et al., 2014), and substantially increases the variability in market weight (Baumgard and Rhoads, 2013). Pigs are particularly sensitive to HS due to their

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inability to sweat and the presence of a thick layer of subcutaneous adipose tissue that prevents heat dissipation (Renaudeau et al., 2006; Fernandez et al., 2015). Commercial pig breeds have been intensely selected for economically important phenotypes, such as increased growth rate and leaner body composition, and this has inadvertently resulted in increased HS susceptibility (Renaudeau et al., 2012) since synthesizing and maintaining lean tissue increases basal heat production.

Genetic variation exists in thermal tolerance among species, between breeds, and within breed (Blackshaw and Blackshaw, 1994; Hoffmann, 2010; Renaudeau et al., 2012), and thus, may provide opportunity to improve thermal tolerance through using genetic tools to identify genomic regions of importance in the response to HS. For instance, recent genome-wide association studies (GWAS) in dairy cattle have identified genomic regions associated with T_R during HS (Dikmen et al., 2013). The development of a high-density Porcine SNP BeadChip has aided the implementation of efficient genomic evaluation and selection in the commercial pig industry (Fernández et al., 2012). Despite the economic and animal welfare effects of HS on pork production and pig health, identifying genomic regions responsible for variation in HS tolerance has not yet been thoroughly explored. In the pig, single nucleotide polymorphism (SNP) markers have been chiefly used for association analysis of growth, meat, and carcass quality traits. The objectives of this study were to conduct GWAS to identify genomic regions associated with thermotolerance traits in crossbred gilts.

MATERIALS AND METHODS

Animals and Experimental Design

The Iowa State University Institutional Animal Care and Use Committee approved all procedures involving animals. Detailed description of experimental designs and how the body temperature variables were calculated during prepubertal and postpubertal development have been described in two other studies that established the HS phenotypes. (Graves et al., 2018; Seibert et al., 2018). Seibert et al. (2018) established the production phenotypes in response to HS while Graves et al. (2018) utilized a subset of the same group of gilts and established the repeatability of the phenotypes later in life and the relationship between the HS response and reproductive success. Collectively, crossbred gilts (n = 235; PIC maternal × Duroc terminal sire) from the same cohort were received on the 24th day of age and arrived immediately after weaning. Due to logistical constraints of the facilities, the experiment was conducted in five replications (n = 44 to 48/replicate). The initial BW from replications 1 to 5 were 59 ± 1.0, 64 ± 1.2, 77 ± 1.2, 88 ± 1.1, and 103 ± 1.6 kg, respectively (Seibert et al., 2018). During the experiment, water and feed were provided ad libitum during the entire experiment. All pigs were fed a standard diet consisting mainly of corn and soybean meal formulated to meet or exceed nutrient requirements (NRC, 2012). The study was divided into three experimental periods (P) for each replicate: P0, P1, and P2. Period 0 (72 h) served as an acclimation period in which all pigs were housed individually in thermoneutral (TN) conditions (21.9 ± 0.5 °C, 62 ± 13% relative humidity [RH]). After P0, pigs remained in TN conditions for 24 h (period 1; P1) and then exposed to HS (29.7 ± 1.3 °C, 49 ± 8% RH) conditions for 24 h (period 2; P2). Pigs were exposed to a 12:12 h light:dark cycle during P0, but continuous light during P1 and P2 to allow for accurate data collection.

T_R (°C) was measured with a lubricated, calibrated digital thermometer (Welch Allyn SureTemp Plus 690, Skaneateles Falls, NY). T_S (°C) was measured using a calibrated infrared thermometer (ST 380A Infrared Thermometer, HDE, Allentown, PA), and RR (breaths per minute) was determined by counting the number of flank movements in 15 s and multiplying by four. During the initial study, FI was measured daily and body temperature indices were monitored during both the 24 h TN (21.9 ± 0.5 °C, 62 ± 13% RH) and HS (29.7 ± 1.3 °C, 49 ± 8% RH) phases. BW were collected at the beginning of the acclimation and TN periods and at the end of the HS period. The difference (Δ) for physiological traits (e.g. T_R, T_S, and RR) was determined by subtracting the TN from the HS value.

Following boar exposure and heat detection, the second study (Graves et al., 2018) utilized 100 cyclic (postpubertal) animals from the initial 235 gilts. Selecting these postpubertal 100 gilts was based on their ability or inability to maintain a minimal T_R during the 24 h HS challenge. During this study, T_R, RR, and T_S were collected at 0800, 1400, 1500, 1600, 1900, 2000, and 2100 h during TN (20 °C) conditions and condensed into a single average to represent each individual's TN thermoregulatory set point. All body temperature indices measured at the same time points during 9 d of HS were condensed into a single average value, representing HS thermortolerance parameters. The difference for

each physiological trait (ΔT_R, ΔT_S, and ΔRR) was calculated by subtracting TN from HS values for each trait.

