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Genome-Wide Association of Myoglobin Concentrations in Pork Loins

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Abstract: Lean color is a major focus for identifying pork loins for export markets, and myoglobin is the primary pigment driving pork color. Thus, increasing myoglobin concentration should increase redness of pork products and the number of loins acceptable for exportation. Therefore, understanding genetic variation and parameters affecting myoglobin concentration is critical for improving pork color. The objective of this study was to identify genetic markers associated with myoglobin concentration in pork loin muscle. Ultimate pH and myoglobin concentrations were measured in *longissimus thoracis et lumborum* samples of pigs (n = 599) from two different commercial finishing swine facilities. A Bayes-C model implemented in GenSel identified regions within 7 chromosomes that explained greater than 63% of the genetic variance in myoglobin concentration. Chromosome 7 had 1 significant region which accounted for 37% of the genetic variance, while chromosome 14 had 4 significant regions accounting for 9.8% of the genetic variance. Candidate genes in the region on chromosome 7 were involved in iron homeostasis, and genes in the significant regions on chromosome 14 were involved in calcium regulation. Genes identified in this study represent potential biomarkers that could be used to select for higher myoglobin concentrations in pork, which may improve lean meat color.

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Introduction

Marketability of pork products, particularly pork loins, is impacted by lean color (Wilson et al., 1959). Loins with darker, redder lean color are preferentially selected for export markets and thus, have greater value. Loins with very pale lean color are discriminated against. Thus, the National Pork Board has set a 5-yr goal to decrease the percentage of pork loin chops scoring below the National Pork Board color score 3 by 10 percentage points (National Pork Board, 2015).

Normal pork quality development is largely regarded to be dependent on the relationship between carcass temperature and pH decline (Bendall and Swatland, 1988; Joo et al., 1999). During the conversion of muscle to meat, the rate and extent of pH decline influences pork color characteristics (Huff-Lonergan and Lonergan, 2005; Lindahl et al., 2006). In extreme cases when pH decreases too drastically, proteins denature affecting light reflectance, i.e., pale color (Joo et al., 1999; Huff-Lonergan and Lonergan, 2005). Thus, most research on pork color has focused on ultimate pH and pH decline immediately postmortem. Consequently, the pork industry has successfully implemented technology and processes to limit pale, soft, and exudative pork. Over the past 20 yr, advances in genetics, animal

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handling, stunning, and pork carcass processing have dramatically reduced the incidence of abnormal pH decline (Berg, 1998; Cassens, 2000; Channon et al., 2000; Sionek and Przybylski, 2016); yet, many loins are still undesirably pale (National Pork Board, 2015). Thus, to accomplish the industry-wide goal of improving pork lean color, an alternative strategy is needed to increase redness and decrease lightness of pork loin lean color.

Novel efforts to improve pork color should address additional mechanisms (Mancini and Hunt, 2005). A logical approach to increase redness of lean color is to increase concentrations of the red pigment present in pork muscle. Because myoglobin is the primary pigment influencing pork lean color, increasing myoglobin concentrations should lead to decreased lightness and increased redness under normal pH (Lindahl et al., 2001; Karamucki et al., 2013; Neethling et al., 2017).

Selection for improved pork quality is difficult because most pork quality attributes can't be measured on a live animal (Rothschild and Ruvinsky, 2011). Numerous studies have been conducted to identify genomic regions associated with pork color and pH in commercial pigs (Nonneman et al., 2013; Sanchez et al., 2014; Bernal Rubio et al., 2015; Liu et al., 2015). A summary of Quantitative Trait Loci (QTL) discovered in pigs can be found at PigQTLdb (https://www.animalgenome.org/cgi-bin/QTLdb/SS/browse). A total of 702 associations have been detected for some measure of post-mortem muscle pH, while 602 associations have been reported for some measure of muscle color. Despite the well-known association of muscle pH and pork color (Bendall and Swatland, 1988; Joo et al., 1999; Lindahl et al., 2006; Neethling et al., 2017), the majority of genomic regions identified in genome wide association studies are only associated with muscle pH or muscle color. As expected, the most common co-localization of associations was found between pH and L^*, where 25 associations for these 2 traits directly overlap. Unfortunately, PigQTLdb does not contain any studies in pigs where myoglobin concentration has been evaluated.

