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Genetic variation in *LUMAN/CREB3* and association with stress and meat quality traits in Yorkshire pigs

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ABSTRACT

LUMAN/CREB3 is a stress regulatory gene that affects activity of the hypothalamic-pituitary-adrenal axis in mice and presents a promising avenue for exploring variable stress-responsiveness in pigs. Pigs with similar characteristics to *LUMAN*-deficient mice, including greater resilience to stress and receptivity to human handling, would be valuable in the pork industry from animal welfare and production efficiency perspectives. We previously identified eight genetic variations and five haplotypes throughout the *LUMAN* locus in Yorkshire pigs. In this study we analysed associations between *LUMAN* variations with behavioural stress response during three tests (open field test, novel object test, and human approach test), physiological stress-responsiveness (cortisol), and carcass/meat quality measurements from purebred Yorkshire pigs. Haplotypes A1 and A2 were associated with decreased activity levels in novel environments and greater plasma cortisol concentrations at slaughter. Haplotype A1 was associated with lower carcass scratch scores and meat with lower cooking losses and greater tenderness. Haplotypes B1 and B2 were associated with the opposite traits including increased activity levels in novel environments and characteristics for lower meat quality including greater cooking losses, lower marbling, and paler coloured meat. We conclude that DNA variations in the *LUMAN* locus could potentially be used as genetic markers for stress resistance and meat quality in pig breeding.

KEY WORDS

genetic marker, *LUMAN*, *CREB3*, pigs, stress response, meat quality

INTRODUCTION

Stress is an important problem for the pork industry given its impact on animal welfare and production performance (e.g. growth, reproduction, and meat quality) for pigs (Warriss 1998; Einarsson et al. 2008; White et al. 2008). Behavioural and neuroendocrine responses to stress are highly variable among pigs and can be attributed to differences in genetic background and prior experience (Martinez-Miro et al. 2016). As much as 62% of the variation in human basal cortisol levels have been attributed to genetics (Bartels et al. 2003), including polymorphisms in several genes involved in the hypothalamic-pituitary adrenal-(HPA) axis (Wust et al. 2004). In fact, a SNP in the swine *NR3C1* gene, which encodes the glucocorticoid receptor, was also found to be associated with basal cortisol concentration in pigs (Murani et al. 2010). Glucocorticoids, in the form of cortisol in pigs, are regulated through the HPA axis, a highly conserved neuroendocrine stress response system in animals that has been linked to overall animal health (Uchoa et al. 2014). A growing number of quantitative trait loci analyses have focused on neuroendocrine responses to stress (Désautés et al. 2002, Ousova et al. 2004; Murani et al. 2012) and behavioural stress response traits (Désautés et al. 2002; Ponsuksili et al. 2015). Ponsuksili and colleagues (2015) identified gene candidates for coping behaviours for stress observed during the back test, in which a pig is manually restrained in the supine position (Hessing et al. 1994). However, the genetic component underlying sensitivity to stress in pigs still remains unclear.

LUMAN, or CREB3 (cyclic AMP responsive element binding protein 3), is an endoplasmic reticulum (ER) bound transcription factor that functions as an important regulator of stress hormone signalling. LUMAN has been shown to affect activity of the HPA axis in mice by modulating the expression of the glucocorticoid receptor (Penney et al. 2017). It is well

known that stress and the HPA axis have a profound impact on overall animal health, immunity, metabolism, reproduction, and a wide range of emotions and behaviours (Christiansen et al. 2007; Marques et al. 2009; Uchoa et al. 2014). *LUMAN*-deficient mice were less susceptible to environmental stress, demonstrating greater stress resilience in response to novel stimuli, and were more receptive to human handling compared to wild-type mice (Penney et al. 2017). Although less severe, similarly blunted stress responses were also observed in *LUMAN*-recruitment factor (*LRF*) gene knockout mice, while heterozygotes were phenotypically normal. *LRF* promotes rapid degradation of *LUMAN* and represses its transactivation activity (Audas et al. 2008). Pigs with characteristics similar to *LUMAN*-deficient and *LRF*-knockout mice would be beneficial in the pork industry from both animal welfare and production perspectives, such as at slaughter, since high levels of peri-mortem stress caused by lairage and human handling prior to slaughter leads to reduced meat quality (Warriss 1998; Rabaste et al. 2007; Dokmanovic et al. 2015). Furthermore, Terlouw (2005) found that genetic background, individual behavioural characteristics (e.g. reactivity to humans), and slaughter conditions could account for much of the variability in meat quality.

Polymorphisms in the swine *LUMAN* gene can plausibly affect stress-responsiveness in pigs by altering *LUMAN* function, including gene expression through promoter/enhancer activity, as well as *LUMAN* protein structure and function, such as altering its ability to localize to the nucleus or to bind nuclear hormone receptors (Lohrer and Tangen 2000; Luciano and Wilson 2000; LeVan et al. 2001). We recently identified eight polymorphisms, including two blocks of high linkage disequilibrium (LD) comprised of two (A1 and B1) and three haplotypes (A2, B2, and C2), as well as evidence of intra-breed diversity within the *LUMAN* locus of Yorkshire pigs (Larson et al. 2020, submitted for publication); however, the relevance and implications of this

genetic variation is unknown. The objective of the present study was to analyse the association between the polymorphisms and haplotypes identified in the *LUMAN* gene with behavioural and physiological stress response as well as carcass and meat quality measures in Ontario Yorkshire pigs. We postulate that naturally occurring variations in the swine *LUMAN* gene may potentially be used as markers for stress resistance and meat quality in pigs, which would have important implications for animal welfare and pork production.

MATERIALS AND METHODS

Animals

A total of 154 purebred Yorkshire pigs from Arkell Research Station's Swine Unit at the University of Guelph were selected for this study. The sample population was selected from 202 pigs that we previously studied (Larson et al. 2020) and included 54 female and 100 castrated male piglets from 27 litters. Piglets with low birth weight were excluded. Experimental pigs were subjected to ear tagging, weighing, and iron injections at birth. Tail docking and teeth clipping were not applied to piglets to minimize stressful experiences and human contact unrelated to the study. Piglets were group housed within their original litters in conventional farrowing crates until weaning at 21 days of age. Litters were then mixed and housed in grower pens. This research project followed the Canadian Council on Animal Care guidelines (CCAC 2009). Procedures and housing conditions of experimental pigs were approved by the Animal Care Committee at the University of Guelph (AUP 3630).