Marker Data|Genotyping and Quality Control

All animals (235) were genotyped using the GGP-Porcine HD BeadChip (GeneSeek, Lincoln, NE), which contains 68,249 SNP that uniformly span the porcine genome according to Illumina's standard protocols (http://www.illumina.com). Autosomal and X chromosome markers were filtered for the call rate ≥95%; Hardy–Weinberg equilibrium (HWE) <0.0001 and minor allele frequency (MAF) ≥0.05. Additionally, of the total animals genotyped, 21 individual samples failed to have at least a call rate of 95% and were excluded. After applying the above quality control criteria, a total of 52,528 SNP for 214 animals remained for the subsequent GWAS analysis. Quality control measures were performed using SNP and Variation Suit v8.3.1 (Golden Helix, Inc., Bozeman, MT, www.goldenhelix.com).

Statistical Analyses

Genome-wide association tests were performed using single-locus mixed linear model Efficient Mixed-Model Association eXpedited (EMMAX), which includes a kinship matrix as random effect and implemented by SNP and Variation Suite Version 8.3.1 software (Golden Helix, Inc.). In GWAS, lack of accounting for population structure may lead to spurious association results (Kang et al., 2010). It has been demonstrated that the EMMAX approach can correct for population stratification and relatedness between samples (Kang et al., 2010). To correct for confounding effects due to population structure and relatedness between individuals; an identity-by-state (IBS) between samples was computed from the genotype data and included as a random effect in the model. The EMMA approach and algorithm have been well described in SNP and Variation Suite Version 8.3.1 documentation (Golden Helix, Inc.).The model used can be expressed as:

$$y = X\beta + Zu + e$$

where y is an $n \times 1$ the vector of observed phenotypic values, X is an $n \times f$ matrix of fixed SNP effects, β is a $q \times 1$ vector representing coefficients of the fixed effect, Z is an $n \times t$ relating the instances of the random effects, u the vector of random effect, and e the residual effect.

Initial BW, replication, and room were included in the analyses as covariates for all of the traits. For each trait, pseudo-heritability, the fraction of phenotypic variance explained by the empirically estimated relationship matrix (Kang et al., 2010; Segura et al., 2012) was estimated with the SNP and Variation Suite (Golden Helix, Inc.).

As for several genome-wide analysis using small sample size (Dockery et al., 2017), we did not detect any SNP that passed Bonferroni adjusted P value threshold; therefore, we considered the top SNP explaining the largest proportion of variance. To reduce the specious noise from single SNP based analyses, the observed phenotypic variance accounted by an individual SNP was smoothed over five SNP sliding windows. This approach has been applied to GWAS studies in cattle and poultry (Dikmen et al., 2013; Fragomeni et al., 2014). As previously demonstrated, SNP windows explaining the largest SNP variance were considered to represent candidate gene regions associated with variation in phenotypes (Dikmen et al., 2013; Fragomeni et al., 2014). In those studies, SNP window thresholds were arbitrarily selected. For instance, Fragomeni et al. (2014) considered the top 10 windows (~200 SNPs) explaining the largest genetic variance using windows of 20 SNP, whereas Dikmen et al. (2013) considered the top 20 loci explaining the largest proportion of variance using three- and five-SNP sliding windows. Therefore, we considered the top 0.1% (25 windows) smoothed variance explained by SNP windows. The candidate genes associated with the top 0.1% SNPs were searched for from the NCBI database (http://www.ncbi.nlm.nih.gov/).

RESULTS AND DISCUSSION

Heritability estimates for prepubetal ΔT_R, ΔRR, postpubetal ΔT_R, and ΔT_S were 0.49, 0.39, 0.83, and 0.00, respectively. There are only limited studies on the heritabilities of thermotolerance traits in pigs to compare with our results. To the best of our knowledge, no prior study reported estimates of heritability for thermotolerance traits in pigs from genome-wide SNP data. Very recently, Gourdine et al. (2017) reported heritability estimates of 0.35 and 0.39 for T_R and RR, respectively, in lactating sows reared in a tropical climate. Generally, the value observed for T_R in the present study is higher than the range of values reported in cattle (0.11 to 0.44) (Da Silva, 1973; Morris et al., 1989) and poultry (0.36) (Taouis et al. 2002). The higher heritability in this study could be partly attributed to small sample size. Concurrent with

this assumption, Baco et al. (1997) showed that the average heritability decreased as the sample size increase from 100 to 400. The moderate and high heritabilities observed in this study imply that there is genetic variation in thermotolerance in pigs that can be exploited to improve heat tolerance.