Marker-assisted selection to improve meat quality traits has been beneficial to the swine industry (Miar et al., 2014), yet greater improvements could result if markers were detected for pigments associated with meat color. The objective of this study was to identify genetic markers associated with myoglobin content in porcine *longissimus thoracis et lumborum* muscle to expand our knowledge of genetic factors controlling the color of pork. The loin was selected as it is the primal cut where color is of greatest economic importance in the U.S. pork industry, and it is the primal targeted by the National Pork Board goals.

Materials and Methods

Data collection

The animals sampled in this experiment did not originate and were not under the control of the U.S. Meat Animal Research Center (USMARC). Animal management and handling was conducted as part of the normal procedures of commercial operations and animals were humanely harvested in USDA-inspected processing facilities. Loins were identified for inclusion in the experiment postmortem. Thus, animal procedures were not reviewed and approved by the USMARC Animal Care and Use Committee.

Source of animals used in this study and sample processing have been described in detail by Shackelford et al. (2012). Data were collected on pigs (n = 599) from 2 different commercial swine grow-finish barns which received weaned pigs from the same commercial sow farm. All pigs were from the same genetic line, approximately 6 mo of age, and comprised 314 females and 285 castrated males. Each barn sent an equal number of pigs to three different processing plants located equal distances from the grow-finish facilities on the same day. All animals were fed the same diets throughout the grow-finish period.

Muscle pH was collected on loin chops from approximately the 14th rib region at 14 d postmortem during pork loin chop evaluation as described in Shackelford et al. (2012). Briefly, pH was measured on *longissimus* muscle using an Omega PHE-2385 (Omega Engineering Inc., Stamford, CT) pH probe with a Reed SD-230 handheld pH meter (Reed Instruments, Wilmington, NC). The pH meter was calibrated with pH 7 and pH 4 buffers according to the manufacturer's instructions at the beginning of each measurement session.

A 2.54-cm thick chop was obtained from the 10th rib region of each loin, placed on a polystyrene tray with a soaker pad, and overwrapped with oxygen permeable polyvinylchloride film [Stretchable meat film 55003815, Prime Source, St. Louis, MO; oxygen transmission rate = 1.4 mL / (cm2 · 24 h) at 23°C]. Packages were placed under continuous fluorescent lighting (color temperature = 3,500K; color rendering index = 86; 32 W; T8 Ecolux bulb, model F32T8/SPX35 GE, GE Lighting, Cleveland, OH). Light intensity at the meat surface was 2,000 lx. Packaged chops were allowed to bloom for at least 2 h before color measurement began. Instrumental color readings (2·LM chop–1·) were taken on each chop using a Hunter Miniscan XE Plus colorimeter (HunterLab, Reston, VA) with a 25-mm port. The colorimeter was set to collect data with Illuminant A and a 10° observer. The CIE L^* (lightness), a^* (redness), and b^* (yellowness)

color-space values were reported as the average of the readings taken on each chop.

A chop, corresponding to the first lumbar vertebrae region, designated for myoglobin content determination was vacuum packaged and stored at –20°C until further analysis. Chops were partially thawed so that external fat and epimysium could be removed. Care was taken to ensure chops did not thaw enough for purge to be lost. The remaining *longissimus lumborum* tissue was pulverized in liquid nitrogen to produce a fine, homogenous powder. Myoglobin was extracted in a phosphate buffer (40 mM K_2PO_4) as described by American Meat Science Association (2012) from 2.5 g of powdered tissue. A 200-µL aliquot of the extract was transferred in triplicate to a 96-well plate, which included a phosphate buffer blank. Absorbance values were obtained at 525 and 700 nm using a SpectraMas plus 96-well plate reader (Molecular Devices, Sunnydale, CA). Extracted myoglobin pigment concentration (mg/g muscle) was calculated by taking the difference between the absorbance at 525 nm and 700 nm, a millimolar extinction coefficient of 7.6 $mM^{-1}cm^{-1}$, the molecular weight of myoglobin (17,000), and the appropriate dilution factor.

