University of São Paulo
"Luiz de Queiroz" College of Agriculture

Association of regulatory polymorphisms of gene expression with color phenotypes, water holding capacity, and pH of Nellore meat

Beatriz Delcarme Lima

Dissertation presented to obtain the degree of Master in Science. Area: Animal Science and Pastures

Piracicaba
2024

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versão revisada de acordo com a Resolução CoPGr 6018 de 2011

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DEDICATION

I dedicate this work to my parents, Silas and Haydêe, who taught me never to stop learning.

I also dedicate his work to my fiancé, Gabriel, who encouraged me and believed that I could do and learn anything.

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EPIGRAPH

"No one knows so much that they have nothing to learn and

No one knows so little that they have nothing to teach."

- Blaise Pascal

SUMMARY

RESUMO

Associação de polimorfismos regulatórios de expressão gênica com fenótipos de cor, capacidade de retenção de água e pH da carne de Nelore

Há uma demanda crescente por carne de alta qualidade, necessitando da avaliação de diversas características influenciadas por fatores intrínsecos e extrínsecos. Estas características, reguladas por múltiplos genes e influências ambientais, representam desafios na medição devido ao seu custo e à necessidade de abate de animais. A genômica surge como uma solução promissora, como uma ferramenta para identificar marcadores genéticos que regulam uma parcela significativa da variação genética aditiva nessas características. Neste trabalho, nosso objetivo foi identificar *locos* de expressão quantitativa (eQTLs) associados a fenótipos de qualidade de carne em bovinos Nelore, incluindo cor, capacidade de retenção de água e pH. Para isso, utilizamos um painel abrangente de polimorfismos de nucleotídeo único (SNPs) de 553.581 marcadores, obtido a partir de um painel imputado de SNPs abrangendo genótipos de 778 progênies, sequenciamento de DNA de 26 touros Nelore não aparentados entre si e dados de sequenciamento de RNA de tecidos musculares de 192 progênies. A análise do eQTL, utilizando genótipos de 192 animais, revelou 51.324 eQTLs. Estudos subsequentes de associação genômica ampla (GWAS) com 374 animais identificaram 838 eQTLs associados à cor, 172 à capacidade de retenção de água e três ao pH. A integração com picos de ATAC-Seq identificou 75 eQTLs associados com a cor da carne e 24 associados com capacidade de retenção de água em regiões abertas da cromatina. A análise de enriquecimento funcional revelou caminhos como resposta imune, processamento e apresentação de antígenos e metabolismo da glutationa. Notavelmente, a maioria dos genes regulados por eQTLs foram associados a estas vias. Esta abordagem abrangente identificou possíveis variantes regulatórias candidatas e genes associados à qualidade da carne, avançando nossa compreensão da genética do *Bos indicus*.

Palavras-chave: eQTL, GWAS, SPNs, Qualidade da carne

ABSTRACT

Association of regulatory polymorphisms of gene expression with color phenotypes, water holding capacity, and pH of Nellore meat

There is a growing demand for high-quality meat, necessitating the assessment of various traits influenced by intrinsic and extrinsic factors. These traits, governed by multiple genes and environmental influences, pose challenges in measurement due to their cost and the need for animal slaughter. Genomics emerges as a promising solution, as a tool to identify genetic markers regulating a significant portion of additive genetic variation in these traits. In this work, we aimed to identify expression quantitative trait loci (eQTLs) associated with meat quality phenotypes in Nellore cattle, including color, water-holding capacity, and pH. For that, we used a comprehensive single nucleotide polymorphisms (SNPs) panel of 553,581 markers, obtained from an imputed panel of SNPs encompassing genotypes from 778 progenies, DNA sequencing from 26 unrelated Nellore sires, and RNA sequencing data from muscle tissues of 192 progenies. The eQTL analysis, using genotypes of 192 animals, revealed 51,324 eQTLs. Subsequent genome-wide association studies (GWAS) with 374 animals identified 838 eQTLs associated with color, 172 with water-holding capacity, and three with pH. Integration with ATAC-Seq peaks identified 75 eQTLs associated with meat color and 24 associated with water-holding capacity in open chromatin regions. Enrichment analysis uncovered pathways such as immune response, antigen processing and presentation, and glutathione metabolism. Notably, most of genes regulated by eQTLs were associated with these pathways. This comprehensive approach identified putative candidate regulatory variants, and genes associated with meat quality, advancing our understanding of *Bos indicus* genetics.

Keywords: eQTL, GWAS, SNPs, Meat quality

FIGURE LIST

TABLE LIST

ACRONYMS LIST

a*: redness

ATAC-Seq: Assay for Transposase-Accessible Chromatin

b*: yellowness

Beta-e: beta values for gene expression

Beta-p: beta values for phenotype

BoLA: bovine leukocyte antigen

cDNA: complementary DNA

DFD: dark-firm-dry

eQTL: expression quantitative trait loci

GATK: Genome Analysis Toolkit

GVCF: Genomic Variant Call Format

GWAS: Genome-Wide Association Studies

L*: lightness

LT: *Longissimus thoracis*

MAF: minor allele frequency

Mb: myoglobin

MHC: Major Histocompatibility Complex

mRNA: messenger RNA

PCA: Principal Component Analysis

PUFA: polyunsaturated fatty acids

QTLs: quantitative trait loci

RFI: residual feed intake

RIN: RNA integrity numbers

RNA- Seq: RNA-Sequencing

ROS: reactive oxygen species

SNPs: single-nucleotide polymorphisms

UCPs: uncoupling proteins

VLCC: voltage-gated L-type calcium channels

WBC: water binding capacity

WHC: water-holding capacity

INTRODUCTION

Consumer preferences have changed in the past few decades, significantly impacting the criteria used to determine the quality of meat products. This evolving consumer demand has prompted a comprehensive exploration of the factors contributing to high-quality meat. Factors influencing a complex trait such as meat quality, include intrinsic and extrinsic aspects controlled by various genes with minor or moderate effects (Ferraz & Felício, 2010; Gao et al., 2021; Mancini & Hunt, 2005; Scollan et al., 2006; Williams, 2008). However, measuring meat quality traits is expensive and can only be done after slaughter. Genomics has emerged as a solution, offering the potential to identify genetic markers for breeding programs (Magalhães et al., 2019; Rezende et al., 2021).

Some traits have a direct influence over meat appearance and other characteristics. This is particularly evident in color, water-holding capacity, and pH. Alterations in pH impact meat color and water-holding capacity (Ramos & Gomide, 2017). Additionally, changes in the water-holding capacity can significantly affect the meat color (King et al., 2023). The interconnected nature of these factors highlights their collective influence on consumer preferences and choices.

Meat color, a multifaceted trait, is intricately influenced by several intrinsic and extrinsic factors, serving as a crucial indicator of meat quality and freshness (Poveda-Arteaga et al., 2023). Complementing this, water-holding capacity (WHC) emerges as an essential determinant of a tissue's ability to retain water, directly impacting visual attributes like meat color, surface appearance, and texture (Toldrá, 2006; Varnam & Sutherland, 1995). WHC also plays a definitive role in determining meat juiciness, a factor that is indirectly quantified (Hamm, 1986; Warner, 2017). Furthermore, pH measurement stands out as a critical parameter in assessing meat quality. The influence of pH extends across various traits, including meat color, water-holding capacity, and texture, establishing its significance as a comprehensive indicator of overall meat quality (Montgomery & Leheska, 2008).

Exploring expression quantitative trait loci (eQTL) analysis holds significant importance in identifying specific genomic regions that exert control over gene expression (de Souza et al., 2020). Through this analysis, it is possible to identify genetic factors capable of modulating complex traits, providing a crucial framework for mapping genetic variations that exert influence over gene expression (Michaelson et al., 2009; Shabalin, 2012; Westra & Franke, 2014). This approach enhances our understanding of the interplay between genetic and environmental factors and serves as a valuable tool for unraveling the genetic elements of complex traits.

An additional analytical approach in revealing the genetic architecture is the Genome-Wide Association Studies (GWAS). This method allows identifying specific genomic regions, markers, and

candidate genes associated with crucial livestock traits. (Goddard & Hayes, 2009; Hayes & Goddard, 2001). Combining the strengths of eQTL and GWAS enhances analytical potency, a synergistic approach not usual in the context of beef cattle (Cesar et al., 2018; Higgins et al., 2018; Leal-Gutiérrez et al., 2020; Wainberg et al., 2019). This work, therefore, offers a comprehensive exploration of polymorphisms influencing gene expression and their association with meat quality traits, including color, water-holding capacity, and pH. By shedding light on these intricate associations, this literature review provides invaluable insights into the genetic foundations of cattle, contributing significantly to our understanding of cattle genetics and its implications for meat quality.

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1. LITERATURE REVIEW

1.1. Phenotypes

Meat quality is an essential important factor that influences the consumer's choices. The current consumer demands a healthy and high-quality product. The measurements that evaluate the meat quality include tenderness, color, fat content, and sensory characteristics like juiciness and flavor (Ferraz & Felício, 2010; Mancini & Hunt, 2005; Scollan et al., 2006; Williams, 2008).

Meat quality traits are expensive to measure since they require slaughtered animals. Several genes control meat quality, so the improvement by traditional selection becomes difficult and takes time. The solution can be found in using genomics as a tool to identify genetic markers that regulate a considerable proportion of additive genetic variation of these traits (Magalhães et al., 2019; Rezende et al., 2021).

Some phenotypes are interconnected, for they influence each other. That is the case of the pH, which can cause changes in color and water-holding capacity (Ramos & Gomide, 2017). Water-holding capacity can cause changes in meat color during storage or display (King et al., 2023). Because they are connected, they are presented together in this work.

2.2. Color

The appearance of beef is a powerful influence on consumers' choices because the color indicates freshness and quality. This visual stimulus has a significant impact on consumers' opinions about meat, since the first perception can define the buying or rejection of a particular meat product (Poveda-Arteaga et al., 2023). Red is highly preferred over purple or brown colors and is considered the most robust value for predicting meat color acceptability (Abril et al., 2001; Holman et al., 2017; Van Rooyen et al., 2017).

Intrinsic and extrinsic factors influence the color. Some of these intrinsic factors are breed, sex, age of the animals, contractile and metabolic muscle properties, muscle position and structure, connective tissue content, intramuscular fat, redox forms and distribution of myoglobin, slaughter weight, and others. And the extrinsic factors are mainly related to the rearing factors, production systems and feeding, premortem stress, transport and animal handling, post-slaughter aging conditions, and to the commercial practices and conditions during retail display such as meat cuts, additives, temperature, chilling rates packaging and type of light (Gagaoua et al., 2018; Poveda-Arteaga et al., 2023). It is proven that these factors and their interactions affect fresh meat color (Mancini & Hunt, 2005; Neethling et al., 2017).

One of the main factors that influence meat color is the concentration and physicochemical state of myoglobin (Mb), the most common pigment in meat (Mancini, 2009; Mancini & Hunt, 2005; Neethling et al., 2017). Myoglobin is a sarcoplasmic heme protein that has as its primary function in living tissues to transport and store oxygen from the blood to the mitochondria in the muscle (Li et al., 2020; Wittenberg et al., 1975; Wu et al., 2015). The Mb concentration differs between muscles and species, owing to the variation in energy demand and metabolism (Wittenberg, 1970).

The meat color will depend on the amount of myoglobin, since the higher the concentration, the darker the meat will be. Physiological changes and oxygen content cause alterations in myoglobin structure, which can be transformed in different oxidation states, especially after slaughtering, when post-mortem muscle metabolism changes from aerobic to anaerobic (Gao et al., 2013; Ke et al., 2017; Li et al., 2020; Varnam & Sutherland, 1995). Different forms of myoglobin can be produced, like deoxymyogobin, which causes a purplish-red color with ferrous iron and no ligand attached; oxymyoglobin that forms a bright cherry-red color with ferrous iron and oxygen attached; and metmyoglobin that causes a dull-brown color, with ferric iron, water attached and absence of oxygen. A meat with high content of oxymyoglobin is associated with a bright red color, which indicates good quality meat, while meat with a predominance of metmyoglobin can cause a consumer refusal (Hernández Salueña et al., 2019; Mancini & Ramanathan, 2014; Varnam & Sutherland, 1995).