Enzyme-Linked Immunosorbent Assays (ELISAs)

Blood samples (2-3 ml) were collected at three time points; 9 days of age, 20 days of age, and at slaughter. Day 9 and 20 samples were obtained from 154 pigs by retro-orbital bleeding. Samples at slaughter were obtained from 91 pigs via the jugular vein during exsanguination. Blood samples were collected between 0830 and 1030 h at all time points and stored on ice in 10 ml Vacutainer tubes (BD, Mississauga, Canada) until processed; serum was separated and stored at -80°C. Cortisol levels were detected using a cortisol ELISA kit (Enzo Life Sciences, Farmingdale, NY, USA) as per the manufacturers' instructions, and detected using a POLARstar Omega plate reader (BMG Labtech GmbH, Offenburg, Germany). Standards were run in duplicate and samples were run in singlet.

Behaviour Testing

All behaviour tests were conducted at the University of Guelph's Arken Research Station's Swine Unit. A total of 149 piglets were individually subjected to three behaviour tests to measure their behavioural coping style and fear to humans: open field test (OFT), novel object test (NOT), and human approach test (HAT) (Donald et al. 2011; Brajon et al. 2016). Tests were videorecorded for subsequent analysis and EthoVision video tracking software (XT8.5, Noldus Information Technology, Wageningen, The Netherlands) was used to measure piglet behaviour. The five piglets not included in behaviour testing died during rearing.

Open Field Test and Novel Object Test

The OFT and NOT were conducted back to back at 41 days of age. Piglets were individually carried to a testing room and placed in the corner of a testing arena with plastic coated slat flooring and solid plastic walls (2.62 m x 2.62 m) for 5 min. Total distance travelled (cm) and

mean velocity (cm/s) were measured using EthoVision software. Frequency of escape attempts (i.e. lifting at least the front legs up against the wall) was manually recorded. In the NOT, a 20.3 cm-diameter sand-filled plastic Jolly Ball © (Jolly Pets, Streetsboro, OH) was lowered from the ceiling into the middle of the arena for 5 min (Brajon et al. 2016). Exploration of the object was recorded using EthoVision software, which divided the arena into two zones. Zone 1 encompassed the circular area directly surrounding the object. Zone 2 comprised the remaining area including the corners. Variables measured for the OFT were also measured for the NOT, in addition to zone 1 duration.

Human Approach Test

The HAT was performed at 42 days of age. Piglets were individually placed in a start corner of the same testing arena. A human experimenter remained seated on a stool in the corner opposite the start corner for the 5-min testing period. EthoVision was used to divide the arena into 7 zones to indicate proximity to the experimenter. Zone 1 encompassed the corner in which the experimenter sat and zone 7 comprised the start corner (i.e. the furthest distance to the experimenter). Zone 7 frequency as well as latency to enter zones 1 and 2 were recorded. Piglets were also manually assigned human approach scores, based on effort to approach, and willingness to be touched by the experimenter (adapted from Brajon et al. 2016). Scoring criteria were based on the following: the pig made no effort to approach the experimenter (score = 4), the pig approached the experimenter but did not allow contact (score = 3), the pig allowed contact with the snout (score = 2), the pig allowed contact with the snout and ear (score = 1), the pig allowed contact with the snout and ear more than once (score = 0).

Genomic Analysis

Detailed procedures are as described in Larson et al. (2020). Briefly, gDNA was isolated from blood samples using the EZ-10 spin column gDNA miniprep kit (BioBasic, Markham, Canada). Polymerase chain reaction (PCR) was performed using gene-specific primers to amplify regions of the *LUMAN* locus from extracted gDNA. Nested PCR was subsequently performed to enhance concentration and specificity of amplicons, and to improve sequencing quality for polymorphism detection. Amplified PCR products from *LUMAN* gDNA fragments were compared to a reference swine *LUMAN* sequence (GenBank Accession No. NC_010443) and sequence variations were confirmed using 4Peaks by Griekspoor and Groothuis (<https://nucleobytes.com>).

Carcass and Meat Quality Evaluation

Pigs were transported to Conestoga Meat Packers in Breslau, Ontario, located 21 km from Arkell Research Station, upon reaching approximately 115 kg live weight. Pigs were transported to slaughter together during one of three slaughter dates and were mixed with unfamiliar pigs during transport as well as in lairage pens at slaughter. Pigs were rendered unconscious in a carbon dioxide chamber prior to death by exsanguination following commercial pork industry procedures. Carcasses were placed in a chill cooler at 1°C approximately 45 min post-mortem. A scratch score was assigned using a three-point scale (0 = no scratches, 3 = severe scratching and bruising). pH in the *longissimus* muscle (LM) was also measured between the 3rd and 4th ribs at 1 h and 24 h post-mortem utilizing a spear-tipped pH meter (Hanna Instruments, Smithfield, RI). At 24 h post-mortem, a primal loin from the left carcass side from each pig was transported to the University of Guelph Meat Laboratory. After a 48-h chilling period, the loin was cut into two

pieces between the 3rd and 4th last ribs to expose the rib interface. Three 3-cm thick chops were cut starting at the 3rd/4th rib interface from the anterior LM section for examination of meat quality traits. Once allowed to oxygenate for 30 min, the first LM chop was utilized for subjective evaluation of colour and marbling as well as objective assessment of colour, ultimate pH, and drip loss. The two remaining LM chops were vacuum packaged, aged for 2 days, and then stored at -20°C until determination of shear force and cooking losses. Slaughter cortisol, loin pH, and scratch score were analyzed in 91 pigs, while all other meat quality measures were analyzed in 86 pigs. The 68 pigs not included in the meat quality evaluation either died during rearing, were exposed to variable slaughter conditions (i.e. inconsistent scalding tank temperature), or could not be identified with complete certainty following slaughter (i.e. indiscernible tattoo, altered slaughter line order). Evaluations were conducted at the University of Guelph and comprised the following procedures.