Genotyping

Genomic DNA was extracted from *longissimus lumborum* tissue, after sampling for myoglobin concentration, using a WIZARD genomic DNA purification kit according to the manufacturer's protocol (Promega Corp., Madison, WI). Genotyping was conducted using the Illumina Porcine SNP60 V2 chip (Illumina, Inc., San Diego, CA) and GGP-Porcine chip (GeneSeek, Lansing, MI). Genotypes were filtered to include only those with minor allele frequency ≥ 5% and a unique map position in the Sscrofa10.2 genome assembly (Groenen et al., 2012). After quality checks 7,755 SNP from the GGP-Porcine chip were used for subsequent analyses. In total, 150 pigs were genotyped using the Illumina Porcine SNP60 V2 chip and 449 pigs were genotyped using the GGP-Porcine chip. Markers that overlapped between the GGP-Porcine chip and the Illumina Porcine SNP 60 V2 chip were used. Averages for missing genotypes were used.

Genomic analysis

Priors for genetic and residual variances for each trait were obtained by running Bayes-Cpi using GenSel (Fernando and Garrick, 2009). The prior proportion of SNP that are assumed to have no effect on myoglobin concentration within an iteration of the Monte Carlo Markov Chain was also determined. A Bayes-C variable selection method, using GenSel software (Fernando and Garrick, 2009), was used to identify and quantify genomic regions associated with myoglobin concentration. A chain of 41,000 iterations was used with the first 1,000 cycles discarded as burn-in. Myoglobin concentration was analyzed with a fixed effect of harvest group (finishing farm and processing plant) and ultimate pH as a covariate. The posterior distribution for the genetic variance was derived using effects sampled every 40 iterations. Genomic regions associated with myoglobin concentration were identified using 1-Mb genome windows.

Further analysis on the most significant SNP from the detected regions in GenSel was conducted and analyzed using a general linear model in R (R Core Team, 2013). Harvest group was included in the model as a fixed effect. Ultimate pH and the most significant SNP from the detected regions were included in the model as covariates.

Gene functions in significant genomic regions

Genes located within 0.5 Mb of significant genomic regions were identified using the NCBI annotation of Sscrofa10.2 (Release 104, Supplementary Table S1). Functions of these genes were determined using the PANTHER classification system (version 11.1; Mi et al., 2013; http://www.pantherdb.org/chart/summary/pantherChart.jsp?filterLevel=1&chartType=1&listType=1&type=5&species=Susscrofa). An enrichment analysis of gene function was performed using the implementation of binomial test of over-representation. Gene ontology (GO) terms were assessed using the default Ensembl Sus scrofa GO annotation as background for the enrichment analysis. Over-representation of GO terms were considered statistically significant at a Bonferroni corrected P-value ≤ 0.05.

Results

All loins included in the present study had normal color and water holding characteristics, and none displayed the pale, soft, and exudative condition. These loins were a subset of those described as 'Commercial' by Bernal Rubio et al. (2015) where QTL scans were conducted for standard measures of pork quality, including color. The mean ± standard deviation for color traits among those 599 loin samples was 57.06 ± 2.94, 15.21 ± 1.45 and 21.83 ± 1.79 for L^*, a^*, and b^*, respectively. In addition, Pearson correlation coefficients between color traits and myoglobin concentrations were –0.556, 0.303 and –0.161 for L^*, a^*, and b^*, respectively. In

the current study, as myoglobin concentration increased, ultimate pH increased, and *L** values decreased.

Genome wide association study

Average myoglobin concentration for the 599 grow-finish pigs used in the study was 0.87 ± 0.005 mg/g. Ultimate pH averaged 5.70 ± 0.006. Detailed results for each 1-Mb window predicted by GenSel to explain a significant percentage of genetic variation (≥ 1.0%) are shown in Table 1. Heritability for myoglobin concentration was low (0.091). Nearly 63% of the genetic variance associated with myoglobin concentration was explained by regions within seven chromosomes.