In the present study, we performed GWAS for ΔRR, prepubertal or postpubertal $\Delta T_{R,}$ and ΔT_S, to identify genomic regions associated with thermoregulatory and production responses to HS in pigs using the Porcine SNP 70 BeadChip technology. Significant SNP were declared when the *P* value was less than the genome-wide type I error rate, adjusted with Bonferroni correction by using α/K, where $\alpha = 0.05$ and K = number of SNPs. We did not detect any SNP displaying the set significant threshold ($0.05/52528 = 9.5187 \times 10^{-7}$) but this was not unexpected given the limited number of observations (214 prepubertal animals and 91 postpubertal animals).

We therefore considered the top 0.1% of the smoothed phenotypic variance explained by five SNP windows. The total number of genes associated with these SNPs were 26, 26, 21, and 14 for ΔRR, prepubertal ΔT_R, postpubertal ΔT_R, and ΔT_S, respectively. The region between 28.85 Mb and 29.10 Mb on chromosome 16 (five SNPs) explained about 0.05% of the observed variation for ΔRR and includes the growth hormone receptor genomic locus (*GHR*; Table 1 and Figure 1). This is not surprising as growth hormone (GH) variables are influenced by HS. For example, HS decreases

Table 1. Phenotypic variance explained by SNP windows for delta respiration rate prior to puberty (prepubertal ΔRR)

SSC[a]	Position start (bp)[b]	Position end (bp)[c]	Variance explained (%)[d]	Candidate gene(s)[e]
14	139721921	139813511	0.077	—
14	139607069	139757205	0.059	*RAB11FIP2*
16	29375218	29645155	0.056	*LOC100524404, CCL28, PAIP1, LOC100524913*
16	29513888	29742940	0.054	*LOC100524404, PAIP1, LOC100524913, NNT*
16	26931779	27129171	0.052	*HEATR7B2, MROH2B*
16	28409425	28629545	0.051	—
16	27848815	28627099	0.049	*OXCT1, FBXO4,LOC102165724*
5	69383487	69487000	0.049	*TSPAN9, TEAD4, TULP3/TUBl3*
16	28850217	29102419	0.047	*GHR*
16	26415650	26619363	0.046	—
5	69487000	69597659	0.046	*TULP3/TUBl3, LOC100524913, LOC102162709, ITFG2, LOC102164154*
16	26861794	27039793	0.045	*LOC100737708, HEATR7B2*
16	29200306	29513888	0.045	*CCL28, LOC100524404*
16	29645155	29881595	0.045	*PAIP1,LOC100524913, PAIP1, NNT*
16	29102419	29375218	0.044	*LOC106506477, CCL28*
16	29742940	30016395	0.044	*NNT*
14	139757205	139906120	0.044	*C14H10orf84*
16	35074836	35176313	0.043	*ARL15*
16	26552965	26755662	0.043	—
16	27039793	27242934	0.043	*MROH2B, LOC106505864, C6*
16	28627099	28800253	0.043	*GHR,LOC102158502, GHR*
5	60978291	61121151	0.043	*ARHGDIB, ART4*
16	26755662	26931779	0.042	*LOC100737708*
16	32429434	32520142	0.041	—

Gene abbreviations: *RAB11FIP2 = RAB11 family interacting protein 2*; *CCL28 = C-C motif chemokine ligand 28*; *PAIP1 = poly(A) binding protein interacting protein 1*; *NNT = nicotinamide nucleotide transhydrogenase*; *HEATR7B2 = maestro heat-like repeat-containing protein family member 2B*; *MROH2B = maestro heat-like repeat family member 2B*; *OXCT1 = 3-oxoacid CoA-transferase 1*; *FBXO4 = F-box protein 4; TSPAN9 = tetraspanin 9*; *TULP3 = tubby like protein 3*; *TEAD4 = TEA domain transcription factor 4*; *GHR = growth hormone receptor*; *ITFG2 = integrin alpha FG-GAP repeat containing 2*; *ARL15 = ADP ribosylation factor like GTPase 15*; *ARHGDIB = Rho GDP dissociation inhibitor beta*; *ART4 = ADP-ribosyltransferase 4*.

[a]Chromosome number of the pig genome for which the SNP window location is mapped.

[b]SNP window positions start location on the chromosome.

[c]SNP window position end location on the chromosome.

[d]Percentage of variance explained by five SNP windows.

[e]Candidate genes located within the SNP window.