The ultimate pH is considered a significant determinant of meat color (Abril et al., 2001; Mahmood et al., 2017). The effect on the redness of beef is probably connected to oxygen consumption. Right after the animal is slaughtered, the mitochondria can still consume oxygen, if the necessary substrates are available. When the pH decreases during the rigor process, the mitochondrial activity gradually decreases, which allows oxymyoglobin levels to increase, and results in an intense red color. Usually, darker beef has lower levels of oxymyoglobin when compared to normal pH beef. This difference is remarkable with the abundance of mitochondria in severe dark-cutting beef compared to beef with a normal pH level. The meat color is considerably more stable at pH values relatively higher due to the influence in the enzyme activity and oxygenation rate (McKeith et al., 2016).

One factor that can negatively affect the meat quality and lead to financial losses is stress before slaughter. When stressed, the glycogen reserves of muscle are depleted and cause, consequently, an insufficient production of lactic acid post-mortem. That low acidity during the aging process causes changes in the color, structure, tenderness, and taste of the meat (Xing et al., 2019). When the ultimate pH reaches values higher than expected, meat color gets darker, and when the

ultimate pH is lower than expected, meat color gets pale, and this consequence is related to modifications at myoglobin and myofibrils (Hughes et al., 2019; Ijaz et al., 2020). The depletion of glycogen reserves in animals during stress in pre-mortem causes the limited lactic acid formation and insufficient pH decline in post-mortem muscle (Ijaz et al., 2020; Mahmood et al., 2017).

A DFD (dark-firm-dry) meat is a condition that happens when the muscle has an ultimate high pH. This kind of beef has a high quantity of myoglobin oxidation products when compared to normal pH muscles, and on the meat surface, the light reflectance is smaller (English et al., 2016; Mahmood et al., 2017; McKeith et al., 2016; Ramanathan et al., 2020).

The darker meat surface color in DFD meat is because of the higher tissue oxygen consumption, due to the high count of mitochondria and their activity, which restricts the myoglobin capacity to oxygenate. Moreover, a DFD meat has as a great water-holding capacity as a characteristic, which causes swollen fibers that increase light scattering from myoglobin fractions and results in a darker superficial appearance, typical of a DFD muscle (English et al., 2016; Gao et al., 2013; Hughes et al., 2017; Ramanathan et al., 2020; Wu et al., 2020).

DFD is a problem because it results in economic losses since their prices tend to be reduced due to meat's unwelcome appearance, and usually turn into processed meat products. Thus, the solution to this issue can prevent the waste of resources, both economically and environmentally (Poveda-Arteaga et al., 2023). The meat exhibits characteristics of an atypical DFD state within the pH range of 5.8 to 6 and transforms into a typical DFD state at pH ≥ 6 (Ferreira et al., 2024). Precise quantification of DFD meat incidence in Brazil and other countries is challenging due to considerable variations influenced by factors such cattle breed, diet, slaughter methods, management practices, and processing. Although absolute numbers are unavailable, international studies underscore the global significance of DFD as a challenge in the beef industry, with prevalence rates ranging from 1.3% to 13.9% (Ponnampalam et al., 2017). In Brazil, limited studies have addressed the incidence of DFD meat, showing regional variations from 4% to 8% (Báron et al., 2021; Rosa et al., 2016).

The cattle breed exerts a direct influence, and evidence supports the assertion that the heightened aggressiveness temperament in pure *Bos indicus* (Brahman) or *Bos indicus* crosses leads to a darker muscle color and elevated pH values in meat in comparison to Angus (*Bos taurus*) cattle, particularly under pasture-fed conditions (Cafe et al., 2011; Gama et al., 2013). Previous studies conducted across diverse regions consistently affirm a heightened consumer preference for bright red meat, indicative of a final pH value <5.8, classified as a normal meat (Carpenter et al., 2001; Viljoen et al., 2002).

2.3. Water-holding capacity

The most abundant meat component is water, which corresponds to about 75% of the meat's total weight and influences meat color, surface appearance, and texture (Toldrá, 2006; Varnam & Sutherland, 1995). Water-holding capacity (WHC) determines the ability of tissue water retention and, consequently, its appearance effects in raw meat (Varnam & Sutherland, 1995). The visual appearance determines the acceptability of the consumer's willingness to purchase the meat product.

The term "water binding capacity" (WBC) refers to water bound by water while it is processed, combined with heating. And the term WHC usually corresponds to the potential ability of raw meat to bind water (Pospiech & Montowska, 2011). There are other descriptions for water released, such as drip, weep, purge, exudate, and cook loss, and they are all inversely related to WHC (Warner, 2014).

A muscle cell has 85% of its water held in the myofibrils, so the drip loss is influenced by factors such as interfilament spacing, extent of lateral and transverse shrinkage of myofibrils at rigor, development of drip channels and extracellular space, postmortem cytoskeletal protein degradation, and the permeability of the cell membrane to water (Hughes et al., 2014). The water's effect on quality is due to its function in molding muscle structure. During heating and cooking, the water is lost, and the proteins become less pliable and more rigid, but when the heating time is longer, sarcoplasmic proteins and collagen get gelatinized, being able to retain water (Warner, 2017).

Other factors can be determined by WHC, including loss of water during slicing, mincing, pressing, transport, storage, process, and cooking. The WHC can also help to evaluate meat juiciness, an important property included in meat texture and quality (Hamm, 1986; Warner, 2017). A poor WHC leads to low cook yields and dry meat (lack of juiciness), items used to measure WHC indirectly. It also results in meat and meat products with a high drip and purge loss, being able to represent a significant loss of weight from carcasses (Warner, 2017).

Another trait that can be measured is free water, which corresponds to the fraction of water that can flow from the structure, unimpeded, when there are conditions to allow this, independent of charged groups. Free water is held by capillary forces between and within myofibrils that are in the sarcoplasmic fluid. In this extra-myofibrillar fraction and the loss of intra-myofibrillar water through shrinkage, WHC usually varies with water. A small part of the intra-myofibrillar and the extra myofibrillar water are mobilized, in the same way as myofibrils and cells shrink in the rigor process.

Although, in cases of high ultimate pH meat or pre-rigor, this water does not flow freely (Pearce et al., 2011).

The definition of meat juiciness consists in the impression of lubrication and moisture when chewing is happening in the mouth, and it is divided into two components. The first component that integrates juiciness is the impression of wetness verified in the initial chews, produced by the meat fluids being released quickly, related to the meat water content. The other component is the impression of juiciness itself during the sustained chewing, probably related to the meat fat content, being a result of the stimulating effect that fat does on salivary flow (Winger & Hagyard, 1994).

Water and fat content have a directly inverse relation, whereas as the fat percentage increases, the water percentage decreases, but despite that, the protein content in the muscle is maintained constantly (Huff-Lonergan & Lonergan, 2005; Juárez et al., 2012). The water of the muscle is mostly associated with myofibrils since 85% of the volume of the muscle cell is myofibrils. The percentage of water in meat tightly bound by proteins is about 1%, and this bound water is resistant to freezing and heating and has reduced mobility (Fennema, 1985). WHC can be related to juiciness, which is an important sensory trait that contributes to consumer's preference and acceptability of meat up to 10%, although juiciness is considered a subjective property of meat (Watson et al., 2008).

Several factors influence the WHC of raw meat, such as animal genetics, pre-slaughter stress, antemortem and postmortem factors, and others. Some biochemical events that happen in pre and postmortem and act in structural components in muscle cells, interfere in the ultimate meat pH and pH decline rate (Hughes et al., 2014; Warner, 2017). Some of the fundamental determinants of WHC of raw, processed, and cooked meat products are pH fall associated with postmortem glycolytic metabolism. The transverse shrinkage in the myofilament network and the water expulsion are associated with the normal pH fall in muscle postmortem, which encompasses the range from 7 to 5.5. This leads to a meat water loss, such as drip, purge, or exudate (Warner, 2017).

The changes in muscle structure are a consequence of chemical charge changes on proteins. The net charge existent on proteins in the myofibrils decreases and the filaments approach, inducing transverse shrinkage in a myofibril, and this process happens during the rigor period when the muscle pH reaches 5.4 or lower. When the ultimate pH reaches the isometric point of muscle proteins, which is the point of minimum charge and happens at pH 5.0 to 5.2, the muscle WHC achieves a minimum value. Therefore, when the muscle pH decreases from 7.0 to 5.5, there is a loss of WHC. In the DFD meat, which the meat has high ultimate pH (higher than 5.8), the myofibrils and

muscle cells shrinkage post-mortem does not occur due to a negative charge. This meat type results from low glycogen levels during slaughter caused by stress before the process, and it experiences less drip than meat with a typical pH level (Warner, 2017).

2.4. pH

One of the most important quality parameters in the meat quality evaluation is the pH measurement. Ultimate pH determines traits of meat quality such as meat color, WHC, and texture (Montgomery & Leheska, 2008).

Before slaughter, in the presence of high levels of stress hormones, occurs the decrease of muscular glycogen reserves, which affects the meat's pH. After death, the circulation stops, and oxygen stops being sent to the animal cells, and reactions begin to happen in anaerobic conditions. The muscle glycogen is hydrolyzed to acid lactic, which leads pH to fall from 7.0 to 5.5, causing the reduction of bacterial growth (Gupta et al., 2007; Odore et al., 2004).

The measure of crucial meat pH value is made 24 hours after slaughter, and it indicates indirectly the levels of muscle glycogen at slaughter (Montgomery & Leheska, 2008). When the pH value is higher than 5.5, changes happen in these traits, such as color alterations, decreased tenderness, and decreased shelf life. To avoid these undesirable effects, the cortisol level must be controlled by good management of animals during transport and slaughter (Secretaría de Agricultura y Desarrollo Rural [SADER], 2011).

When meat pH reaches values higher than 6, it is considered DFD meat, which has a shorter shelf life and is related to poor-quality meat (Ferreira et al., 2024). Due to reduced levels of ATP present in muscle, the actin-myosin cross-bridges are formed, and it leads to rigor bonds related to an increase in the toughness of the meat (Maltin et al., 2003).

The tenderization postmortem may be influenced by protein synthesis and degradation rates, while the rate and extent of postmortem glycolysis are influenced by levels of stored glycogen, and the rate of temperature that declines postmortem may be influenced by levels of fat in the depots. All these factors cause rigor development and activities of proteolytic enzymes (Juárez et al., 2012).

Water holding capacity and pH are correlated since WHC is affected by the same postmortem changes as pH (Warriss, 2000). After slaughter, the lactic acid accumulates because its removal stops, and this causes a decrease in pH until the glycogen stock runs out or the glycolysis is inhibited due to

lower pH. When there exists an excessive decline of postmortem pH, it results also in a lower WHC, since the shrinking of myofibrils is led by the electrostatic repulsion between the filaments which are affected by pH (Geletu et al., 2021).

2.5. Gene expression

2.5.1. Gene expression in cattle

Meat quality is affected by factors that change gene expression, such as breed, genotype, age, and nutrition. Gene expression, on the other hand, affects phenotypes that compose beef quality traits (Chen et al., 2019). Studies and analyses like GWAS and differential gene expression help to investigate the gene function in phenotypes considered complex, that involve interactions between genes and regulatory mechanisms (Diniz et al., 2019).

The current bioinformatics tools and analytical technologies contribute to clarifying the biological process involving genetic expression and metabolic responses that affect the final quality of the meat product with more detail (Acevedo-Giraldo et al., 2020; Bogdanowicz et al., 2018; Domingo et al., 2015). Understanding the differences in gene expression and its effect on metabolic processes and animal development can help to explain different meat quality traits according to different breeds and other factors that can diverge to management conditions (Munekata et al., 2021).