Table 1. Windows identified in the GenSel analysis that explain at least 1.0% of genetic variation in myoglobin concentration

Chromosome	Position[1], Mb	% of genetic variance explained	Number of SNPs	Frequency of iterations with, $P > 0$
7	4	36.97	9	0.89
16	77	5.98	3	0.24
X	11	4.66	4	0.20
14	146	4.52	2	0.19
14	82	2.03	3	0.11
14	108	2.03	3	0.10
4	14	1.59	4	0.08
1	86	1.51	3	0.07
15	141	1.40	5	0.08
14	54	1.19	3	0.06
1	140	1.10	3	0.05

[1]Positions are based on the Sscrofa 10.2 genome.

Chromosome 7 had one region that accounted for 37.0% of the genetic variance. Chromosome 14 had four regions cumulatively accounting for 9.8% of the genetic variance in myoglobin concentration. Chromosome 16 had one region that accounted for 6.0% of the genetic variance while chromosome X had one region that accounted for 4.7% of the genetic variance.

Output from linear model is shown in Table 2. Contemporary group was not significant, however the best-fit model included contemporary group ($R^2 = 0.41$). Ultimate pH was significantly associated with myoglobin concentration ($P < 2.2E\text{-}16$). As ultimate pH increased, myoglobin concentrations also increased (regression coefficient 4.52 ± 0.28). Two SNP from the general linear model exceeded a Bonferroni correction factor assuming 7755 tests of significance ($P > 6.45E\text{-}06$). These two SNP are ALGA0038081 located on SSC 7 at position 4406736 and ALGA0082929 located on SSC 14 at position 146970022. Five other SNP had highly significant nominal significance levels ($P < 0.001$).

Discussion

The impact of muscle pH on pork lean color is well documented and has been extensively studied in an effort to mitigate incidence of the Pale, Soft, and Exudative (PSE) lean condition (Berg, 1998; Cassens, 2000; Channon et al., 2000; Sionek and Przybylski, 2016). As pH declines, increased internal reflectance (Bendall and Swatland, 1988) and increased lightness of meat occurs (Joo et al., 1999). Change in genetics of market animals, animal handling, stunning, and chilling have reduced losses due to inferior

Table 2. Linear model output for analysis of myoglobin concentration using most significant SNP from windows identified in GenSel

Effect/SNP	rs#	Map Position[1]	Estimate (± SE)	*P*-value
Contemporary Group				0.1047
Ultimate pH			4.52 ± 0.28	2.20E-16
ALGA0038081	80949003	7_4406736	-0.036 ± 0.006	2.35E-07
ALGA0082929	80787250	14_146970022	0.028 ± 0.011	1.34E-06
ASGA0080798	80945548	X_11060922	-0.024 ± 0.006	1.17E-05
ASGA0003560	80995769	1_86991865	0.021 ± 0.007	6.39E-05
ALGA0091756	81462291	16_77815248	0.027 ± 0.006	1.26E-04
H3GA0041684	80856633	14_108719339	-0.014 ± 0.007	3.77E-04
MARC0039560	80782447	1_140541767	0.017 ± 0.007	8.62E-04
H3GA0012050	80940346	4_14722391	0.018 ± 0.006	0.0016
ASGA0064592	80975788	14_82509062	0.023 ± 0.007	0.0031
H3GA0045233	80939746	15_141419118	-0.022 ± 0.007	0.0051
ASGA0063381	80978128	14_54230949	-0.031 ± 0.012	0.0111

[1]Positions are based on the Sscrofa 10.2 genome.

pork quality (Sionek and Przybylski, 2016), so further efforts to improve pork color should address additional mechanisms, including myoglobin concentration. Muscle color is affected by numerous attributes, perhaps most notably by pigment concentration (Karamucki et al., 2013; Neethling et al., 2017). Lindahl et al. (2001) reported that pigment concentration and myoglobin redox state explained 86 and 90% of the variation in *L** and a* values of pork *longissimus* and *biceps femoris*.