Meat Colour

Meat colour scores were subjectively assessed based on the Canadian Pork Quality Standards (CPI 2013) seven-point scale (0 = pale pinkish gray to white; 6 = dark purplish red) and the Japan Meat Grading Association colour standards (Nakai et al. 1975) six-point scale (1 = extremely pale pink to gray; 6 = dark purplish red). Meat colour was also objectively measured at 3 locations on the chop utilizing a Minolta CR-400 colourimeter with Spectra QC-400 software (Folio Instruments, Kitchener, ON) and illuminant D65 (2° viewing angle). Colour data were collected according to the International Commission on Illumination (CIE) $L^*a^*b^*$ scale (L^* = luminosity; a^* = continuum from red to green; b^* = continuum from yellow to blue) and $L^*C^*h^*$ scale (C^* = chroma; h^* = hue angle) (CIE 1978). The colorimeter was standardized to a white ceramic tile ($Y = 93.18$, $x = 0.3165$, and $y = 0.3328$).

Marbling

The amount of marbling present in the LM was assessed based on the National Pork Producers Council (NPPC 2000) ten-point scale (1 = devoid of marbling; 10 = very abundant marbling) as well as the Canadian Pork Quality Standards (CPI, 2013) seven-point scale (0 = devoid of marbling; 6 = very abundant).

Ultimate pH and Drip Loss

Ultimate pH was determined from the average of three measurements taken from the chop at 48 h post-mortem utilizing a spear-tipped foodcare pH electrode (Hanna Instruments, Smithfield, RI). Drip loss was also determined in duplicate by means of the E-Z cup method described by Rassmussen and Andersson (1996).

Warner-Bratzler Shear Force and Cooking Loss

Two LM chops were thawed overnight in a cooler at $\leq 4^{\circ}\text{C}$, trimmed of subcutaneous fat, weighed, and cooked utilizing a Garland Grill (ED-30B broiler, Garland Commercial Range Ltd., Mississauga, ON). Chops were turned at an internal temperature of 40°C and weighed once an internal end point temperature of 74°C was reached for determination of cooking losses. Chops were stored at $\leq 4^{\circ}\text{C}$ for 24 h prior to coring, at which time they were brought to room temperature. Six 1.27-cm meat cores were then removed parallel to the muscle fibres from each chop using a drill press mounted corer. Cores were sheared by a Warner-Bratzler blade on a TA-XT Plus texture analyzer (Texture Technologies Corp., Scarsdale, NY) with the crosshead speed set at 3.3 mm s^{-1} . A custom macro program in Stable Microsystems Exponent software was utilized to determine peak shear force. The six peak force measurements per loin

chop were averaged.

Statistical Analysis

Genetic Diversity Analysis

Detailed procedures are as described in Larson et al. (2020). Briefly, genetic diversity parameters, including genotypic and allelic frequencies were determined by direct counting according to the genotyping result. Haploview 4.2 software (Barrett et al. 2005) was used to generate LD statistics using the squared correlation of alleles at two loci (r^2). Haplotypes and the respective frequencies were also reconstructed for each LD block using Haploview 4.2 software.

Association Analysis

The effects of genotype on stress response and meat quality were analysed using general linear mixed models using Proc GLIMMIX in SAS version 9.4 University edition. The statistical model ($\text{StressResponse}_{ij} = \mu + \text{genotype}_i + \text{litter}_j + \varepsilon_{ij}$) was developed to assess whether the response to stress differs significantly among genotype. Based on LD analysis, genotypes were independently analysed for block 1, marker 4 (c.266C>G), block 2, and marker 8 (c.*97delC). Genotypes for block 1 (i.e. A1A1, A1B1 and B1B1) and block 2 (i.e. A2A2, A2B2 A2C2, B2B2, B2C2) were analysed based on observed haplotype combinations. Genotypes for markers 4 and 8 were analysed based on homozygosity and heterozygosity of alleles (e.g. for marker 4: CC, CG, GG). Genotype was included as a fixed effect and litter as a random effect. Slaughter date was included as a random effect for the statistical analysis of meat quality traits. Variables including HAT score and scratch score were analysed using multinomial analysis. ε represents the residual random error of the model. Contrast statements

were used to analyse the effect of homozygosity versus presence of another haplotype (e.g. A1A1 vs. A1B1 and B1B1) as well as the carrier and null genotypes (e.g. A2 carriers: A2B2 and A2C2; A2 nulls: B2B2 and B2C2). Model residuals were assessed using a scatterplot of the studentized residuals for homoscedasticity, linear predictor for linearity, and the Shapiro-Wilk test for normality. To meet model assumptions, variables (Y) were transformed using logarithm (stress response: cortisol concentration at 9 days of age, attempts to escape the OFT and NOT, velocity during the NOT; and meat quality: loin hue) and square root (stress response: latency during the HAT, frequency in zone 7 during the HAT; and meat quality: loin pH at 24 h, loin shear, loin cook loss, loin drip loss, and cortisol concentration at slaughter). The p-value for pairwise comparisons between genotypes were adjusted for multiple analyses using the Tukey test. Pearson correlation coefficients were used to reduce the number of variables and to analyse inter-test consistency. Results were considered statistically significant for comparisons with a p-value (P) lower than 0.05 and tendencies for those with a p-value (P) lower than 0.10 but higher than or equal to 0.05.

RESULTS

To investigate the significance of the polymorphisms and haplotypes previously discovered, we analysed the association between genotype and physiological and behavioural stress-responsiveness as well as meat quality. Block 1 genotype associations are presented in Table 1 for stress-responsiveness measures and in Table 2 for meat quality measures. In both tables, Block 1 genotype comparisons are presented in two contrast analyses, i.e., Contrast 1: A1 homozygous (A1A1) vs. B1 presence (A1B1 and B1B1) and Contrast 2: B1 homozygous (B1B1) vs. A1 presence (A1B1 and A1A1). During the OFT, A1 homozygotes tended to show a lower distance travelled and velocity compared to individuals featuring the B1 haplotype (both

$P = 0.061$; Contrast 1, Table 1). A1 homozygotes also made significantly more escape attempts during the NOT than B1 homozygotes ($P = 0.043$; Table 1). In contrast, distance travelled and velocity were significantly greater during the OFT (both $P = 0.038$) and tended to be greater during the NOT ($P = 0.099$ and $P = 0.083$, respectively) for B1 homozygotes compared to individuals with A1 present (Contrast 2, Table 1). B1 homozygotes also tended to score lower in the HAT than those featuring the A1 haplotype ($P = 0.094$; Contrast 2, Table 1).