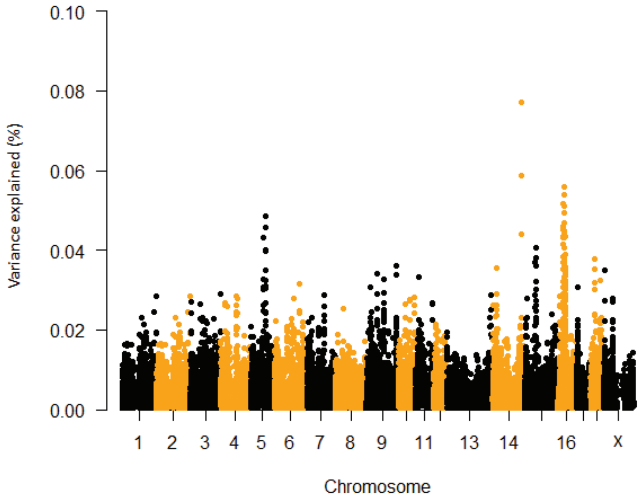

Figure 1. Manhattan plot of delta respiration rate during first HS challenge prior to puberty (prepubertal ΔRR) percentage of variance explained by SNP windows in crossbred gilts. The variance accounted by an individual SNP was smoothed over five SNP sliding windows.

GHR mRNA abundance in hepatic tissue of lactating Holstein dairy cows (Deane and Woo, 2005; Rhoads et al., 2010) and avian species (Gasparino et al., 2014; Del Vesco et al., 2015), and is independent of the heat-induced feed intake reduction (Collier et al., 2008). Additionally, although not always observed (Rhoads et al., 2009), circulating GH levels decline in HS compared to TN cattle (Farooq et al., 2010); this decrease in circulating GH is attributed to reduced GH secretion at the pituitary gland. Furthermore, primiparous cattle treated with growth hormone-releasing hormone (GHRH) during HS had increased BW gain, milk yield, pregnancy rates, and circulating prolactin (PRL), and reduced mortality (Brown et al., 2008). Polymorphisms within *GHR* have known to significantly affect growth traits including in pigs and goats (An et al., 2011; Tian et al., 2014). Considering the critical physiological and metabolic role of GHR, SNPs within this gene are likely potential selection candidates for HS tolerance.

Another important candidate gene with close proximity to *GHR* is poly(A) binding protein interacting protein 1 (*PAIP1)* which falls within a five SNP window that explained about 0.06% the variance on SSC16 at 29.37 to 29.64 Mb. Based on an in vitro experiment using HeLa cells, the abundance of PAIP1 protein decreases in response to HS (Datu and Bag, 2013). In mammals, HS increases free radical formation (reactive oxygen species; ROS) and induces oxidative stress (Lord-Fontaine and Averill-Bates, 2002). HS also induces oxidative damage in pigs (Montilla et al., 2014) and fish (Heise et al., 2006) and oxidative stress is involved in heat-induced cell death (Davidson et al., 1996). Interestingly, we detected SNP on chromosome 16 that explain 0.05% of the variance for ΔRR and contained the nicotinamide nucleotide transhydrogenase (*NNT*) gene (Table 1 and Figure 1). The *NNT* gene product is necessary to prevent ROS accretion (Arkblad et al., 2005; Nickel et al., 2015) and loss of its activity has been implicated in increased mitochondrial oxidative damage, ultimately resulting in overall increased sensitivity to oxidative stress (Arkblad et al., 2005; Navarro et al., 2012). Moreover, *Nnt* knockdown in mice leads to increased ROS production and a stronger inflammatory response in macrophages (Ripoll et al., 2012). Interestingly, it has been reported that a mutated *Nnt* gene in mice results in loss of B-cell lymphoma 2 (BCL-2) (Navarro et al., 2012), a major antiapoptotic protein implicated in the prevention of heat-induced cell death (Setroikromo et al., 2007). In vitro heat shock downregulates *Bcl-2* expression (Khar et al., 2006), which may inhibit its activity to prevent permeability of the outer mitochondrial membrane and ultimate release of apoptogenic factors (Beere, 2004). The effect of HS-induced autophagy signaling in the pig ovary demonstrated that BECN1 abundance correlates with an increase in phosphorylation of BCL2 (Hale et al., 2017). Thus, *NNT* could be involved in variation of HS-induced oxidative stress and autophagy in pigs.