Some studies explored how gene expression is associated with meat quality traits as tenderness, pH, meat color, and shear force (Bongiorni et al., 2016; Cinar et al., 2012; Edwards et al., 2003). To improve the knowledge about processes that influence high-quality meat, the foodomics approach can be useful (Munekata et al., 2021). Several studies have observed that exists a moderate to high genetic correlation within these traits, which supports the idea that are important pleiotropic effects regulating the phenotypic expression of meat quality traits, considering the number of common candidate genes in these traits (Herd et al., 2018; Tonussi et al., 2015).

Genotyping became common, with the use of DNA microarray chips, to identify genomics associations from molecular markers with gene expression and complex phenotypes (Dikeman & Devine, 2004; Lu et al., 2013). These genetic markers have been used to develop genetic maps that indicate chromosomal regions and genes that can be useful for selection. Several studies identified quantitative trait locus (QTL) regions that affect important economic production traits (Louda et al., 2009).

A lot of these markers are already used in commercial tests targeting specific traits. Meat quality traits are controlled by several genes, considering that some genes have a greater effect on these traits than others. Therefore, the analysis of genes associated with gene expression and phenotypes of interest is important in meat quality and performance evaluation (Hozáková et al., 2020).

The most accurate methodology for genome-wide gene expression study is the RNA-Sequencing (RNA-Seq) analysis. It has become an important tool that efficiently sequences millions of nucleotides, expanding the understanding of transcriptomics research (van Dijk et al., 2014). RNA-Seq technology has contributed to identifying elements that affect the expression of complex traits, like candidate genes, genetic variation, and regulatory elements (Berton et al., 2016; Cesar et al., 2015; dos Santos Silva et al., 2019; Fonseca et al., 2020). Studies that do not consider gene expression like genomic sequencing using SNPs or GWAS have limitations when predicting efficiency, in this regard, the association of these approaches with gene expression is very interesting (Hayes & Goddard, 2010).

2.5.2. eQTL

The use of expression quantitative trait loci (eQTL) is important to supply information about genetic factors that regulate complex phenotypes, transcriptional regulation, functional interpretation for trait-associated SNPS, and it's also important to mapping genetic variations and gene expression that are integrated at the whole-genome level (Michaelson et al., 2009; Shabalin, 2012; Westra & Franke, 2014).

Meat quality traits are controlled by a complex genetic architecture that can be better known by findings of analysis like eQTL. Using variants from RNA-Seq increases the chances of discovering putative mutations next to or in the QTL (Suárez-Vega et al., 2015). Studies have suggested that SNPs that are associated with complex traits are probably eQTL. Using the eQTL information associated with GWAS, the power of this analysis is increased, and therefore, this strategy is being used to locate candidate variants. eQTL analysis can identify the genotype effect on gene expression levels, which helps to understand the process of gene regulation and annotating functional sequence variation (Nica & Dermitzakis, 2013).

eQTL are specific genomic regions that can control some gene expression of greater or lesser effect intrinsically as well as in response to the environment. When the effect on gene expression occurs on the same DNA molecule, it is classified as cis eQTL, and when the effect occurs on

physically distant genes, it is considered a trans eQTL (de Souza et al., 2020). Gene expression is classified as medium-highly heritable, based on some estimates (Stranger et al., 2007).

Several studies have found genes that were eQTL master regulated associated with a great proportion of meat quality genes in beef cattle. One of them pointed out that the top GWAS association of traits was significantly enriched for regulatory QTLs (Leal-Gutiérrez et al., 2020; Liu et al., 2022). Another study detected several candidate genes by doing an overlap of the genes detected from GWAS and eQTL analyses (Wang et al., 2022). In general, studies of eQTL in beef cattle remain scarce, and the association between analyses like eQTL and GWAS are even more rare (Cesar et al., 2018; Higgins et al., 2018; Leal-Gutiérrez et al., 2020; Wainberg et al., 2019). Studies of this line of research are important to have more knowledge of the genetic architecture that composes meat quality.

2.6. GWAS

Some important economic traits are difficult to measure and need the slaughter, like carcass and meat quality traits. This fact causes difficulty and increases the cost of breeding programs that objectify the improvement of these characteristics. These traits are controlled by multiple genes, with moderate or minor effects (Gao et al., 2021). Single-nucleotide polymorphisms (SNPs) high-density arrays are becoming more accessible. Thus, even if most meat quality traits have low or moderate heritability, the Genome-Wide Association Studies (GWAS) are being used to identify candidate genes through these high-density panels which allow large-scale genotyping (Hermesch et al., 2000; Suzuki et al., 2005).

Due to GWAS, it's possible to detect specific genomic regions, markers, and candidate genes associated with livestock important traits (Goddard & Hayes, 2009; Hayes & Goddard, 2001). The results obtained from GWAS contribute with information about genetic architecture and pleiotropic effects of complex traits such as carcass and meat quality characteristics, attributes that contain economic importance in the industry for they determine consumer acceptance and demand. If genetic markers show themselves responsible for a considerable proportion of additive genetic variance, genomic selection presents itself as a great selection method (Rezende et al., 2021).

In species like poultry and pigs, GWAS for meat color traits has been used to identify genomic regions and quantitative trait loci (QTL) that influence these characteristics (Luo et al., 2012; Rohrer et al., 2006; Sun et al., 2013; Yang et al., 2017; Zhang et al., 2015). However, there is a shortage of

GWAS studies, especially in Bos indicus, regarding bovine meat color (Reardon et al., 2010; Tizioto et al., 2013).

The polymorphisms cause pleiotropic effects and add to the result of linkage disequilibrium of genes associated with traits, their consequence generates genetic correlation (Fortes et al., 2013). Meat color traits are affected by many small effect quantitative trait loci. Studies suggest applying multi-traits GWAS for analyzing meat color traits since this model directly considers the genetic relations among them (Marín-Garzón et al., 2021).

Genetic variants associated with meat quality traits have been explored to explain variations, but other external mechanisms also affect these traits, although these effects are little understood. Studies suggest that an ideal strategy to improve meat quality traits is genomic selection, which explores simultaneously many gene variabilities since these traits are controlled mainly by many small effects QTLs (Tizioto et al., 2013).

The magnitude of estimated QTL effects is influenced by sample size, so it is necessary to have more studies to validate the results of previous studies, especially with Nellore cattle. More studies will also help to develop models for genetic merit prediction, used to implement genomic selection for meat quality (Tizioto et al., 2013).

Causal mutations of interest are unknown, but the association of SNPs with candidate regions can be used for marker-assisted selection (Goddard & Hayes, 2009). Genetic markers can be used to improve important economic traits (Otto et al., 2007; van der Steen et al., 2005).

2.7. Conclusion

This literature review exposed the importance of some phenotypes including color, water-holding capacity, and pH as indicators of meat quality. It also strengthened the relevance of techniques such as eQTL and GWAS as tools that help to indentify polymorphisms that regulate gene expression and are associated with phenotypic variation related to meat quality traits. This overview is important to understand the exploration of the genetic architecture of beef cattle and its influence on meat quality.

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3 ASSOCIATION OF REGULATORY POLYMORPHISMS OF GENE EXPRESSION WITH COLOR PHENOTYPES, WATER HOLDING CAPACITY, AND PH OF NELLORE MEAT

Abstract

Consumers' preferences for meat quality have increased the significance of understanding the multifaceted traits governing this choice. This study explore the complexity of meat quality, influenced by intrinsic and extrinsic factors, with focusing on genomic analyses to identify key regulatory variants. We aimed to identify expression quantitative trait loci (eQTLs) associated with meat quality phenotypes in Nellore cattle, including color, water-holding capacity, and pH. For that, we used a comprehensive single nucleotide polymorphisms (SNPs) panel of 553,581 markers, obtained from an imputed panel of SNPs encompassing genotypes from 778 progenies, DNA sequencing from 26 unrelated Nellore sires, and RNA sequencing data from muscle tissues of 192 progenies. The eQTL analysis, using genotypes of 192 animals, revealed 51,324 eQTLs. Subsequent genome-wide association studies (GWAS) with 374 animals identified 838 eQTLs associated with color, 172 with water-holding capacity, and three with pH. Integration with ATAC-Seq peaks identified 75 eQTLs associated with meat color and 24 associated with water-holding capacity in open chromatin regions. Enrichment analysis uncovered pathways such as immune response, antigen processing and presentation, and glutathione metabolism. Notably, most of genes regulated by eQTLs were associated with these pathways. This comprehensive approach identified putative candidate regulatory variants, and genes associated with meat quality, advancing our understanding of *Bos indicus* genetics.

Keywords: eQTL. GWAS. SNPs. Meat quality.

3.1. Introduction

Consumer preferences have changed in the past few decades, significantly impacting the criteria used to determine the quality of meat products. This evolving consumer demand has prompted a comprehensive exploration of the factors contributing to high-quality meat. Factors influencing a complex trait such as meat quality, include intrinsic and extrinsic aspects controlled by various genes with minor or moderate effects (Ferraz & Felício, 2010; Gao et al., 2021; Mancini & Hunt, 2005; Scollan et al., 2006; Williams, 2008). However, measuring meat quality traits is expensive and can only be done after slaughter. Genomics has emerged as a solution, offering the potential to identify genetic markers for breeding programs (Magalhães et al., 2019; Rezende et al., 2021).

Some traits have a direct influence over meat appearance and other characteristics. This is particularly evident in color, water-holding capacity, and pH. Alterations in pH impact meat color and water-holding capacity (Abril et al., 2001; Mahmood et al., 2017; Warner, 2017). Additionally, changes in the water-holding capacity can significantly affect the meat color (King et al., 2023). The interconnected nature of these factors highlights their collective influence on consumer preferences and choices.

Meat color, a multifaceted trait, is intricately influenced by several intrinsic and extrinsic factors, serving as a crucial indicator of meat quality and freshness (Poveda-Arteaga et al., 2023). Complementing this, water-holding capacity (WHC) emerges as an essential determinant of a tissue's ability to retain water, directly impacting visual attributes like meat color, surface appearance, and texture (Toldrá, 2006; Varnam & Sutherland, 1995). WHC also plays a definitive role in determining meat juiciness, a factor that is indirectly quantified (Hamm, 1986; Warner, 2017). Inadequate WHC results in diminished cooking yields, yielding dry meat lacking juiciness, which can be indirectly quantified. Additionally, poor WHC leads to high drip and purge losses in meat and meat products, culminating in significant weight reduction from carcasses (Warner, 2017). Furthermore, pH measurement stands out as a critical parameter in assessing meat quality. The influence of pH extends across various traits, including meat color, water-holding capacity, and texture, establishing its significance as a comprehensive indicator of overall meat quality (Montgomery & Leheska, 2008).

Nellore cattle have some particularities that differs from *Bos taurus* cattle. Research indicates that *Longissimus Thoracis* steaks obtained from Nellore animals (*Bos indicus*) exhibited heightened redness (a* value) and lightness (L* values) compared to steaks from crossbred cattle *Bos indicus* × *Bos taurus*, 50% Nellore and 50% Aberdeen Angus (Miguel et al., 2014). Another study noted that *Longissimus Thoracis et lumborum* muscle in Nellore appeared darker than those in two other *Bos taurus* breeds, Caracu and Holstein Friesian (Rotta et al., 2009). Additionally, owing to increased fat deposition in *Bos taurus* animals, the muscles displayed a brighter color compared to the meat of *Bos indicus* cattle (Café et al., 2011). Cattle breed exerts a direct influence in meat quality, and evidence supports the assertion that the heightened aggressiveness temperament in pure *Bos indicus* (Brahman) or *Bos indicus* crosses leads to a darker muscle color and elevated pH values in meat in comparison to Angus (*Bos taurus*) cattle, particularly under pasture-fed conditions (Cafe et al., 2011; Gama et al., 2013).