At slaughter, A1 homozygotes had significantly lower plasma cortisol concentrations than pigs with B1 present ($P = 0.037$; Contrast 1, Table 2) and significantly lower carcass scratch scores than B1 homozygotes ($P = 0.033$; Table 2). The pH at 1-h post-mortem tended to be greater ($P = 0.087$) and shear force tended to be lower ($P = 0.085$) in the LM from A1 homozygotes than from pigs featuring the B1 haplotype (Contrast 1, Table 2). The LM from A1 homozygotes also showed significantly lower pH at 48 h post-mortem ($P = 0.009$) as well as higher drip loss ($P = 0.045$) than the LM of heterozygotes (Table 2). In contrast, B1 homozygotes displayed higher carcass scratch scores than A1 homozygotes ($P = 0.033$). Furthermore, the LM from B1 homozygotes had higher a^* ($P = 0.017$), b^* ($P = 0.037$), and C^* values ($P = 0.016$) than the LM from heterozygotes (Table 2), as well as less marbling ($P = 0.040$), greater cooking losses ($P = 0.033$), and a tendency for lower CPQS colour scores ($P = 0.073$) compared to the LM from pigs with A1 present (Contrast 2, Table 2).

Block 2 genotype comparisons (Table 3 and 4) included five contrast analyses, i.e., Contrast 1: A2 homozygous (A2A2) vs. A2 carrier (A2B2 and A2C2); Contrast 2: A2 presence (A2A2, A2B2, and A2C2) vs. A2 null (B2B2 and B2C2); Contrast 3: B2 homozygous (B2B2) vs. B2 carrier (A2B2 and B2C2); Contrast 4: B2 presence (B2B2, A2B2, and B2C2) vs. B2 null (A2A2 and A2C2); Contrast 5: C2 presence (A2C2 and B2C2) vs. C2 null (A2A2, A2B2, and B2B2). Individuals with A2 present (A2 homozygotes and carriers) showed lower distance

travelled and velocity during the OFT (both $P = 0.022$) and NOT ($P = 0.009$ and $P = 0.018$, respectively) than A2 nulls (Contrast 2, Table 3). Individuals featuring A2 also spent less time in the zone around the novel object than A2 nulls ($P = 0.018$; Contrast 2, Table 3), whereas individuals with the B2 haplotype tended to spend more time here than B2 nulls ($P = 0.082$; Contrast 4, Table 3). Compared to B2 nulls, individuals featuring B2 also tended to have lower basal cortisol levels at 20 days of age ($P = 0.078$) and showed significantly higher activity levels, including greater distance travelled and velocity during the OFT (both $P = 0.047$) as well as greater distance travelled during the NOT ($P = 0.048$; Contrast 4, Table 3). During the NOT, B2 homozygotes made significantly fewer escape attempts than B2 carriers ($P = 0.038$; Contrast 3, Table 3).

At slaughter, individuals featuring A2 tended to show lower plasma cortisol concentrations than A2 nulls ($P = 0.085$; Contrast 2, Table 4). In contrast, individuals with B2 showed significantly greater plasma cortisol concentrations at slaughter ($P = 0.011$) and greater LM pH at 48 h post-mortem ($P = 0.010$) than B2 nulls (Contrast 4, Table 4). This significant contrast in loin pH can mainly be attributed to the pH value for the A2B2 genotype being greater than pH values for A2A2 ($P = 0.036$) and A2C2 genotypes ($P = 0.008$) in particular. Carcass scratch scores for B2 homozygotes and carriers also tended to be greater than those for B2 nulls ($P = 0.081$; Contrast 4, Table 4). B2 homozygotes had significantly greater carcass scratch scores ($P = 0.038$) and Minolta a^* ($P = 0.027$), b^* ($P = 0.032$), and C^* values ($P = 0.030$) with a lower Japanese colour score ($P = 0.049$) compared to B2 carriers (Contrast 3, Table 4). The LM from B2 homozygotes also tended to have lower pH 24 h post-mortem ($P = 0.094$) and greater cooking losses ($P = 0.095$) than the LM from B2 carriers (Contrast 3, Table 4). Finally, C2 carriers tended to have lower plasma cortisol concentrations at slaughter ($P = 0.066$) as well as significantly lower carcass scratch scores ($P = 0.029$) and LM pH at 48 h post-mortem ($P = 0.010$) compared

to C2 nulls (Contrast 5, Table 4).

No differences were observed between block 1 and block 2 genotypes for the following variables: basal plasma cortisol at 9 days, escape attempts during the OFT, zone frequency and latency during the HAT, L^* value, hue angle, and CPQS marbling score. No differences were observed between marker 4 and marker 8 genotypes.

DISCUSSION

In our previous survey of the *LUMAN* locus for Ontario Yorkshire pigs (Larson et al. 2020), we identified eight genetic variants, including two LD blocks each comprised of 3 variants (Block 1: c.-345G>T, c.80G>C, and c.130-24T>C; Block 2: c.318C>A, c.611+213A>G, and c.*80G>A) that constituted two haplotypes (A1 and B1) and three haplotypes (A2, B2, and C2), respectively. The present study provides evidence indicating that genetic variations within the swine *LUMAN* locus are associated with variability in physiological (i.e. cortisol) and behavioural stress-responsiveness (i.e. responses to novelty and human interaction) as well as carcass and meat quality traits in pigs. The genetic selection of pigs that have greater stress resilience in response to novel stimuli and greater receptivity to human handling would be especially beneficial around slaughter, given the high levels of stress induced by transportation, lairage, and human handling, even under best management practices. Therefore, our objective was to identify genetic markers that can be used to reduce animal stress, thereby improving animal welfare and meat quality.

Throughout this paper, we discuss significant results ($P < 0.05$) as well as results with a ‘tendency’ to differ ($0.05 \leq P < 0.10$) to provide a wide base of reference for guiding subsequent studies, as important information could be lost by not reporting traits with P values near 0.05. Considering our relatively low sample sizes for two genotypes (A2C2 and B2C2), future studies

with larger sample sizes may have the necessary power to identify true differences between these genotypes.