For ΔRR and prepubertal ΔT_R, the SSC 5: 69.38 to 69.48 Mb region accounted for 0.05% the observed variance and contained TEA domain transcription factor 4 (*TEAD4)* or related transcription enhancer factor-1 (*RTEF-1*) (Tables 1 and 2; Figures 1 and 2). However, this region was not detected for postpubertal ΔT_R. The lack of detecting a common significant region for prepubertal ΔT_R and postpubertal ΔT_R could be ascribed to differences in either animal age or sample size or both. TEAD4 protein prevents oxidative stress in blastocoels (Kaneko and DePamphilis, 2013). Also, hypoxic inducible factor 1 alpha (*HIF-1α*) gene expression was decreased when *RTEF-1* was knocked down in endothelial cells (Jin et al., 2011). *HIF-1α* can interact with *HSP90*, which mediates heat-induced stabilization of *HIF-1α* (Katschinski et al., 2002). The region extending from 136.70 Mb to 139.10 Mb (10 loci) on SSC 14 accounted for about 0.05% of the observed variance for the prepubertal ΔT_R and encompasses the attractin-like 1 (*ATRNL1*) gene locus. Previous studies suggest selecting certain alleles in this gene may improve high-altitude adaptation (Simonson et al., 2010). Thus, *TEAD4* and *ATRNL1* represent gene candidates that could be explored as targets to improve heat tolerance in pigs.

Table 2. Phenotypic variance explained by SNP windows for the change in T_R during heat stress prior to puberty (prepubertal ΔT_R)

SSC[a]	Position start (bp)[b]	Position end (bp)[c]	Variance explained (%)[d]	Candidate gene(s)[e]
5	69383487	69487000	0.049	*TSPAN9, TEAD4*
5	72245513	72424166	0.043	*MICAL3, LOC102162673*
5	69487000	69597659	0.041	*TULP3, LOC102162709, ITFG2, LOC102164154*
14	136702381	136891524	0.040	*ATRNL1, LOC102161079*
14	13443000	13564731	0.040	*FZD3*
5	69437477	69555670	0.039	*TEAD4, TULP3, LOC102162709*
14	39275817	39773984	0.038	—
5	69333042	69437477	0.038	*TSPAN9, TEAD4*
5	72352991	72500090	0.037	*LOC102162673*
14	139721921	139813511	0.037	—
5	72141748	72352991	0.036	*BID, MICAL3*
5	69555670	69691307	0.035	*LOC102162709, ITFG2, LOC10216415, LOC102164154, LOC106510369, LOC100512907*
14	13564731	13666604	0.035	*LOC102157783, EXTL3*
14	136824061	136939847	0.035	*ATRNL1*
14	13356571	13497286	0.034	*FBXO16, FZD3*
18	27560376	27761494	0.033	*ING3, TSPAN12*
14	39616077	39904195	0.033	*LOC102157597*
14	13497286	13621432	0.033	*FZD3*
1	283482216	283609728	0.032	*SUSD1*
7	111019262	111170076	0.032	
14	136939847	137099653	0.032	*ATRNL1*
5	70488420	70775116	0.032	*ERC1, RAD52*
13	215584218	215697149	0.031	*C2CD2, LOC102161849*
5	69597659	69759629	0.031	*LOC102164154, LOC106510369, LOC100512907, IQSEC3*

Gene abbreviations: *TSPAN9 = tetraspanin 9*; *TEAD4 = TEA domain transcription factor 4*; *MICAL3 = microtubule associated monooxygenase, calponin and LIM domain containing 3*; *ITFG2 = integrin alpha FG-GAP repeat containing 2*; *EXTL3 = exostosin like glycosyltransferase 3*; *ATRNL1 = attractin-like 1*; *FBXO16 = F-box protein 16*; *FZD3 = frizzled class receptor 3*; *ING3 = inhibitor of growth family member 3*; *TSPAN12 = tetraspanin 12*; *SUSD1 = sushi domain containing 1*; *ERC1 = ELKS/RAB6-interacting/CAST family member 1*; *RAD52 = RAD52 homolog, DNA repair protein*; *C2CD2 = C2 calcium dependent domain containing 2*; *IQSEC3 = IQ motif and Sec7 domain 3*.

[a]Chromosome number of the pig genome for which the SNP window location is mapped.

[b]SNP window positions start location on the chromosome.

[c]SNP window position end location on the chromosome.

[d]Percentage of variance explained by five SNP windows.

[e]Genes located within the SNP window.