Exploring expression quantitative trait loci (eQTL) analysis holds significant importance in identifying specific genomic regions that exert control over gene expression and exhibit responsiveness to environmental stimuli (de Souza et al., 2020). Through this analysis, it is possible to identify genetic factors capable of modulating complex traits, providing a crucial framework for mapping genetic variations that exert influence over gene expression (Michaelson et al., 2009; Shabalin, 2012; Westra & Franke, 2014). By incorporating variants from RNA-Seq data, the likelihood of identifying potential mutations proximal to or within the QTL is significantly enhanced (Suárez-Vega et al., 2015). This approach enhances our understanding of the interplay between genetic and environmental factors and serves as a valuable tool for unraveling the genetic elements of complex traits.

An additional analytical approach instrumental in revealing the genetic architecture is the Genome-Wide Association Studies (GWAS). This method allows identifying specific genomic regions, markers, and candidate genes associated with important livestock traits (Goddard & Hayes, 2009; Hayes & Goddard, 2001). Combining the strengths of eQTL and GWAS enhances analytical potency, a synergistic approach uncommon in the context of beef cattle (Cesar et al., 2018; Higgins et al., 2018; Leal-Gutiérrez et al., 2020; Wainberg et al., 2019).

This work, therefore, offers a comprehensive exploration of polymorphisms influencing gene expression and their association with meat quality traits, including color, water-holding capacity, and pH, through the utilization of eQTL and GWAS techniques. This approach allowed for the identification of mutations that participate in the regulation of important gene expressions, such as genes that are involved in immune response, glutathione metabolism, and detoxification processes. By shedding light on these intricate associations, the findings enhance the understanding of the mechanisms involved genetic foundations of cattle, contributing significantly to our understanding of cattle genetics and its implications for meat quality.

3.2. Materials and Methods

3.2.1. Animals

This study utilized an experimental population of 374 Nellore steers from the Brazilian Agricultural Research Corporation (EMBRAPA), originating from 34 unrelated bulls representing key Brazilian Nellore genealogies. These animals were raised between 2009-2011, utilizing grazing systems, and finished in uniform feedlots conditions with identical handling and nutritional protocols. Ethical procedures related to animal welfare were approved in accordance with Institutional Animal Care and Use Committee Guidelines from EMBRAPA (CEUA 01/2013). These animals, with an average weight of 452 kg and an average age of 25 months, were slaughtered in a commercial slaughterhouse. Additional procedural details are available in Cesar et al. (2016) and Tizioto et al. (2013).

3.2.2. Samples and phenotypes

RNA Sequencing analysis was conducted on a subset of 192 animals. Tissue samples weighing approximately 5 g were collected from the *Longissimus thoracis* (LT) muscle on the right side of each

carcass, between the 12th and 13th ribs, immediately after the animal's death and stored at -80°C until the analysis. Phenotypic data were collected from carcasses vertically sectioned and refrigerated at 2°C for 24 hours. A sample of approximately 10 g was also taken to determine the water- holding capacity. Two of the three 2.5 cm steak samples were vacuum packed and aged in a cold chamber at an average temperature of 2°C, for 7 and 14 days. After the maturation period, the steaks were frozen for subsequent analysis of meat quality characteristics. The steak that was not matured had its quality parameters measured on the same day the sample was taken, that is, after 24 hours of cooling the carcasses in a refrigerated room. For the evaluation of the matured samples, they were previously thawed under refrigeration (5°C) for around 18 hours until they reached an internal temperature of approximately 4°C. Thirty minutes before conducting the determinations, a transverse cut was made through the muscle to expose the myoglobin to oxygen, leading to the consequent transformation into oxymyoglobin.

In the exposed portion, meat color was measured using a portable colorimeter (HunterLab, Miniscan XE plus model), at three different points in the sample. The colorimeter was calibrated by placing the light shielding devices, a calibrated black tile, and a calibrated white tile into the reflectance port. The color coordinates were calculated utilizing the illuminant D65 (daylight, 6,500K), an observer angle of 10°, and the CIELAB color system (L*= luminosity, a*= red content, and b*= yellow content). The pH of the *Longissimus* muscle portion was measured using a digital pH meter (Testo, model R 230), with measurements taken at a minimum of three different points in the sample. Water-holding capacity, expressed as a percentage of total moisture, was obtained by the weight difference of a meat sample (approximately 2 g) before and after subjecting it to a 10 kg pressure for 5 minutes, accounting for the sample's humidity. Following color and pH measurements, the steaks were weighed and cooked in an electric industrial oven (Tedesco, model TC06/ELT) at 170°C until reaching an internal temperature of 70°C at the geometric center, monitored using a digital thermocouple. After cooling, the steaks were reweighed, and cooking losses (comprising exudation and evaporation) were calculated as the difference in weight before and after cooking.

3.2.3. Genotyping

Genotyping analysis of the steers and their sires utilized the BovineHD 770 k BeadChip (Infinium BeadChip, Illumina, San Diego, CA, USA), encompassing 783,450 SNPs. The analysis was conducted at the Bovine Functional Genomics Laboratory ARS/USA and ESALQ Genomics Center (Piracicaba, São Paulo, Brazil), adhering to Illumina's protocol. To ensure data quality, SNPs located

on sexual chromosomes, with a call rate ≤ 95%, minor allele frequency (MAF) ≤ 5%, and those not mapped in the *Bos taurus* ARS-UCD1.2 reference genome, were excluded from further analyses. For more details about this analysis, please refer to Tizioto et al. (2013) and Cesar et al. (2016).

3.2.4. RNA-Sequencing

All RNA Sequencing analyses were conducted at the ESALQ Genomics Center (Piracicaba, São Paulo, Brazil). RNA extraction involved processing a 100 mg sample of the *Longissimus thoracis (LT)* muscle with the Trizol reagent (Life Technologies, Carlsbad, CA, USA). RNA integrity was verified using the Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA), with samples possessing RNA integrity numbers (RIN) greater than 7 considered for subsequent analyses. Complementary DNA libraries (cDNA) were prepared using 2μg of RNA from each sample, following the Illumina protocol outlined in the TruSeq RNA Sample Preparation kit v2 guide (Illumina, San Diego, CA, USA). Sequencing was carried out using the Illumina HiSeq 2500 ultra-high-throughput sequencing system. The SeqyClean package v. 1.4.13 (https://github.com/ibest/seqyclean) was utilized to eliminate low complexity reads and adapter sequences during the library preparation process. Sequencing quality was assessed using FASTQC v. 0.10.1 software (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Further details are described in Cesar et al. (2015).

3.2.5. SNP annotation

For SNPs identification in the gene expression data, GATK (Genome Analysis Toolkit) v. 4.1.0.0 was used in Genomic Variant Call Format (GVCF) mode, according to the program manual (Brouard et al., 2019; McKenna et al., 2010). Known variants were referenced from the Ensembl *Bos taurus* SNP database (release 96), and variants were individually called by haplotype using data from 192 animals with RNA-seq information. Variant quality was assessed using Phred scaled polymorphism probability (Phred). SNPs with Phred values > 30 and minimum variant coverage > 10 were filtered for Minor Allele Frequency (MAF) > 5% and call rate > 95%. Non-biallelic SNPs located on sex chromosomes were excluded from the analysis. Detailed procedures for this step are outlined in Silva-Vignato et al. (2022).

3.2.6. Imputed data

SNPs found in the genomic DNA sequence of 26 sires were imputed in their progenies using genotypic data acquired from the BovineHD BeadChip 770k panel (Illumina, San Diego, CA, USA). The programs used was Eagle (Loh et al., 2016) for phasing, followed by Minimac3 (Das et al., 2016) for imputation. Imputation accuracy was assessed through leave-one-out cross-validation. In this approach, each sequenced animal was excluded once from the reference population and incorporated into the target population, along with the progenies genotyped using the high-density panel. Subsequently, imputation efficiency was measured by comparing imputed alleles with those observed in the DNA sequence of each sire. The allelic imputation error rate was quantified as the proportion of incorrectly imputed alleles relative to the total number of alleles imputed, and the imputation accuracy was defined as the correlation between imputed and actual genotypes. Subsequent analysis retained SNPs with an allelic imputation error rate of < 2% or an accuracy exceeding 0.98, while monomorphic and sexual SNPs with a call rate of < 95% and an allele frequency of < 5% were removed. SNPs with an R2 value calculated by Minimac3 > 0.90 were retained. Additionally, filtering based on allele frequency removed SNPs with MAF < 5% and monomorphic SNPs. RNA sequencing-detected SNPs were imputed to a panel composed of imputed DNA-Seq variants and SNPs from the high-density panel using Eagle for phasing and Minimac3 for imputation. Following imputation, SNPs with an R2 value (calculated by Minimac3) greater than 0.90 were retained. Additionally, SNPs were subjected to allele frequency filtering, eliminating monomorphic variants and those with a Minor Allele Frequency (MAF) less than 5%. A complete description of SNP imputation procedures is provided in Garcia et al., under revision at Scientific Reports.

3.2.7. Expression quantitative trait loci (eQTL)

Before the eQTL analysis, the Principal Component Analysis (PCA) was used to investigate the population stratification, using the PLINK software (Purcell et al., 2007). We examined population stratification through principal component analysis (PCA) using genotypes obtained from the BovineHD 770k BeadChip, focusing on our dataset of 192 animals for eQTL mapping and 374 animals for association analysis. Initial filtering of variants included a Minor Allele Frequency (MAF) threshold of > 5% and a call rate exceeding 95%. Subsequently, we employed the PLINK software to assess population stratification, utilizing 446,498 genotypes. To validate sample clustering, we specifically utilized the sires, considering our population's origin from 34 unrelated bulls. The complete list of the PCA results can be found at Garcia et al., under revision at Scientific Reports. For eQTL analysis, the

panel of imputed SNPs assigned was screened within a subset of 192 animals with available RNA-Seq data.

Quality control retained SNPs with call rate > 95% and MAF > 5%, resulting in 4,436,504 SNPs for analyses. In the set of imputed SNPs, we employed PLINK v. 1.07 for selection based on linkage disequilibrium (LD) calculation and variant pruning. For variant pruning, we set parameters with pairwise connections requiring a minimum r^2 of 0.8 and a window size of 100 SNPs. The window shifted 10 SNPs in each step, resulting in the identification of a subset of informative SNPs (tag-SNPs) within LD blocks. Cis and trans-eQTL identification was conducted using the Matrix eQTL v. 2.3 (Shabalin, 2012), an R package establishing links between SNP genotypes and gene expression. The expression data for 12,991 muscle genes were normalized in log2-CPM (Counts per million of mapped reads) and adjusted for lane and flow cell effects. The model included as covariates the first two principal components (PC1 and PC2) to adjust for potential effects from population stratification, and the contemporary group (CG), encompassing the animals from the same year, farm, and slaughter date. Imputed SNPs were further refined based on linkage disequilibrium (LD) using PLINK v. 1.07, resulting in a panel with 553,581 SNPs.

In this study, the cis-eQTLs were defined as SNPs located up to 1 Mb away from the regulated gene, while trans-eQTLs were SNPs located > 1 Mb away from the gene. Linear regression was employed to test the additive effect of each gene-SNP pair, and the false discovery rate (FDR) was determined using the Benjamini-Hochberg methodology (Benjamini & Hochberg, 1995). Separate FDR calculations were performed for cis and trans-eQTLs (Shabalin, 2012). All cis and trans eQTL (FDR < 5%) were individually annotated using VEP v.101.0 (McLaren et al., 2016). The complete list of annotations is available in Supplementary Tables 1 and 2.

3.2.8. Genome wide associations study (GWAS)

The genome wide association study (GWAS) was used to associate the significant eQTLs (FDR < 5%) with the studied phenotypes. For the analysis, a population of 374 animals was used, previously used in other studies by the same research group (Cesar et al., 2014). GWAS was conducted using PLINK v. 1.07 (Purcell et al., 2007), employing a linear model and an SNP-by-SNP approach with multiple tests adjustment, that incorporated the same covariates used in eQTL mapping, like CG, PC1, PC2, and hot carcass weight, which was included as a covariate (Cesar et al., 2014). A total of 30,581 eQTLs were tested for phenotypes that were measured in *Longissimus*

thoracis muscle, including color phenotypes (L* for lightness, a* for redness, and b* for yellowness at 0, 7, and 14-day intervals); water-holding capacity phenotypes (including free water, humidity, cooking loss, and water-holding capacity at 0, 7, and 14-day intervals), and pH phenotype (measured at 0 days, 24 hours *postmortem*). Consequently, SNPs considered significant exhibited an association with the phenotype at a threshold of FDR < 10% (Benjamini & Hochberg, 1995).