Block 1 Genotypes and Stress-Responsiveness

In the present study, we show there are individual differences in behavioural coping styles for stress that are associated with *LUMAN* genotypes. Our findings indicate that piglets with B1 homozygosity displayed significantly higher activity levels (i.e. mean velocity and distance travelled) during the OFT and a tendency for higher activity levels during the NOT than those with genotypes featuring the A1 haplotype. These findings reflect a higher proactive coping style in response to novelty among B1 homozygotes. Since pigs did not encounter the novel environment by choice, activity levels measured during the OFT and NOT likely reflect fear and anxiety to novelty rather than voluntary exploration (Wemelsfelder et al. 2000). However, a combination of different behavioural dimensions need to be considered when assessing emotionality in pigs (Donald et al. 2011). Interestingly, B1 homozygotes also attempted to escape less often than A1 homozygotes during the NOT. Increased activity levels and fewer escape attempts are characteristics that can suggest lower neophobia (Donald et al. 2011) and reduced emotional reactivity (Brajon et al. 2017) to novelty, respectively. The combination of these characteristics in B1 homozygotes can be interpreted as indicating reduced fear/anxiety towards novelty (Donald et al. 2011). Furthermore, B1 homozygous piglets show numerically shorter latencies and tended to be more likely to approach the experimenter voluntarily during the HAT than pigs featuring the A1 haplotype. In this experiment, an animal's willingness to approach could reflect a lower fearfulness of humans and a willingness for human interaction (Brajon et al. 2016) or a combination of fear and curiosity to explore the novel environment (Murphey et al. 1981;

Marchant et al. 1997). These results indicate that B1 homozygous piglets were more proactive and curious toward an unfamiliar person than A1 homozygotes.

In contrast, A1 homozygotes tended to display lower activity levels during the OFT than those featuring B1, reflecting lower exploration of the novel arena (Donald et al. 2011). A1 homozygotes also attempted to escape more often during the NOT than piglets featuring B1, suggesting higher emotional reactivity (Brajon et al. 2017) to the novel arena and object. The combination of lower exploration and higher emotional reactivity to novelty could suggest higher neophobia (Donald et al. 2011).

Block 1 Genotypes and Meat Quality

This study also suggests that *LUMAN* genotypes are associated with variability in cortisol levels at slaughter as well as meat quality traits and may therefore have implications in pork production. Our results indicate that A1 homozygosity is associated with significantly lower plasma cortisol levels at slaughter compared to genotypes featuring the B1 haplotype. Furthermore, the carcasses from A1 homozygotes were significantly more likely to have lower scratch scores than those of B1 homozygotes, which may reflect less arousal and aggression among A1 homozygotes in response to the mixing of unknown pigs during transportation to slaughter and lairage that can induce social stress as new dominance hierarchies are established (Pitts et al. 2000; Séguin et al. 2006). Our findings also indicate that A1 homozygotes tended to display higher loin pH 1-h post-mortem compared to pigs featuring the B1 haplotype and significantly lower ultimate loin pH than heterozygotes. However, pH differences between genotypes were not very pronounced and all scores remained above 5.50 and would therefore not classify as pale, soft, exudative (PSE) meat (PIC 2003). Low pH in warm muscle post-mortem accelerates protein denaturation and consequently reduces water-holding capacity (WHC) of muscle (den Hertog-

Meischke et al. 1997). Meat with lower WHC is typically more exudative and leads to an unappealing loss of moisture prior to cooking as well as lower juiciness after cooking (Bowker and Zhuang 2013). A1 homozygotes were more likely to display meat with greater drip loss % than heterozygous individuals. However, individuals featuring the A1 haplotype were significantly more likely to display meat with lower cooking losses than B1 homozygotes, which is a characteristic of more desirable meat with greater WHC. Furthermore, A1 homozygosity was also associated with a tendency to display meat with lower shear force than genotypes featuring B1, which would reflect more desirable tender meat. It is worth noting that differences in shear force were not very pronounced between genotypes. While these results are somewhat inconsistent with ultimate pH, other findings indicate that pH is not a reliable predictor for overall pork quality (Tomovic et al. 2014), suggesting that the relationship between pH and meat quality is still unclear.

In contrast, individuals featuring the B1 haplotype exhibited significantly higher plasma cortisol concentrations at slaughter and B1 homozygotes exhibited higher carcass scratch scores compared to A1 homozygotes, which may reflect higher arousal during the mixing of pigs around slaughter that can lead to higher levels of peri-mortem stress (Dokmanovic et al. 2015). Individuals featuring the B1 haplotype also tended to display lower loin pH 1-h post-mortem than A1 homozygotes, which can accelerate protein denaturation and consequently reduce WHC of muscle (den Hertog-Meischke et al. 1997). Indeed, B1 homozygotes are significantly more likely to yield meat with higher cooking losses compared to individuals featuring the A1 haplotype. B1 homozygotes were also significantly more likely to have lower NPPC marbling scores, which is associated with less flavourful meat, compared to individuals featuring the A1 haplotype (Ibarburu et al. 2007). An association with intramuscular fat content is not surprising considering a single missense variant identified within *LRF*, which is a negative regulator of LUMAN, is strongly

associated with body mass index and human obesity, promoting increased fat storage and decreased cellular energy use (Minster et al. 2016). Therefore, it is possible that homozygosity for haplotype B1, including an arginine amino acid at missense variant p.Arg27Pro, could contribute to increased cellular metabolism and reduced intramuscular fat content. However, it is worth noting that the significant 0.3-0.4 score difference in marbling is not very pronounced on the 10-point NPPC scale. B1 homozygosity was also associated with higher Minolta a*, b*, and C* values compared to heterozygosity, indicating more vivid red-yellow coloured meat (CIE 1978). Although significant, these differences may not be detected by the human eye. Furthermore, while B1 homozygotes tended to display paler coloured meat according to the CPQS tool than individuals featuring A1, all scores remained indicative of an ideal reddish-pink coloured meat that is generally preferred by consumers (Tomovic et al. 2014).

Block 2 Genotypes and Stress-Responsiveness

This study also shows that genetic variation in LD Block 2 of the *LUMAN* locus may contribute to inherent differences in stress induced HPA activation and stress-responsiveness among pigs. Our findings indicate that A2 homozygotes and carriers displayed significantly lower activity levels during the OFT and NOT, and spent less time around the novel object than A2 nulls. The reduced locomotor activity and exploration of the arena and novel object observed among A2 homozygotes and carriers are characteristics that can suggest greater neophobia (Donald et al. 2011).