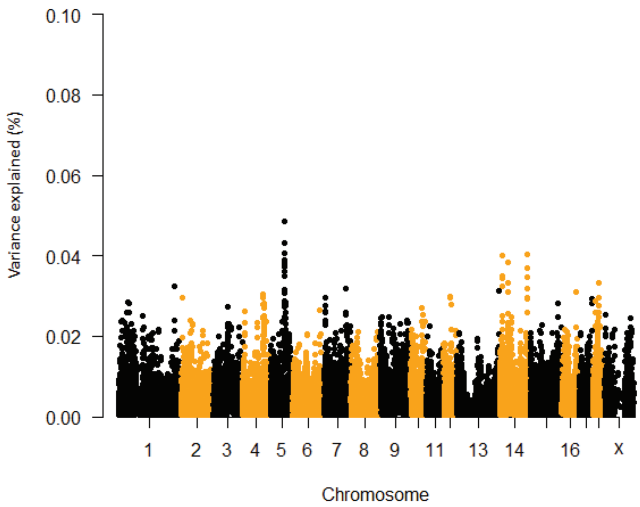

Figure 2. Manhattan plot of delta T_R first HS challenge (prepubertal ΔT_R) percentage of variance explained by SNP windows in crossbred gilts. The variance accounted by an individual SNP was smoothed over five SNP sliding windows.

Phenotypic variances explained by SNP windows postpubertal ΔT_R are shown in Table 3 and Figure 3. The region between 65.86 and 66.79 Mb encompassed the LIM and senescent cell antigen-like domains 2 (*LIMS2*) gene. Hepatic *LIMS2* is differentially expressed in response to high ambient temperature (Coble et al., 2014). Another candidate region on SSC15 extending from 65.58 to 66.44 Mb contained the transthyretin (*TTR*) gene. Studies have revealed that the expression patterns of *Ttr* was altered by chronic stress in different rat strains (Andrus et al., 2012). In addition, various stress stimuli upregulate *Ttr* and calcium binding-related genes in the prefrontal cortex of the cerebrum in mice. Our single marker based analyses also detected the death-domain association protein (*DAXX*) candidate gene for postpubertal ΔT_R (Supplementary Table S1 and Supplementary

Table 3. Phenotypic variance explained by SNP windows for the change in T_R during the heat stress challenge following puberty (postpubertal ΔT_R)

SSC[a]	Position start (bp)[b]	Position end (bp)[c]	Variance explained (%)[d]	Candidate gene(s)[e]
18	45115484	45291167	0.116	*DST*
15	65866223	66790298	0.104	*FMNL2, GPR17, LIMS2*
15	65585973	66442766	0.096	*UGGT1, GPR17, LIMS2*
16	45509140	45700636	0.091	—
6	20211611	20449928	0.086	*LOC102165723*
6	110474964	110566591	0.085	—
18	45058469	45173869	0.084	—
15	66442766	66907405	0.083	*GPR17, LIMS2, FMNL2*
6	108129231	108545750	0.083	*TTR, LOC106510687, RNF125*
6	109474291	109870440	0.083	*ASXL3*
6	108138639	108646700	0.081	*LOC106510687, RNF125*
18	20063484	20338092	0.081	*LOC100516838, LOC100737195, STRIP2, AHCYL2*
6	115147146	115788549	0.081	—
16	37793490	38004706	0.081	—
6	107975607	108138639	0.080	—
16	48647762	48792525	0.079	*LOC106504115, MAST4*
4	13581654	13825367	0.077	*LOC102164882*
16	47786015	47968058	0.077	*ERBB2IP, LOC102167060*
15	65327301	65866223	0.074	*UGGT1*
2	46429654	46587388	0.074	—
7	34708292	34803564	0.072	—
2	159763907	159898148	0.071	—
1	16981336	17160649	0.071	*CCDC170*
2	51076948	51244889	0.070	—

Gene abbreviations: *DST = dystonin*; *FMNL2 = formin like 2*; *GPR17 = G protein-coupled receptor 17*; *LIMS2 = LIM zinc finger domain containing 2; TTR = transthyretin*; *ASXL3 = additional sex combs like 3*; *STRIP2 = striatin interacting protein 2*; *AHCYL2 = adenosylhomocysteinase like 2*; *MAST4 = microtubule associated serine/threonine kinase family member 4*; *ERBB2IP = erbb2 interacting protein*; *UGGT1 = UDP-glucose glycoprotein glucosyltransferase 1*; *CCDC170 = coiled-coil domain containing 170*.

[a]Chromosome number of the pig genome for which the SNP window location is mapped.

[b]SNP window positions start location on the chromosome.

[c]SNP window position end location on the chromosome.

[d]Percentage of variance explained by five SNP windows.

[e]Genes located within the SNP window.