3.2.9. ATAC-Seq

The ATAC-Seq analysis was employed to isolate and identify euchromatin regions, referred to as ATAC-seq peaks. This analysis was performed in a previous study, conducted on two *Longissimus thoracis* (*LT)* muscle samples obtained from Nellore males sourced from a commercial slaughterhouse. The technique, as described in Buenrostro et al. (2013), was implemented with four technical replicates. The paired-end sequencing (2x100) was made using HiSeq 2500, generating approximately 40 million reads per library, and to remove adapters from read ends the Trimmomatic (v:0.36) was used (Bolger et al., 2014). Quality control was performed using the FastQC tool (Leggett et al., 2013; Martin, 2011). Subsequently, ATAC-Seq pileup files was generated by FASTA files using the nfcore/atacseq pipeline implemented in Nextflow (https://nf-co.re/atacseq). A consensus peak mapping was derived from the four samples, considering only regions with counts higher than zero in all. In these regions, the FRIP (Fraction of Reads In Peak) score was computed for each replicate. An ATAC-Seq Peak region was defined when the average FRIP score across the four replicates exceeded 0.2, following recommendations from the ENCODE (Encyclopedia of DNA Elements) consortium (ENCODE, 2012). Utilizing deepTools v.3.5.1 (Ramírez et al., 2016), we constructed matrices, heatmaps, and line plots to assess overlapping peaks with transcription start sites (TSS) from individual and merged replicates as an additional quality control (Alexandre at al., 2021).

As a result, a consensus peak mapping was formed from the four replicate samples that passed through additional quality control steps (Alexandre et al., 2021; Ramírez et al., 2016; The ENCODE Project Consortium, 2012). An R script based on the function *subsetByOverlaps* from the GenomicRanges R/Bioconductor package was used to analyze the genomic overlap between the eQTLs associated with phenotypes and ATAC-seq peaks (Lawrence et al., 2013). For visualization of the peaks, the Integrative Genomics Viewer (IGV – v.2.15.4) was used (Robinson et al., 2011). The Atac-Seq dataset analyzed in this study is available in the ENA repository (EMBL-EBI) under the accession code: PRJEB64479.

3.2.10. Functional enrichment

The annotation of eQTL-regulated genes was carried out using the Ensembl Biomart tool (Ensembl Genes 104). For functional enrichment analysis, the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (https://david.ncifcrf.gov) was employed. This resource delineates Biological Processes, Molecular Functions, GO Terms, and Kegg Pathways associated with genes regulated by the representative eQTL.

3.3. Results

3.3.1. Phenotypes

Table 1 displays the minimum, maximum, average, and standard deviation for all phenotypes considered in this study.

Table 1. Minimum, maximum, average and standard deviation for color, water-holding capacity, and pH phenotypes in a Nellore cattle population.

Phenotypes	Minimium	Maximum	Average	Standart Deviation
pH	5.33	5.97	5.60	0.14
humidity	26.30	83.50	73.22	2.89
freewater	7.14	36.82	26.56	4.26
WHC	63.18	92.86	73.44	4.26
CL	14.42	66.90	26.06	4.44
L	33.50	46.22	37.90	1.94
a	12.02	40.21	15.35	1.69
b	9.48	17.17	12.82	1.06
pH7	5.42	5.77	5.56	0.07
freewater7	26.53	72.14	38.83	4.82
WHC7	27.90	73.50	61.17	4.82
CL7	13.47	48.59	26.39	4.51
L7	33.83	47.47	39.73	2.40
a7	13.31	20.36	17.14	1.24
b7	11.08	20.00	15.09	1.48
pH14	5.38	5.82	5.57	0.07
freewater14	26.81	68.00	38.32	4.28
WHC14	32.00	73.19	61.68	4.28
CL14	6.32	45.86	25.26	5.70
L14	33.82	47.62	40.42	2.41
a14	12.35	22.01	17.40	1.35
b14	10.10	23.04	15.40	1.36

Subtitle: L: lightness; a: redness; b: yellowness; CL: cooking loss; WHC: water-holding capacity.

3.3.2. eQTL analysis

The eQTL analysis is used to identify variants located in regions that exert control over gene expression (Westra & Franke, 2014). To enhance the eQTL and GWAS analyses, minimize the need for numerous tests, and prevent dataset inflation from SNPs with similar effects, we employed linkage disequilibrium to refine the SNP panel. Following quality control and pruning steps, the analysis focused on 553,581 tag-SNPs. As a result, the analysis identified 51,324 eQTLs (FDR < 5%), including 36,916 cis-eQTLs, 14,408 trans-eQTLs, and 3,823 eQTLs functioning both in cis and trans, distributed across the 29 autosomal chromosomes of *Bos taurus* (BTAs). Considering a single SNP count, there were 25,896 cis-eQTLs, (including 2,381 novel SNPs), 4,685 trans-eQTls, (including 663 novel SNPs), and 1,950 acting as both cis and trans-eQTLs. These cis-eQTLs affected the expression of 5,142 genes, while trans-eQTL influenced the expression of 4,707 genes. For further details, like beta value from expression, which indicates how the SNP affects the gene expression, and SNP annotation information, please refer to Supplementary Table 1 and 2.

3.3.3. GWAS analysis

Variations in regulatory regions of gene expression caused by polymorphisms can give rise to phenotypic diversity (Mattick, 1994; Piraino & Furney, 2017; Weischenfeldt et al., 2013). Consequently, a GWAS analysis was performed to investigate the potential link between the identified eQTLs and variations in phenotypes. The results revealed 838 eQTLs associated with color phenotypes, 172 eQTLs associated with water-holding capacity phenotypes, and three eQTLs associated with pH phenotypes. Values from beta, which indicates how the alternative SNP affects the phenotype magnitude are in the Supplementary Table 4.

3.3.4. Overlap with regulatory regions

The Assay for Transposase-Accessible Chromatin, commonly referred to as ATAC-Seq, serves as an additional instrument capable of enhancing the causal gene regulatory variants mapping. This analysis can unveil accessible chromatin regions, enabling the discovery of eQTLs within these regulatory domains (Kumasaka et al., 2016; Thurman et al., 2012). For more precise identification of potential causal variants, an overlap analysis was conducted between the eQTLs associated with the studied traits and the peaks obtained through ATAC-Seq data. This analysis

revealed that 99 eQTLs were situated within open chromatin regions, with 75 of them related to color phenotypes, and 24 associated with water-holding capacity phenotypes. Consequently, these regions were deemed candidate regulatory regions. We utilized the Integrative Genomics Viewer (IGV – v.2.15.4) for visualizing peaks, and specific genes are illustrated in Figures 1 to 6. IGV enables consultation of the ChromHMM model-based profile of chromatin states for cattle muscle from the Functional Annotation of Animal and Genomes consortium database (FAANG). This model utilizes established epigenetic signals from histone marks and CTCF sites to characterize regulatory elements across the chromatin (Kern et al., 2021). For a complete list of the eQTLs and their corresponding ATAC-Seq peaks, please refer to Supplementary Table 3.

3.3.5. Functional enrichment

Functional enrichment analysis was conducted using the Database for Annotation, Visualization, and Integrated Discovery (DAVID). Among all the phenotypes evaluated, pathways related to immune response, Major Histocompatibility Complex (MHC) classes I/II, antigen process and presentation, ATP binding, and glutathione metabolism were the most prominent ones identified. Supplementary Table 5 and 6 contains all the information about the functional enrichment.

3.4. Discussion

For a clearer understanding of the relationship between eQTLs and meat quality traits, the discussion will be organized by phenotypes, addressing significant genes and their potential impact on each specific trait.

3.4.1. Color genes

Of the entire set of eQTLs, 75 were found to be associated with color phenotypes and were located within ATAC-Seq peaks, indicating their presence within open chromatin regions. Notably, ten of these eQTLs were identified to regulate novel genes. Beyond the Major Histocompatibility Complex (MHC) genes, other genes were found to be influenced by eQTLs residing in ATAC-Seq

peaks, including BLM, PTER, CACNG4, EIF2AK1, EIF2AK4, and TMEM186. For a comprehensive overview of the ATAC-Seq peaks data, please refer to Supplementary Table 3.

The BLM, classified as a BLM RecQ-like helicase, has been previously linked to meat quality, specifically demonstrating a significant impact on texture and water content (Chen et al., 2007; Guo et al., 2022; Li et al., 2022; San et al., 2021; H. Wang et al., 2022; Wen et al., 2022; Zhao et al., 2019). Notably, water content, a parameter known to exert a direct influence on meat color, has been underscored in prior research (Varnam & Sutherland, 1995). The alternatives SNPs, responsible for the eQTL SNP located on BTA 21:22841828 and BTA 21:21722576, decreased the expression of BLM (beta-e = -0.35 and -0.31), and increased L* on day 7 (beta-p = 0.34 and 0.23) and b* on day 14 (beta-p = 0.32), which shows the influence in meat color.

The PTER, identified as a phosphotriesterase, has been correlated with meat tenderness and juiciness, displaying a negative association with chewiness (Zhu et al., 2021). While literature does not indicate a direct association with meat color, the negative beta-e value for PTER gene expression (-0.18) and similarly negative beta-p values (-0.24 for L* on day 7 and -0.25 for b* on day 7) suggest potential indirect implications for color-related attributes.

CACNG4 is a calcium voltage-gated channel auxiliary and belongs to the CACNG gene family responsible for encoding gamma subunits of voltage-dependent calcium channels. In Landrace pigs, Lee et al. (2018) reported a positive additive effect and a negative dominant effect of this gene on final weight. This suggests a significant role for CACNG4 in influencing weight-related traits, particularly in the context of fat accumulation. It is noteworthy that the regulation of ion transport processes can exert a substantial impact on fat accumulation. In this regard, it is crucial to emphasize that biological processes governing ion transport and its regulation may exhibit heterozygous advantages, including forms of overdominance and underdominance, influencing fat accumulation dynamics. Furthermore, it is observed that CACNG4, when in a homozygote state, has the capacity to decrease adipose tissue accumulation, underscoring its potential as a key player in modulating fat-related phenotypes (Lee et al., 2018).

Research indicates that CACNG4 plays a critical role in muscle development in cattle. Remarkably, its expression has been observed to overlap with MyoD during myoblast differentiation in chicks, suggesting a potentially intricate role for CACNG4 in the processes of muscle development (Kious et al., 2002; Wei et al., 2022). In the present investigation, three SNPs influenced the expression of CACNG4 (beta-e = 0.48, 0.35, -0.33). All of them were associated with L* on day 7 (beta-p = 0.27 and 0.23). The association between meat color and the CACNG4 gene, as revealed by

these specific SNPs, has not been documented in the literature. However, this study establishes an association, indicating that the influence of CACNG4 on fat content or muscle development may interfere in meat color. This can be plausible given that meat color is influenced by multiple intrinsic factors, including intramuscular fat and muscle properties (Gagaoua et al., 2018; Poveda-Artaega et al., 2023).

EIF2AK4, also recognized as GCN2, is implicated in crucial cellular processes such as thermo-tolerance, DNA damage repair, and apoptosis (Wang et al., 2019). Belonging to the kinase family, EIF2AK4 plays an important role in the activation and mediation of cellular responses to various stressors, including DNA-damaging agents such as UV radiation, indicating its potential involvement in environmental adaptation. EIF2AK4 also assumes a central position in preventing oxidative damage (Liang et al., 2016). Additionally, it plays a key role in adaptation to hypoxic stress by modulating p53 levels and transcriptional activity (Liu et al., 2010). The phosphorylation of eIF2a, a typical response of eukaryotic cells to diverse cellular stresses, further underscores the multifaceted nature of EIF2AK4's involvement in cellular homeostasis (Clemens, 2001).