In contrast, B2 homozygosity and heterozygosity are associated with significantly greater activity levels during the OFT, as well as greater distance travelled and a tendency to spend more time around the novel object during the NOT, compared to genotypes lacking the B2 haplotype. The increased locomotor activity and exploration of the arena and novel object suggest that the B2

haplotype is associated with reduced neophobia (Donald et al. 2011). Additionally, we found that B2 homozygotes and carriers tended to display lower basal cortisol levels at 20 days of age than B2 nulls. These findings are consistent with those of Hessing et al. (1994), who characterized pigs with more proactive behavioural coping styles for stress as having lower basal cortisol levels. It is possible that the B2 haplotype is associated with a slower but more prolonged cortisol response (Hessing et al. 1994).

Intermediate behaviours were also observed for some genotypes. No associations were observed between haplotype C2 and a particular coping style for stress during the behaviour tests performed. These findings are supported by van Erp-van der Kooij et al. (2000) who describe a unimodal distribution of coping behaviour, noting that Hessing and colleagues (1994) disregarded intermediate results by classifying pigs as either active or passive coping pigs.

Block 2 Genotypes and Meat Quality

This study also provides evidence to suggest that Block 2 genotypes may be associated with variability in cortisol levels at slaughter as well as carcass and meat quality traits. At slaughter, A2 homozygotes and carriers tended to display lower plasma cortisol concentrations than A2 nulls, whereas B2 homozygotes and carriers showed significantly higher plasma cortisol concentrations than B2 nulls. Higher scratch scores also tended to be more frequent among carcasses of haplotype B2 homozygotes and carriers than B2 nulls. Furthermore, these scores were significantly higher in B2 homozygotes than carriers. These findings may reflect higher aggression among pigs featuring the B2 haplotype, especially B2 homozygotes. Our findings surrounding the B2 haplotype are consistent with others who report that lower basal cortisol levels are associated with higher activity levels in response to environmental stress (Bergeron et al. 1996; Foury et al. 2007) and increased aggression (Murani et al. 2010). Our findings also indicate that ultimate loin pH was significantly

higher among B2 homozygotes and carriers compared to B2 nulls; however, differences were not very pronounced between genotypes. Furthermore, this significant contrast can mainly be attributed to the most frequent A2B2 genotype in particular. We found that B2 homozygotes tended to display meat with greater cooking losses than heterozygotes, which is characteristic of less desirable meat with lower WHC and lower juiciness after cooking. Similar to the B1 haplotype, B2 homozygosity was associated with greater Minolta a^* , b^* , and C^* values compared to B2 heterozygosity, indicating more vivid red-yellow coloured meat (CIE 1978). B2 homozygosity was also associated with less ideal paler coloured meat on the Japanese scoring system compared to B2 heterozygosity (Nakai et al. 1975). Although significant, these colour differences may not be detected by the human eye.

Finally, our findings indicate that C2 heterozygotes tended to display lower plasma cortisol concentrations at slaughter and significantly lower carcass scratch scores than C2 nulls. This suggests that the C2 haplotype may be associated with lower arousal and aggression at slaughter and perhaps lower levels of peri-mortem stress. The carcasses from C2 heterozygotes also displayed lower ultimate pH than C2 nulls; however, this difference can mainly be attributed to pigs with the A2C2 genotype. While the low average ultimate loin pH of 5.47 exhibited by these pigs is a characteristic of PSE meat (PIC 2003), there were no differences in drip loss, cooking loss, or Minolta L^* values that would support the PSE classification.

The absence of genotype differences for several variables (i.e. day 9 basal plasma cortisol, OFT escape attempts, HAT zone frequency, HAT latency, L^* value, hue angle, and CPQS marbling score) as well as at markers 4 and 8 altogether, could suggest that the tests/variables used are not closely connected to the stress phenotype or to the identified SNPs. It is also possible that

the underlying genetics is too multivarious to be delineated by the current experimental design and/or current sample size.

Conclusion

In conclusion, our studies provide valuable insight into the genetic component underlying variable stress response in pigs. Mice deficient in *LUMAN* and *LRF* show a blunted stress response that is accompanied by increased activity of the glucocorticoid receptor (Martyn et al. 2012; Penney et al. 2017). As such, *LUMAN* and *LRF* have been postulated to modulate HPA axis reactivity (Penney et al. 2017) and may represent a genetic circuit that regulates animal stress. Our findings suggest that genetic variation in the *LUMAN* locus may contribute to inherent differences in stress induced HPA activation and stress-responsiveness as well as carcass and meat quality traits among pigs. Based on the identified associations, our results imply that *LUMAN* variants could potentially be used as markers for physiological and behavioural response to novel environments, including pig emotionality, as well as markers for meat quality traits, including drip loss and cooking loss. Further detailed investigations into the functional mechanisms of each identified genetic variation as well as potential combined effects of multiple variants including haplotypes are warranted. Furthermore, *LUMAN*, *LRF*, and associated genes provide promising targets for the identification of genetic markers for animal stress that would be valuable in the pork industry for both animal welfare and production efficiency.

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Table 1. The effects of Block 1 (markers 1, 2, 3) genotypes on basal cortisol concentrations and behaviour of pigs during open field test (OFT), novel object test (NOT), and human approach test (HAT)

	Genotype (Lsmeans±SE)			P-values for Contrasts ^a	
	A1A1 (n _c =54, n _b =53) ^b	A1B1 (n _c =71, n _b =68)	B1B1 (n _c =29, n _b =28)	Contrast 1: A1A1 vs. (A1B1 & B1B1)	Contrast 2: (A1A1 & A1B1) vs. B1B1
Basal cortisol 9d (ng/mL) ^c	15.1±2.08	14.2±2.54	18.3±4.06	0.500	0.174
Basal cortisol 20d (ng/mL)	22.9±4.04	18.6±3.80	18.5±4.47	0.224	0.576
OFT distance travelled (m)	52.7±3.80	56.5±3.53	64.5±4.53	<i>0.061</i>	0.038*
OFT velocity (cm/s)	17.6±1.27	18.8±1.18	21.5±1.51	<i>0.061</i>	0.038*
OFT escape attempts ^d	0.7±0.47	0.4±0.44	1.2±0.59	0.376	0.834
NOT distance travelled (m)	38.2±3.37	40.9±3.13	46.4±4.00	0.130	<i>0.099</i>
NOT velocity (cm/s) ^d	12.2±0.62	13.0±0.61	15.3±0.93	0.144	<i>0.083</i>
NOT escape attempts ^{d, e}	0.6±0.08a	0.3±0.04ab	0.1±0.01b	0.022*	0.033*
NOT zone 1 duration (s)	59.3±5.80	72.0±5.50	70.3±9.35	0.159	0.215
HAT zone 1 + 2 latency (s)	241.8±11.67	244.1±10.81	210.2±18.29	0.960	0.149
HAT zone 7 frequency	6.1±0.86	7.1±0.80	8.5±1.06	0.109	0.116
HAT score	3.2±0.22	3.4±0.21	2.8±0.27	0.730	<i>0.094</i>

^a Significant p-values ($P < 0.05$) are indicated by asterisk, and a tendency for significance ($0.05 \leq P < 0.10$) is indicated by italics.