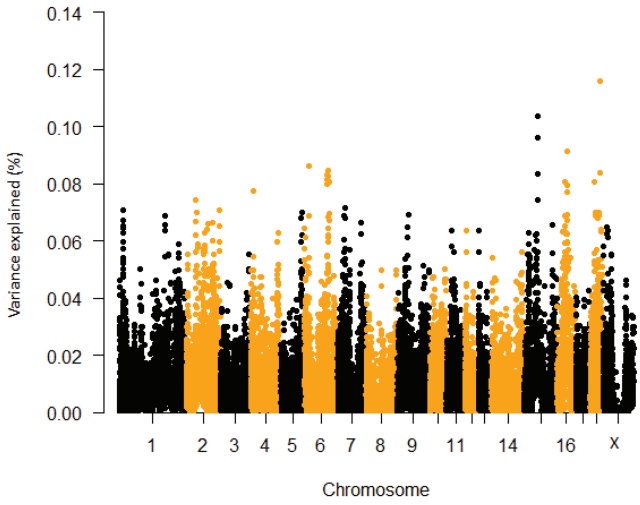

Figure 3. Manhattan plot of delta T_R during the second HS challenge (postpubertal ΔT_R) percentage of variance explained by SNP windows in crossbred gilts. The variance accounted by an individual SNP was smoothed over five SNP sliding windows.

Figure S1). This gene product plays a key role as a mediator of heat shock factor 1 (*HSF1*) activation (Nefkens et al., 2003; Boellmann et al., 2004). Other studies have reported that heat shock protein (HSP) expression is modulated by *DAXX* (Boellmann et al., 2004). Thus, taking into account the known direct and indirect association of these genes (*LIMS2*, *TTR*, and *DAXX*) with stress, they represent potential candidates for HS tolerance in pigs.

In Table 4 and Figure 4, phenotypic variance explained by SNP windows for ΔT_S is presented. The region of interest is flagged on SSC 15 (125.96 to 126.47 Mb) comprising the erb-b2 receptor tyrosine kinase 4 (*ERBB4*) genomic locus, which is a member of the tyrosine kinase family and is involved in the DNA damage response (Gilmore-Hebert et al., 2010). Expression of this gene can

Table 4. Phenotypic variance explained by SNP windows for delta T_S prior to puberty (prepubertal ΔT_S)

SSC[a]	Position start (bp)[b]	Position end (bp)[c]	Variance explained (%)[d]	Candidate gene(s)[e]
3	121853700	122019392	0.044	*LOC100521960, FKBP1B, ATAD2B*
15	126029433	126285452	0.042	*ERBB4*
15	25747414	25910541	0.041	—
1	271950519	272028559	0.038	—
9	140242929	140385683	0.038	—
15	125958213	126098646	0.037	*ERBB4*
3	121766459	121896763	0.037	*ITSN2, LOC100521960, FKBP1B*
9	142991886	143124394	0.036	—
4	12197136	12282429	0.035	—
15	127761392	127886086	0.035	*IKZF2, LOC100737978*
15	126098646	126467297	0.034	*ERBB4*
8	19747348	19780058	0.034	—
15	136273347	136352463	0.034	—
17	59360910	59447126	0.034	*ATP9A*
1	271984966	272080412	0.034	*LOC100153054*
9	143029683	143164935	0.033	—
4	12025385	12095880	0.033	—
15	136764149	136868267	0.033	*EPHA4, LOC102159610*
7	9126277	9208606	0.033	—
9	143124394	143242124	0.033	*RPS6KC1*
15	136981095	137108151	0.033	*LOC106506372*
17	59314509	59400449	0.032	*NFATC2, ATP9A*
4	12237013	12315209	0.032	—
15	136181273	136319027	0.032	—

Gene abbreviations: *FKBP1B = FK506 binding protein 1B; ATAD2B = ATPase family, AAA domain containing 2B; ERBB4 = erb-b2 receptor tyrosine kinase 4; ATP9A = ATPase phospholipid transporting 9A (putative); EPHA4 = EPH receptor A4; RPS6KC1 = ribosomal protein S6 kinase C1; NFATC2 = nuclear factor of activated T-cells 2; ATP9A = ATPase phospholipids' transporting 9A.*

[a]Chromosome number of the pig genome for which the SNP window location is mapped.

[b]SNP window positions start location on the chromosome.

[c]SNP window position end location on the chromosome.

[d]Percentage of variance explained by five SNP windows.

[e]Candidate genes located within the SNP window.