The eukaryotic initiation factor -2 (eIF2α) alpha subunit is a molecule participating in the control of the initiation of protein synthesis in eukaryotes (Morimoto & Baba, 2012). This essential initiation factor undergoes phosphorylation by protein kinases activated in response to a spectrum of stress conditions, including oxidative stress, heme deficiency, osmotic shock, and heat shock (Krishna & Kumar, 2018). The EIF2AK4 gene serves as a key regulator, repressing the translation of most mRNAs in response to stress-induced signals. Activation of EIF2AK4 is noted in instances of viral infection and oxidative stress (Harding et al., 2000; Roobol et al., 2015; Taniuchi et al., 2016). Associated with thermo-tolerance, DNA damage repair, and a potential relevance in the adaptation of zebu cattle to episodes of nutritional deficiencies, this gene assumes a critical role in gene expression regulation in response to amino acid and glucose deprivation, suggesting its involvement in cellular adaptations under conditions of nutritional stress (Edea et al., 2018; Liang et al., 2016). Additionally, EIF2AK4 has been linked to resistance against apoptosis induced by UV irradiation (Jiang & Wek, 2005). This gene plays a central role in the integrated stress response by regulating the sensing of starvation conditions (Masson, 2019).

The EIF2AK4 gene fulfills a compensatory role as a heme-regulated inhibitor (HRI) in response to thermal stress (Yoon et al., 2017; Zhan et al., 2004). This gene has been associated with thermal tolerance, suggesting its potential relevance in the adaptation of Chinese cattle to high temperatures (Edea et al., 2018; Wang et al., 2019). Given the profound impact of heat stress on animal production, particularly in tropical and subtropical regions, understanding the genetic basis of

thermal tolerance is crucial (Zeng et al., 2023). Conversely, the EIF2AK1 gene is involved in the regulation of protein synthesis in response to stress and has been linked to body mass index and growth traits in pigs (Gong et al., 2019; Poklukar et al., 2023). Its participation in the response to external stimuli and negative regulation of cell proliferation further underscores its role in cellular homeostasis (X. Wang et al., 2022).

In our study, both EIF2AK4 and EIF2AK1 demonstrated associations with a* on day 0 (beta-p = -0.14, 0.17, 0.18, 0.19). Two SNPs influenced EIF2AK1 expression (beta-e = -0.13, 0.07), and four influenced EIF2AK4 expression (beta-e = -0.20, 0.26, 0.28, 0.29). Despite the absence of documented associations between these genes and meat color in the current literature, it can be hypothesized that the response to oxidative stress processes might indirectly influence meat color. This is based on the premise that reactive oxygen species can impact myofibrillar proteins, potentially causing cellular structures damages and, consequently, affecting meat quality, including its color (Martinaud et al., 1997). Additionally, TMEM186, a transmembrane protein, and other members of the same gene family have been associated with meat production (Kawahara-Miki et al., 2011). In our investigation, the beta-e value for TMEM186 was -0.11, and the beta-p for L* on day 7 was -0.23, providing insights into its potential impact on meat characteristics.

3.4.2. WHC genes

In the context of eQTLs associated with water-holding capacity phenotypes, 24 loci were found within ATAC-Seq peaks, with eleven demonstrating regulatory effects on novel genes. Further than MHC genes, regulation was observed for genes such as UCP2 and GSTA4 within ATAC-Seq peaks. Uncoupling proteins (UCPs) play a pivotal role in regulating energy homeostasis and are part of the superfamily inner mitochondrial membrane anion carrier (Krauss et al., 2005). UCPs, in general, reduce metabolic efficiency by dissipating stored energy as heat, thereby increasing energy expenditure, a factor recognized as a potentially significant determinant of body composition (Echtay et al., 2002). Their involvement spans critical processes, including the control of ATP synthesis, regulation of reactive oxygen species (ROS) production, and modulation of fatty acid metabolism (Echtay, 2007; Ledesma et al., 2002). Among the three distinct UCP isoforms (UCP1, UCP2, and UCP3), UCP2 emerges as the principal isoform in muscular tissues and white adipose, emphasizing its specific role in these physiological contexts (Boss et al., 2000; Fleury et al., 1997).

UCP2, a gene extensively expressed across various mammalian tissues, plays a vital role in regulating energy homeostasis at multiple levels, encompassing gene expression, transcription, and post-translational regulation (Donadelli et al., 2014). Functionally, UCP2 modulates the synthesis of fatty acids (FA) in white adipose tissue, exerts negative regulation on insulin secretion and lipogenesis, and is implicated in metabolic regulation, contributing to diet-induced thermogenesis and weight loss (Ricquier et al., 1991; Rossmeisl et al., 2000; Zhang et al., 2001). The central role of uncoupling proteins lies in calorie burning and heat generation, achieved by creating a pathway for the dissipation of the electrochemical gradient of protons across the inner membrane of mitochondria, uncoupled from any energy-consuming process. This pathway has been demonstrated to regulate energy expenditure, body composition, and metabolism of glucose (Adams, 2000). It also serves to prevent the formation of ROS and is activated during fever responses to infections, showcasing its association with immune function (Nordfors et al., 1998).

In humans, UCP2 emerges as a promising candidate gene for obesity, and in cattle and chicks, it has been linked to growth-related traits (Liu et al., 2007; Liu & Lai, 2016; Nordfors et al., 1998; Oh et al., 2006; Zhao et al., 2006). Specifically, in the bovine UCP2 gene, SNPs have been associated with body weight, intramuscular fat content, lean meat yield, and have demonstrated positive effects on growth and carcass traits (Liu & Lai, 2016; Sherman et al., 2008). A synonymous mutation in beef cattle within the same gene has been correlated with carcass weight and eye muscle area (Ryu et al., 2012). These associations extend to chicks, where SNPs in the UCP2 gene have been linked to body weight, carcass weight and fatness traits (Liu et al., 2007; Oh et al., 2006; Zhao J et al., 2006).

UCP2 is multifunctional, exerting influences on insulin secretion, glucose metabolism, and overall body development and growth. These effects may account for the observed associations between polymorphisms in the UCP2 gene and traits related to growth and carcass characteristics (Boss et al., 2000). Consistent findings have been reported in various studies, reinforcing the link between UCP2 and growth and carcass traits (Ryu et al., 2012; Sherman et al., 2008). Moreover, high expression levels of UCP2 have been documented in Nellore cattle populations exhibiting lower residual feed intake (RFI), indicative of high feed efficiency. This suggests a potential role for UCP2 in influencing feed efficiency in beef cattle (Tizioto et al., 2015). Associations of UCP2 with meat quality traits have been identified in Qinchuan cattle (Wang et al., 2016). Additionally, previous investigations have linked UCP2 to hot carcass weight, backfat thickness, fat accumulation, and body weight in cattle (Brennan et al., 2009; Ferraz et al., 2009; Sherman et al., 2008).

Polymorphisms in the UCP2 gene have been correlated with the pH levels observed 24 hours after the slaughter of the *Longissimus* muscle and hind leg muscle in rabbits (Liu & Lai, 2016). pH is a critical meat quality parameter, influencing other traits such as color and water-holding capacity (Gou et al., 2002). The adipose oxidative metabolism's potential to elevate reactive oxygen species (ROS) production underscores the physiological significance of UCP2's uncoupling activity in modulating ROS generation within mitochondria of certain cell types (Li et al., 2001). As previously mentioned, pH can influence WHC characteristics, such as cooking loss (Warner, 2017). This relation can explain the association of this gene with cooking loss in the present study, where beta-e values from UCP2 were 0.60 and 0.38, and beta-p values for the cooking loss phenotype were recorded at 0.21 and 0.22, indicating an influence at meat quality.

Figure 1 and 2 represents the genomic location of the novel SNP chr15:53759536, situated within an ATAC-Seq peak. The visualization utilized the Integrative Genomics Viewer (IGV). Regions containing eQTLs within ATAC-seq peaks are commonly denoted as chromatin states (Ernst & Kellis, 2010). These chromatin states facilitate the identification of regulatory regions, contributing to a more comprehensive understanding of gene expression regulation (Ernst & Kellis, 2017). They serve as effective tools for discerning various genomic elements, including active enhancers, transcription start sites, and insulators. In this case, this SNP is situated within the chromatin state, exhibiting characteristics indicative of a predicted insulator. Insulators represent DNA regions where certain elements can bind, safeguarding gene expression by shielding it from signals emanating from the surrounding genomic regions. Insulators execute this protective function through two distinct mechanisms: as enhancer-blocking element or as barriers preventing the progression of condensed chromatin that could otherwise silence expression (West et al., 2002). An eQTL residing in a cis-regulatory element, such as insulators, has the potential to influence gene expression (Brown et al., 2013).

Figure 1. Visualization of eQTL BTA 15:53759536 in ATAC-Seq peak and its chromatin state, generated by software IGV.

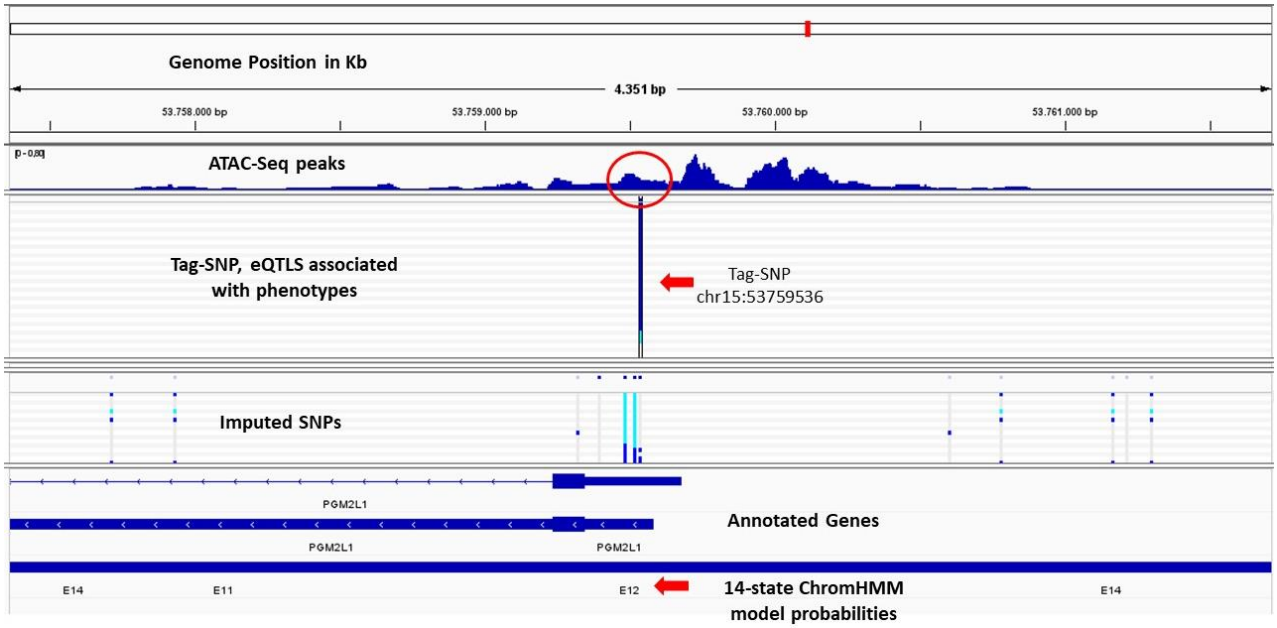
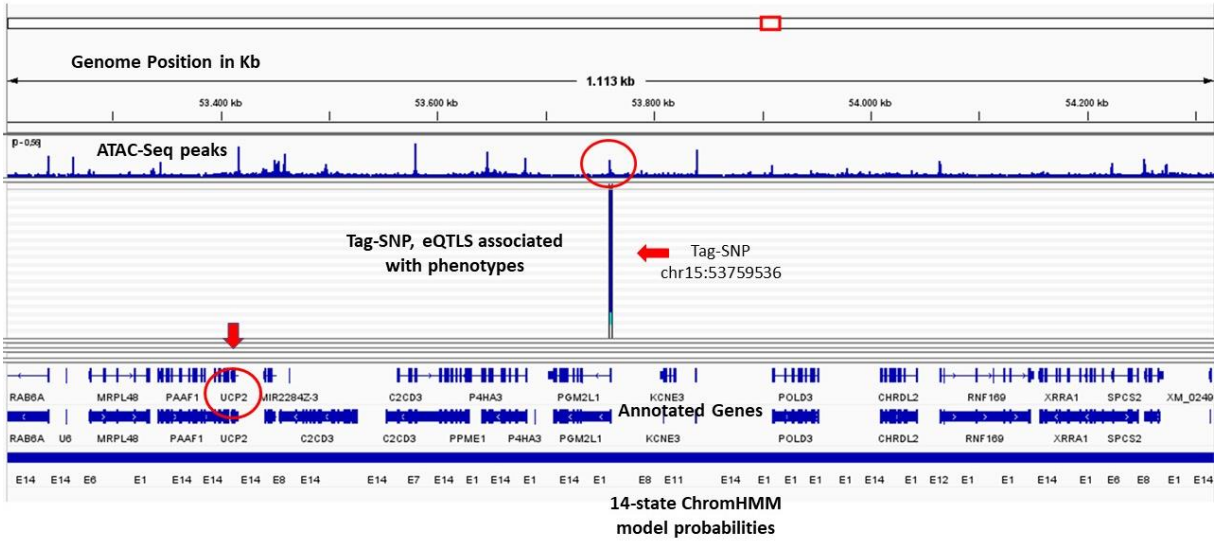


Figure 2. Visualization of eQTL BTA 15:53759536 and its regulated gene, UCP2. Image generated by software IGV.