^b 'n_c' indicates sample size for basal cortisol. 'n_b' indicates sample size for behaviour variables.

^c Data were transformed for statistical analysis; values presented are average±SE.

^d Data were transformed for statistical analysis; values presented are backtransformed Lsmeans±SE.

^e Lsmeans within a row with different lowercased letters (a, b) differ significantly ($P < 0.05$).

Table 2. The effects of Block 1 (markers 1, 2, 3) genotypes on slaughter cortisol concentrations and meat and carcass quality traits for finisher pigs

	Genotype (Lsmeans±SE)			P-values for Contrasts ^a	
	A1A1 (n _c =33, n _m =31) ^b	A1B1 (n _c =38, n _m =36)	B1B1 (n _c =20, n _m =19)	Contrast 1: A1A1 vs. (A1B1 & B1B1)	Contrast 2: (A1A1 & A1B1) vs. B1B1
Slaughter cortisol (ng/mL) ^c	22.3±2.90	32.8±2.94	34.5±4.22	0.037*	0.179
Scratch score ^d	0.7±0.21b	1.0±0.2ab	1.5±0.26a	0.030*	0.017*
pH 1 h post-mortem	6.41±0.079	6.31±0.077	6.31±0.086	<i>0.087</i>	0.429
pH 24 h post-mortem	5.69±0.016	5.70±0.018	5.63±0.035	0.509	0.134
pH 48 h post-mortem	5.51±0.011b	5.55±0.010a	5.53±0.014ab	0.016*	0.714
Colour (CPQS)	3.3±0.30	3.2±0.30	3.0±0.31	0.207	<i>0.073</i>
Colour (JSS) ^e	2.9±0.25	3.0±0.25	2.9±0.26	0.946	0.223
Luminosity (L*)	48.5±0.36	48.1±0.34	48.1±0.42	0.275	0.730
Red-green axis (a*)	6.7±0.28ab	6.5±0.26b	7.2±0.31a	0.578	0.018*
Yellow-blue axis (b*)	3.8±0.13ab	3.4±0.12b	3.9±0.17a	0.513	<i>0.084</i>
Chroma (C*)	7.7±0.25ab	7.3±0.23b	8.2±0.30a	0.864	0.028*
Hue angle (h*)	29.3±1.40	27.7±1.36	28.5±1.48	0.187	0.981
Marbling (NPPC)	2.0±0.19	1.9±0.19	1.6±0.20	0.107	0.040*
Marbling (CPQS)	2.1±0.23	2.1±0.22	1.9±0.26	0.707	0.474
Drip loss (%)	4.0±0.32a	3.2±0.25b	4.0±0.34ab	0.215	0.258
WBSF (kg) ^f	4.3±0.10	4.5±0.08	4.7±0.18	<i>0.085</i>	0.152
Cooking loss (%)	19.4±0.50	19.6±0.61	22.0±0.50	0.425	0.033*

^a Significant p-values ($P < 0.05$) are indicated by asterisk, and a tendency for significance ($0.05 \leq P < 0.10$) is indicated by italics.

^b 'n_c' indicates sample size for cortisol, loin temperature, loin pH, and scratch score. 'n_m' indicates sample size for remaining meat quality variables.

^c Data were transformed for statistical analysis; values presented are average±SE.

^d Lsmeans within a row with different lowercased letters (a, b) differ significantly ($P < 0.05$).

^e JSS: Japanese scoring system (Nakai et al. 1975)

^f WBSF: Warner-Bratzler shear force

Table 3. The effects of Block 2 (markers 5, 6, 7) genotypes on basal cortisol concentrations and the behaviour of pigs during open field test (OFT), novel object test (NOT), and human approach test (HAT)

	Genotype (Lsmeans±SE)					P-values for Contrasts ^a				
	A2A2 (n _c = 42, n _b =41) ^b	A2B2 (n _c = 59, n _b =57)	A2C2 (n _c = 12, n _b =12)	B2B2 (n _c = 29, n _b =28)	B2C2 (n _c = 12, n _b =11)	Contrast 1: A2A2 vs. (A2B2 & A2C2)	Contrast 2: (A2A2, A2B2 & A2C2) vs. (B2B2 & B2C2)	Contrast 3: B2B2 vs. (A2B2 & B2C2)	Contrast 4: (B2B2, A2B2 & B2C2) vs. (A2A2 & A2C2)	Contrast 5: (A2C2 & B2C2) vs. (A2A2, A2B2 & B2B2)
Basal cortisol 9 d (ng/mL) ^c	14.4±2.51	13.5±2.98	17.7±3.07	18.3±4.06	17.2±3.17	0.904	0.192	0.482	0.633	0.653
Basal cortisol 20 d (ng/mL)	21.0±4.23	19.3±3.95	28.8±6.96	18.4±4.45	15.7±6.10	0.506	0.105	0.850	0.078	0.618
OFT distance travelled (m)	54.5±4.17	55.4±3.86	47.1±7.38	64.4±4.55	60.3±6.50	0.538	0.022*	0.230	0.047*	0.455
OFT velocity (cm/s)	18.2±1.39	18.5±1.29	15.7±2.46	21.5±1.52	20.1±2.17	0.539	0.022*	0.230	0.047*	0.455
OFT escape attempts ^c	0.7±0.54	0.2±0.50	0.8±1.0	1.2±0.60	0.8±0.86	0.287	0.992	0.959	0.674	0.897
NOT distance travelled (m)	40.0±3.65	38.8±3.38	32.9±6.42	46.1±3.97	48.3±5.65	0.357	0.009*	0.588	0.048*	0.840
NOT velocity (cm/s)	13.2±1.21	12.9±1.12	12.8±2.12	15.4±1.31	16.5±1.87	0.831	0.018*	0.666	0.140	0.629
NOT escape attempts ^c	1.6±0.45	0.4±0.42	1.0±0.74	0.1±0.48	1.45±0.651	0.317	0.582	0.038*	0.160	0.139
NOT zone 1 duration (s)	62.9±6.90	69.9±6.28	47.0±9.15	70.3±9.36	82.8±9.57	0.676	0.018*	0.971	0.082	0.761
HAT zone 1 + 2 latency (s)	254.2±12.37	251.4±11.23	199.6±26.14	210.2±18.29	206.3±30.34	0.550	0.761	0.160	0.411	0.469
HAT zone 7 frequency	6.1±0.98	7.0±0.90	6.2±1.77	8.5±1.08	7.3±1.56	0.705	0.191	0.347	0.210	0.754
HAT score	3.4±0.24	3.5±0.22	2.4±0.44	2.8±0.27	3.1±0.39	0.178	0.526	0.177	0.450	0.148