The strong positive relationship between myoglobin concentration and muscle pH was surprising (Table 2). We speculate that the correlation between muscle pH and myoglobin content are likely a result in increased glycolytic metabolism associated with increased proportion of white muscle fibers (i.e., white muscle fibers more dependent on glycolytic metabolism would be expected to have lower pH due to greater lactate accumulation, as well as lesser myoglobin content). Ryu and Kim (2005) reported that increases in the percentage of white (Type IIb) muscle fibers in pork *longissimus* muscle was associated with increased glycolytic rate, and increased *L** values at 45 min and 24 h postmortem, and decreased *a** values at 45 min postmortem. England et al. (2016) reported greater myoglobin content in pork *masseter* muscle, which is predominately composed of slow (red) muscle fibers, compared to *longissimus* muscle, which has a greater proportion of fast (white) muscle fibers. Genes located within the QTL for myoglobin content, which will be discussed in detail below, support this hypothesis.

Additional QTL identified for myoglobin content may also help explain the relationship between muscle pH and myoglobin content. Proto-oncogene tyrosine-protein kinase, located in region SSC 1_86 Mb regulates intracellular pH in cardiomyocytes through ATP activation (Pucéat et al., 1998). Qiao et al. (2011) identified a QTL in region SSC 15_141 Mb, with a strong effect on 24-hour postmortem pH and color. Thus, QTL for myoglobin content in the present experiment and QTL for muscle pH reported by other investigators overlap to some degree. This suggests that the mechanism by which this gene influences pH, may also be related to myoglobin concentration. Alternatively, the genes influencing myoglobin concentration may simply be segregating along with genes regulating muscle pH.

Heritability for myoglobin concentration in this study was low (0.091). Newcom et al. (2004) estimated the heritability for soluble myoglobin concentration to be 0.27 from a data set representing 7 different sire breeds in a progeny test for the 1999 National Barrow Show. An estimate of heritability for myoglobin concentration was much higher (0.63) in Duroc barrows (Allen et al., 1966). The high heritability estimate from Newcom et al. (2004) is likely due to study design in which environmental variation was limited and purebred animals from 7 different breeds were used. Thus, Newcom et al. (2004) had minimized environmental variation while maximizing genetic variation. Allen et al. (1966) only had 55 Duroc barrows and also studied 87 Yorkshire barrows. They were unable to estimate heritability in the Yorkshire population and their heritability estimate in Duroc barrows (0.63) wasn't significantly different from 0 due to an extremely high standard error (0.61). In the current study pigs were from the same terminal cross such that genotypic variation in the data set is reduced. While pigs were reared on 2 different finishing farms and harvested in 3 different processing facilities, these environmental factors were balanced such that they could be adequately accounted for in the statistical model.

This is the first study to conduct a Genome Wide Association Study for myoglobin content in pork *longissimus* muscle. Myoglobin, through comparative mapping, has been mapped to SSC 5_9 Mb. No QTL were found in this region. However, a major QTL, located on chromosome 7 (SSC 7_4), was identified that explained approximately one third of the genetic variation in myoglobin concentration. Bone morphogenetic protein 6 (*BMP6*), located in the region SSC 7_4 Mb, regulates a range of biological processes, including iron homeostasis, ovulation, and bone and fat development. This gene has been shown to impact meat quality (Lee et al., 2014). As a member of the hedgehog-signaling pathway, *BMP6* may be important in slow oxidative fiber clustering in pigs (Lee et al., 2014). Hence, selection for desirable alleles of *BMP6* may increase iron homeostasis and increase oxidative fiber clustering, which could in turn increase myoglobin concentration in the meat.