The glutathione S-transferase alpha 4 (GSTA4) protein plays an important role in glutathione metabolism, a crucial pathway with significant implications, particularly in tissue detoxification from reactive oxygen species, and in removing harmful products (Ribeiro et al., 2022; Wu et al., 2004). Glutathione, renowned for its diverse biological functions in skeletal muscle, regulates cell redox state, acts as a cysteine reservoir for protein synthesis, and provides protection against ROS and

oxidants (Sen, 1998). GSTA4 actively participates in the catalysis of conjugation reactions involving endogenous substances, fatty acids, heme, xenobiotics, and oxidative processes products (Listowsky et al., 1988). The critical role of glutathione S-transferase lies in converting cytotoxic damage into less toxic forms, thereby facilitating detoxification (Ketterer et al., 1983). Understanding this process, an observed increase in the content of cysteine-glutathione disulfide, formed through S-glutathionylation of cysteine exposed to oxidative stress, was noted in wooden breast broilers muscles, suggesting a potential defense mechanism within the *Pectoralis major* muscle against oxidative stress (Abasht et al., 2016; Soglia et al., 2021).

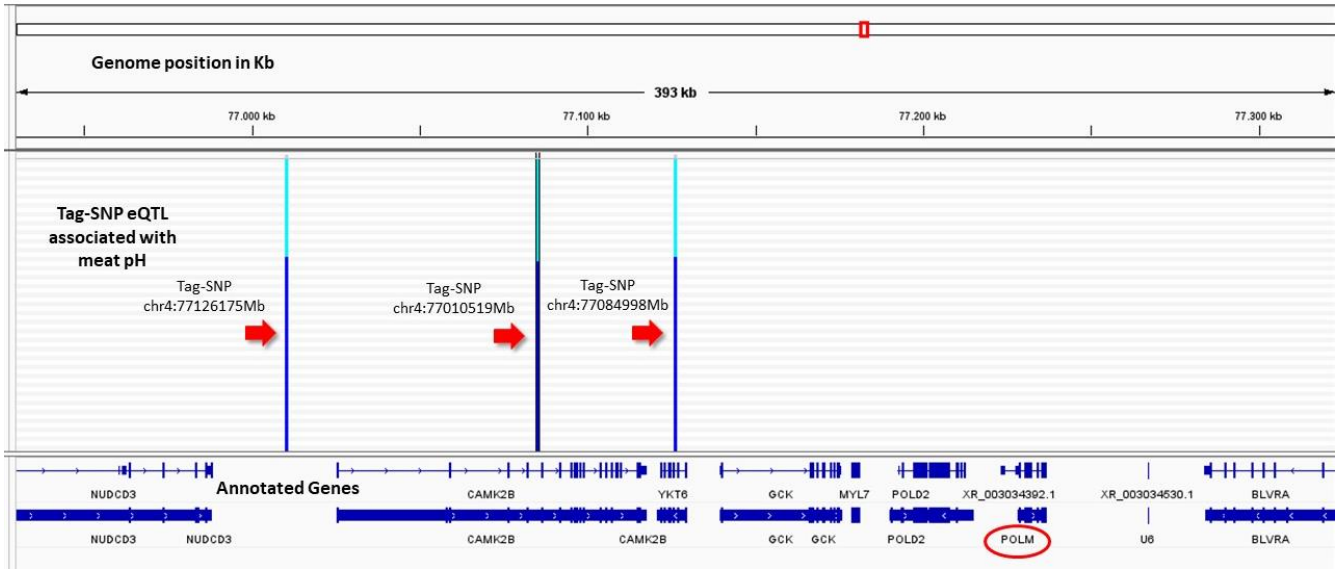
Within the investigation of Ribeiro et al. (2022), pigs subjected to a diet that exhibited elevated dietary availability of polyunsaturated fatty acids (PUFA) compared to control pigs showed a heightened abundance of GSTA4, suggesting a potential adaptative to enhance tissue ROS detoxification (Ribeiro et al., 2022). Previous studies observed the downregulation of GSTA4 in a high-boar-taint group in pig liver, along with another member of the same family, GSTO1. GSTO1 has been associated with eQTLs related to various growth and health parameters in pigs, including backfat thickness, ham weight, feed intake, and linolenic acid content (Drag et al., 2018). In chickens, GSTA4 emerged as a promising candidate gene affecting antioxidant enzyme activity in chicken embryos at day 16 and day 20, suggesting its upregulation in response to oxidative stress (Shearn et al., 2016; Yang et al., 2018). Additionally, GSTA4 has been linked to high gain (low intake) in beef steers (Kern et al., 2017). Other members of the GSTA family, namely GSTA1 and GSTA2, were also associated with color phenotypes in the present study. The beta-e GSTA4 was recorded at 0.16, with beta-p values noted at -0.20 for free water and 0.20 for water-holding capacity. It is noteworthy that GSTA4 may exert an influence on meat quality traits. While there isn't direct evidence of its association with WHC registered in literature, it is known that GSTA4 plays a role in oxidative stress situations, and the detoxification processes could potentially impact meat pH and WHC (Warner, 2017). Therefore, as in this work the gene expression was increased by the presence of eQTL, it can be hipothysed that the expression of GSTA4 increase the meat water-holding capacity, which inversely descrease meat free water.

3.4.3. pH gene

The three eQTLs associated with pH meat 24 hours *postmortem* were identified in chromosome BTA 4. These eQTLs were found to regulate the expression of the DNA polymerase μ (POLM) gene. The phenotypic association revealed beta-p values of 0.11, while the gene expression

exhibited beta-e of -0.20. This indicates that the alternative allele is associated with higher pH levels and lower expression of the POLM gene. Figure 3, generated using IGV software, shows the three eQTLs and the corresponding regulated gene.

Figure 3. Visualization in IGV software of the three eQTL associated with meat pH.



The POLM gene encodes DNA polymerase Pol, a pivotal player in DNA damage repair (Pryor et al., 2015). POLM is associated with TdT, a terminal transferase enzyme involved in V(D)J joining and the creation of junctional diversity. This gene functions as a hypermutation polymerase, exhibiting predominant expression in lymphocytes, B and T cells, and peripheral lymphoid tissues such as lymph nodes, spleen, and thymus. This pattern of expression suggests potential involvement in somatic hypermutation, facilitating the expansion and maturation of antibody repertoires (Aoufouchi, 2000; Dominguez, 2000; Friedberg et al., 2000). Notably, the high expression of POLM in developing germinal centers provides evidence of its lymphoid function (Gearhart & Wood, 2001).

Researchers suggest the potential to reduce the DNA repair capacity for double-strand breaks by epigenetically suppressing genes involved in repair pathways due to oxidative stress (Zhong et al., 2017). DNA double-strand break is considered the most severe type of DNA damage, induced by reactive oxygen species (ROS) (Huelsmann et al., 2019). The DNA repair polymerase, Pol, encoded by POLM, often fails to perform accurate translesional synthesis, leading to error introduction. Pol typically deletes bases or erroneously incorporates deoxyadenosine opposite 8-oxodG instead of the correct deoxycytosine, resulting in a C:G to A:T transversion mutation (Shibutani et al., 1991; Zhang et al., 2002).

In an experiment involving POLM knockout mice, it was observed that the absence of POLM likely decreased the mutagenic impact of ROS by indirectly enhancing the accuracy of oxidative DNA damage repair (Huelsmann et al., 2019). The rate of oxidative DNA damage is directly linked to metabolic rate and inversely associated with organism lifespan. Potential mechanisms of oxidative damage include nitration of protein tyrosine residues, lipid peroxidation, DNA degradation, and formation of oligonucleosome fragments (Hemnani & Parihar, 1998).

The interrelation between pH and ROS has been established in studies. It is proposed that the fast decrease in pH contributes to the denaturation of sarcoplasmic proteins and myosin, leading to ROS accumulation in *postmortem* muscle. The weakening of antioxidant enzymes and other defense systems is one of the factors contributing to ROS production in postmortem muscle (Kurz et al., 2007; Ma et al., 2022). During meat aging and storage, myofibrillar proteins are impacted by ROS, potentially causing damage to cellular structures, and affecting meat quality (Martinaud et al., 1997).

For meat to achieve the criteria of high quality, it must maintain an ultimate pH within the range of 5.4 to 5.6. Beyond a pH of 5.8, there is a noticeable decline in the ability to sustain good quality during cooling processes. A high pH is unsuitable for procedures such as meat sorting, processing, and vacuum packaging. Meat with elevated pH levels may exhibit traits such as increased water-holding capacity, a gummy texture, and a diminished specific taste (Pipek et al., 2003; Villarroel et al., 2003).

In literature, no established association has been documented between the POLM gene and meat quality. Given POLM's role in DNA repair in the presence of ROS, it is conceivable that under conditions of insufficient glycogen reserves and concurrent oxidative stress, POLM may engage in DNA damage repair. An inferred connection related to identified mutations suggests that animals carrying these mutations might experience reduced POLM expression, leading to elevated ROS levels in muscle tissue. Consequently, this elevated oxidative stress could contribute to a higher meat pH value. Given that the observed pH level in the animals was 5.6, the slightly elevated pH resulting from the mutation remains marginally above the mean. However, it does not surpass the threshold considered indicative of low-quality meat, which is 5.8 or higher (Ferreira et al., 2024).

3.4.4. MHC genes

In Functional Enrichment, the Major Histocompatibility Complex (MHC) genes emerged as the predominant pathway among genes associated with color and WHC phenotypes. Due to their

significance, we will discuss them separately in this section. The MHC is a ubiquitous feature across all mammalian species and holds a central role in the immune system. This complex is responsible for protein expression in specific cells, facilitating their presentation to antigens. It plays a critical role in determining susceptibility or resistance to various diseases, as well as in intracellular recognition and self-discrimination processes (Abbas et al., 2014; Behl et al., 2012).

In cattle, the major histocompatibility complex is known as bovine leukocyte antigen (BoLA). The BoLA gene family encompasses over 154 closely linked genes within a 4000 kb region of chromosome 23, spanning positions 25Mbp to 29Mbp. While these BoLA genes function collectively as a system, they are organized into distinct groups; some loci are nearby, while others are relatively distant (Behl et al., 2012; Maiorano et al., 2022; Nam et al., 2021). The MHC complex comprises a highly polymorphic group of genes, crucial for enabling the immune system to recognize and combat a wide array of pathogens effectively (Pinto De Melo et al., 2017).