^a Significant p-values ($P < 0.05$) are indicated by an asterisk, and a tendency for significance ($0.05 \leq P < 0.10$) is indicated by italics.

^b 'n_c' indicates sample size for basal cortisol. 'n_b' indicates sample size for behaviour variables.

^c Data were transformed for statistical analysis; values presented are backtransformed Lsmeans±SE.

Table 4. The effects of Block 2 (markers 5, 6, 7) genotypes on slaughter cortisol concentrations and meat and carcass quality traits for finisher pigs

	Genotype (Lsmeans±SE)					P-values for Contrasts ^a				
	A2A2 (n _c = 26, n _m = 26) ^b	A2B2 (n _c = 29, n _m = 29)	A2C2 (n _c = 7, n _m = 5)	B2B2 (n _c = 20, n _m = 19)	B2C2 (n _c = 9, n _m = 7)	Contrast 1: A2A2 vs. (A2B2 & A2C2)	Contrast 2: (A2A2, A2B2 & A2C2) vs. (B2B2 & B2C2)	Contrast 3: B2B2 vs. (A2B2 & B2C2)	Contrast 4: (B2B2, A2B2 & B2C2) vs. (A2A2 & A2C2)	Contrast 5: (A2C2 & B2C2) vs. (A2A2, A2B2 & B2B2)
Slaughter cortisol (ng/mL) ^c	23.8±3.64	32.6±3.38	17.6±3.18	34.5±4.22	33.1±6.06	0.390	<i>0.085</i>	0.477	0.011*	<i>0.066</i>
Scratch score	0.8±0.22	1.1±0.21	0.3±0.38	1.5±0.25	0.61±0.349	0.496	0.225	0.038*	<i>0.081</i>	0.029*
pH 1 h post-mortem	6.41±0.082	6.31±0.079	6.39±0.112	6.31±0.086	6.29±0.105	0.407	0.246	0.907	0.137	0.940
pH 24 h post-mortem	5.69±0.018	5.69±0.019	5.73±0.028	5.63±0.035	5.75±0.038	0.726	0.863	<i>0.094</i>	0.759	0.225
pH 48 h post-mortem ^d	5.51±0.013b	5.56±0.012a	5.47±0.025b	5.53±0.014ab	5.51±0.021ab	0.875	0.553	0.886	0.010*	0.010*
Colour (CPQS)	3.3±0.32	3.3±0.31	3.2±0.39	3.0±0.32	3.0±0.37	0.810	0.119	0.349	0.362	0.762
Colour (JSS) ^e	3.0±0.25	3.0±0.25	2.8±0.28	2.9±0.25	3.1±0.27	0.546	0.298	0.049*	0.294	0.506
Luminosity (L*)	48.5±0.37	48.2±0.34	48.4±0.70	48.1±0.41	47.5±0.60	0.634	0.189	0.515	0.236	0.527
Red-green axis (a*)	6.7±0.28ab	6.4±0.26b	6.9±0.049ab	7.2±0.30a	6.6±0.42ab	0.916	0.438	0.027*	0.833	0.993
Yellow-blue axis (b*)	3.7±0.14	3.4±0.14	4.0±0.32	3.9±0.17	3.5±0.28	0.996	0.875	0.032*	0.167	0.747
Chroma (C*)	7.6±0.25	7.3±0.23	8.1±0.54	8.2±0.29	7.5±0.45	0.888	0.565	0.030*	0.559	0.764
Hue angle (h*)	29.0±1.47	27.6±1.44	30.6±2.06	28.5±1.52	27.9±1.86	0.941	0.393	0.525	0.106	0.464
Marbling (NPPC)	2.0±0.20	1.9±0.20	2.0±0.29	1.6±0.21	1.8±0.26	0.920	0.108	0.235	0.230	0.808
Marbling (CPQS)	2.0±0.25	2.1±0.24	2.4±0.44	1.9±0.27	1.9±0.37	0.328	0.247	0.719	0.350	0.603
Drip loss (%)	3.9±0.20	3.5±0.21	3.3±0.21	4.6±0.32	3.6±0.52	0.284	0.860	0.10	0.266	0.858
WBSF (kg) ^f	4.3±0.11	4.4±0.09	4.3±0.21	4.7±0.18	4.8±0.22	0.460	0.117	0.752	0.214	0.629
Cooking loss (%)	19.5±0.56	19.5±0.64	18.9±1.15	22.1±0.64	19.8±1.17	0.968	0.205	<i>0.095</i>	0.7565	0.834

^a Significant p-values (P < 0.05) are indicated by asterisk, and a tendency for significance (0.05 ≤ P < 0.10) is indicated by italics.
^b ‘n_c’ indicates sample size for cortisol, loin temperature, loin pH, and scratch score. ‘n_m’ indicates sample size for remaining meat quality variables.
^c Data were transformed for statistical analysis; values presented are average±SE.
^d Lsmeans within a row with different lowercased letters (a, b) differ significantly (P < 0.05).
^e JSS: Japanese scoring system (Nakai et al. 1975)
^f WBSF: Warner-Bratzler shear force