Another candidate gene, located in SSC 15_141 Mb, was insulin receptor substrate 1 (*IRS1*). This gene has been shown to be highly associated with slow-twitch and fast-twitch oxidative fibers (Liu et al., 2016). ADAM metallopeptidase domain 12 (*ADAM12*), a candidate in SSC 14_146 Mb has been shown to have higher expression in slow-oxidative muscle fibers in cattle (Coles et al., 2014). Red oxidative muscle fiber types have higher myoglobin content than white glycolytic muscle fiber types (Beecher et al., 1965). Muscles with increased myoglobin concentrations are likely to be more dependent on oxidative metabolism than those with lesser myoglobin content. Greater myoglobin would be needed to supply oxygen for aerobic metabolism, while less anaerobic metabolism would ultimately result in less lactate in postmortem muscle (higher ultimate pH). Thus, we speculate that the positive correlation between muscle pH and myoglobin concentration

is the result of differences in muscle fiber type composition affecting both traits. Thus, selection for increased myoglobin content should also increase ultimate pH.

Over-representation of GO terms for the set of genes located in significant genomic regions was analyzed using PANTHER. In this set of genes, cellular component terms: organelle part, cytoplasmic part, and cytoplasm were significantly over-represented (Table 3). Many of the candidate genes annotated by enriched GO terms were located on chromosome 14, in particular region SSC 14_82 Mb. One of the genes, calcium-calmodulin dependent protein kinase II (*CAMK2G*), located in SSC 14_82 Mb, is responsible for sarcoplasmic reticulum calcium transport into skeletal muscle, thus assisting in calcium release in slow and fast twitch muscles. Another candidate gene, myozenin 1 (*MYOZ1*), located in SSC 14_ 82 Mb, is a sarcomeric calcineurin binding protein of striated muscles (Frey et al., 2008). Protein phosphatase 3 catalytic subunit β (*PPP3CB*), located in SSC 14_82 Mb, is a calcium-dependent, calmodulin-stimulated protein phosphatase and plays a role in calmodulin activation of calcineurin.

All 3 of the above mentioned candidate genes (*CAMK2G, MYOZ1, PPP3CB*) regulate calcium or calmodulin, which control activation of calcineurin. Calcineurin is a calcium and calmodulin dependent serine phosphatase, which has been shown to regulate skeletal muscle fiber type switching (Chin et al., 1998; Olson and Williams, 2000; Parsons et al., 2003). This occurs through targeting transcriptional factors of the nuclear factor of activated T cells (McCullagh et al., 2004). Kanatous et al. (2009) established the calcineurin pathway as a potent transcriptional regulator of myoglobin gene expression. Selection on functional variants within these candidate genes could increase myoglobin concentrations through a change in muscle fiber type.

Conclusion

Candidate genes associated with myoglobin concentration in swine *longissimus thoracis et lumborum* muscle were identified. Gene ontology terms organelle part, cytoplasmic part, and cytoplasm were among those over-represented in the set of candidate genes. Chromosome 14 had three candidate genes involved in the regulation of calcineurin, thus regulating muscle fiber type. Additionally, the major QTL on chromosome 7 harbored a gene involved in iron homeostasis. Selection for genetic markers and candidate genes identified in this study may result in higher myoglobin concentrations in pork *longissimus*, which would improve lean meat color.

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Table 3. List of gene ontology terms that were significantly over-represented in the set of genes located in significant windows from the GenSel analysis.

Ontology term	Gene set (n genes) Annotated genes[1] (21,398)	Genes[2] (93)	Genes expected	*P*-value
Biological Process				
Hydrogen peroxide metabolic process	17	4	0.08	7.79E-03
Social behavior	26	4	0.12	4.13E-02
Intraspecies interaction between organisms	26	4	0.12	4.13E-02
Regulation of hormone levels	237	10	1.06	8.21E-04
Cellular Component				
Organelle part	5211	42	1.80	2.63E-02
Cytoplasmic part	5319	46	23.86	9.03E-04
Cytoplasm	6878	52	30.86	7.26E-03

[1]Number of genes in the background *Sus scrofa* annotation set with given GO term. Total number of annotated genes is shown in parentheses.

[2]Number of genes with given GO term. Total number of genes with annotations in the background *Sus scrofa* annotation set is shown in parentheses.

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