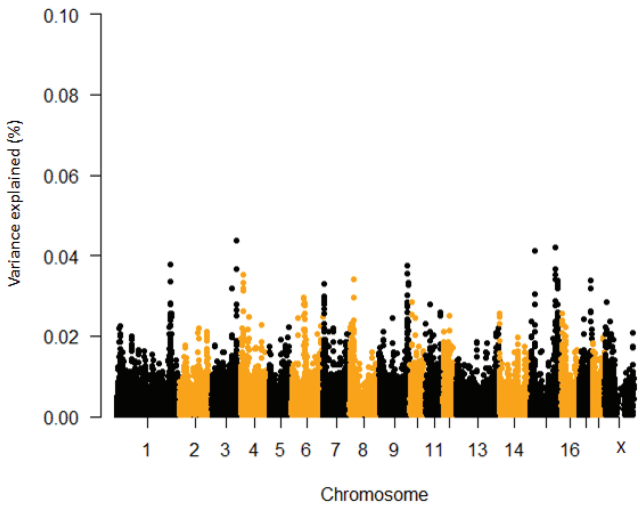

Figure 4. Manhattan plot of delta T_S first HS challenge (prepubertal ΔT_S) percentage of variance explained by SNP windows in crossbred gilts. The variance accounted by an individual SNP was smoothed over five SNP sliding windows.

be induced in response to various cellular stresses and it plays a key role in preventing apoptosis (Hua et al., 2012). Furthermore, this gene induces HSPs in a HSF1-dependent manner (Khaleque et al., 2005) and is associated with maximum lifespan in rodents (Edrey et al., 2012). Another potential candidate region associated with ΔTs is the SSC 17: 59.36 to 59.44 Mb, which includes the ATPase phospholipid transporting 9A (*ATP9A*) gene. ATPases move ions across cellular membranes (Altshuler et al., 2012) and are involved in maintaining ion homeostasis during heat stroke or stress (Kourtis et al., 2012). For instance, HSP-16.1 functions with the Ca^{2+}- and Mn^{2+}-transporting ATPase calcium-transporting (PMR-1) to maintain Ca^{2+} homeostasis under heat stroke (Kourtis et al., 2012). Moreover, it has been shown that mutant protein lacking ATPase domain resulted in loss of key activities of HSP72 (Volloch et al., 1999). The SNPs on SSC15 at 127.76 Mb to

127.88 Mb accounted for 0.04% of the observed SNP variance and contained the IKAROS family zinc finger 2 (*IKZF2*) gene. This is a stress-related gene and expressed in various lymphomas and leukemia (Antica et al., 2008) and is also associated with QTL regions for T lymphocyte subpopulations in swine (Lu et al., 2012). On SSC 3, the highest proportion of phenotypic variance explained (0.04%) by SNP windows was observed at 121.85 to 122.02 Mb and encompassed the FK506 binding protein 1B (*FKBP1B*) locus, which is differentially expressed in response to HS in catfish (Liu et al., 2013). In addition, members of the *FKBP* protein family are involved in modulating thermotolerance by interacting with HSP90.1 and are essential for survival at high temperatures (Meiri and Breiman, 2009). Another potential candidate gene detected on SSC17 (59.31 to 59.40 Mb) is nuclear factor of activated T-cells 2 (*NFATC2*). The *NFAT* gene family mediated transcription is induced in epidermal cells in response to UV light (Horsley and Pavlath, 2002). *NFATC2* is a novel *HSF1* target that strongly inhibits polyglutamine aggregation (polyQ) and is required for *HSF1*-mediated suppression of ployQ aggregation (Hayashida et al., 2010). Single marker based analyses for ΔT_S identified a SNP on SSC 6 explaining 0.05% of the observed variance and located within the U6 snRNA gene (Supplementary Table S1 and Supplementary Figure S1). U6 snRNA is essential for mRNA splicing and interestingly enough, this gene has been associated with T_R under HS in Holstein cattle (Dikmen et al., 2013).

LIMITATIONS AND CONCLUSIONS

To identify loci associated with thermotolerance traits in pigs, we employed a classical GWAS approach. GWAS using a large number of markers require thousands of samples to attain an adequate statistical power (Spencer et al., 2009; Hong and Park, 2012). As indicated in several studies, GWAS undertaken using smaller sample size, have little power to identify loci with small polygenic effects and only loci with very large effects are expected to reach the genome-wide significant threshold (Davenport et al., 2015). As expected, with our small sample size, no SNPs reached the set genome-wide significance threshold. Therefore, we conclude that the results of the present study are suggestive and warrant further replication and follow-up study using reasonable sample sizes.

Despite the above-indicated limitation of this study, we have identified some genes that are known to be involved in physiological adaptation to general stressors. The SNPs explaining the largest proportion of variance and associated with or located within (*GHR, PAIP1, TEAD, NNT, ERBB4, FKBP1B,* and *NFATC2*) and related to apoptosis and cellular stress and may prove to be potential candidates for further validation studies.

Conflict of interest statement. Any opinion, findings, conclusions, or recommendations expressed in this publication are those of the authors and do not necessarily reflect the view of the National Pork Board. No conflicts of interest, financial, or otherwise are declared by the author(s).

SUPPLEMENTARY DATA

Supplementary data are available at *Journal of Animal Science* online.

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