BoLA genes serve as markers for polymorphic DNA sequences that are responsible for the formation of defense responses in cattle through genetic polymorphism analysis. Consequently, these genes have been identified as indicators of selection in the Nellore breed, suggesting their significant involvement in the breed's adaptation (Fernandes Júnior et al., 2020). Previous research has highlighted the importance of the bovine lymphocyte antigen complex in host immunity, demonstrating its association with parasitic diseases (Behl et al., 2012; Kim et al., 2017). Additionally, it is proposed that heightened expression of these immune genes might contribute to a more favorable feed efficiency phenotype (Lindholm-Perry et al., 2016).

Various MHC genes, including BOLA, BOLA-DQB, BOLA-DQA5, BOLA-NC1, and JSP.1, have been identified in association with color and water-holding capacity phenotypes. SNPs that regulate MHC gene expression are associated with specific phenotypic traits, such as L* (lightness on days 0 and 7), a* (redness on day 0), b* (yellowness on days 7 and 14), cooking loss (on days 0 and 14), humidity, water-holding capacity (at day 0) and free water. Moreover, these MHC genes have been detected in ATAC-Seq peaks, including the genes BOLA, BOLA-DQB, and JSP.1. The BOLA gene was found in the peak position chr 23:28551309, associated with a* phenotype on day 0. SNPs regulating BOLA-DQB gene expression were in trans, leading to the ATAC-Seq peak position chr21:22841828, associated with L* on day 7 and b* on day 14. The JSP.1 gene appeared in peaks at positions 23:28551191 (associated with L* on day 7) and 23:28551891 (associated with a* on day 0).

The beta values representing phenotypic association (beta-p) varied significantly across different genes and phenotypes, with 39 values increasing the phenotypes and 29 values decreasing

them. Specifically, the BOLA gene, associated with L* on day 7, exhibited beta-p values ranging from 0.29 to 0.24 in cis and from -0.25 to 0.25 in trans. For a* on day 0, beta-p values ranged from -0.17 to -0.16. The lowest beta-p value was associated with humidity and regulated by the JSP.1 gene (-0.42), whereas the highest beta-p value, regulated by BOLA-DQB in trans, was 0.34 and associated with the L*7 phenotype. Regarding beta values derived from eQTL and representing values for gene expression (beta-e), these values also displayed variation based on specific genes and phenotypes. Between them, 55 were negative, indicating a decrease in gene expression, while 13 were positive values. The lowest beta-e value was found regulated by gene BOLA-DQB in trans, measuring -2.10, associated with b* on day 7. In contrast, the highest beta-e value, 1.21, was regulated by BOLA-DQA5 in cis, associated with a* on day 0. To confer the description of all values, please check Supplementary Tables 1, 2, and 4.

Genes belonging to the BoLA family have previously been linked to various meat quality traits. Specifically, the BOLA-DQB gene has been associated with immune responses and adaptation to tropical environments. Within *Bos taurus*, there is substantial evidence indicating the presence of over 50 BoLA-DQB alleles distributed across at least five DQB loci, rendering this genomic region one of the most complex in the BoLA gene family (Behl et al., 2012; Fernandes Júnior et al., 2020). In a study by Fonseca et al., (2017), higher expression of the BoLA-DQB transcript was observed in tougher meat samples.

Elevated levels of gene expression for JSP.1 and BoLA-BQB were observed in the high marbling group of Nellore meat (Fonseca et al., 2020). In a study focused on meat color, six non-synonymous deletions were identified within coding regions of messenger RNAs (mRNAs), including JSP.1-204, in the group of desirable meat colors. All identified variants had a significant impact on the protein structure. The JSP.1 gene was specifically associated with the meat color trait, with minor genetic variants located within its coding regions. Notably, in this study, JSP.1-204 exhibited differential expression in animals with desirable flesh color compared to those in the undesirable flesh color groups (Muniz et al., 2022). In the current study, the beta-e values exhibited variability in the expression of JSP.1, with three SNPs positively regulating gene expression and eight SNPs negatively regulating. The beta-p also varied, demonstrating positive associations with the phenotypes of L* on day 7, a* on day 0, WHC, and cooking loss on day 14. The beta-p was negative for phenotypes of L* on day 0 and 7, a* on day 0, free water, and humidity. JSP.1 significantly impact various meat quality traits, including color and water-holding capacity phenotypes. Figures 4 and 5 represent the eQTL regulating JSP.1, located within an ATAC-Seq peak. The identified SNP is situated within a chromatin state, suggestive of a predicted insulator (Ernst & Kellis, 2010). As mentioned in

the topic of WHC genes, this observation strengthens the notion of its influence in gene expression (Brown et al., 2013).

Figure 4. Visualization of eQTL BTA 23:28551891 in ATAC-Seq peak and its chromatin state, generated by software IGV.

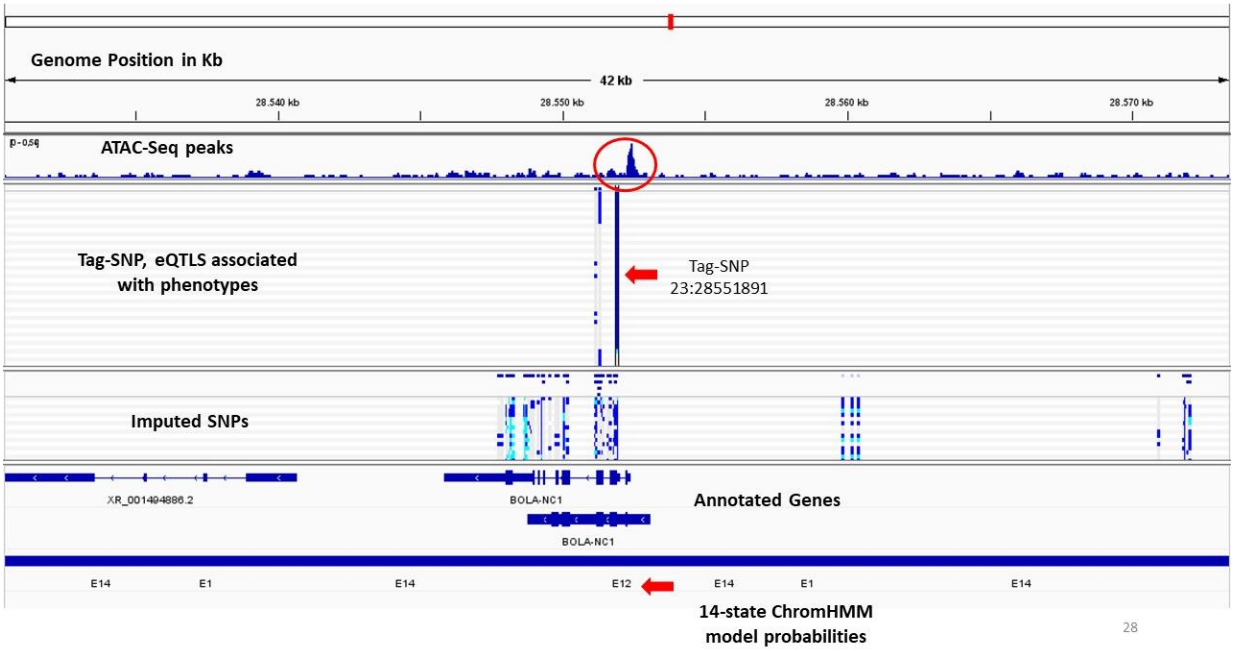
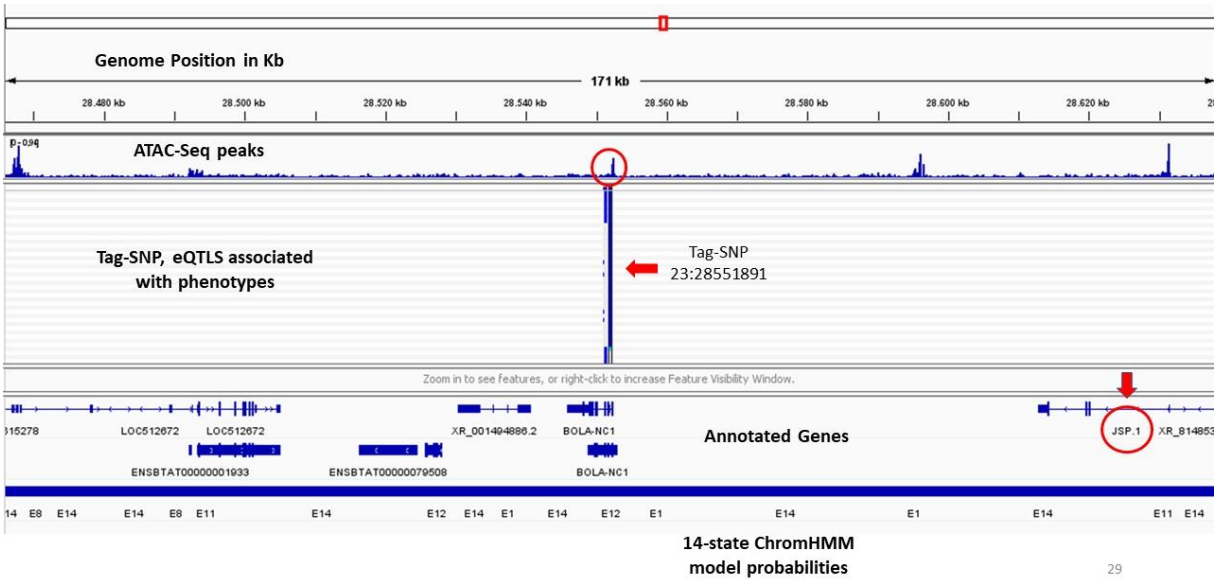


Figure 5. Visualization of eQTL BTA 23:28551891 and its regulated gene, JSP.1. Image generated by software IGV.



Fonseca et al.'s (2020) study utilized the identical database employed by Muniz et al. (2022), to investigate marbling traits. Color and marbling characteristics in Nellore beef cattle are probably influenced by overlapping genetic mechanisms, indicating the potential for simultaneous enhancement. Within these studies, specific mRNA isoforms were identified as potential candidates, including JSP.1-204, which was linked to both meat marbling and color traits. These regions feature minor genetic variants at their splice sites, which play a crucial role in determining the expression and functionality of these mRNA isoforms (Muniz et al., 2022).

As previously mentioned, MHC genes are involved not only in immune responses but also influences meat quality. Within this study, MHC genes emerged as the most prevalent, demonstrating their significant involvement in regulating gene expression. Although the precise biological pathways through which these genes influence meat quality remain incompletely elucidated, a confirmed connection between them has been established.

3.5. Conclusions

In this study, it was found various polymorphisms associated with gene expression regulation and phenotypic variation related to meat quality. The identified mutations were involved in the regulation of genes that participate in immune response, antigen process and presentation, and glutathione metabolism. These genes also demonstrated influence over meat quality traits, impacting phenotypes of color, water-holding capacity, and pH. Certain genes, including PTER, GSTA4, BOLA-DQB, and JSP.1, have previously been linked to meat quality. Conversely, genes like EIF2AK4 and POLM, not directly associated with meat quality in existing literature, demonstrated significant associations in this study. This exploration enhances the understanding of genetic architecture that controls meat quality traits.

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Supplementary Material

Supplementary Table 1. Cis-eQTL (Partial).

snps	gene	statistic	pvalue	FDR	beta
19_55193069	ENSBTAG00000053829	28.50775193	2.86E-67	1.76E-60	1.594918097
20_41490368	ENSBTAG00000049212	27.00681581	6.00E-64	1.85E-57	2.634427078
7_40398885	ENSBTAG00000015727	-23.23882581	4.35E-55	8.94E-49	-2.72611033
16_6213025	ENSBTAG00000040409	22.86869657	3.55E-54	5.47E-48	1.827688078
6_85830182	ENSBTAG00000007816	21.58596398	5.86E-51	6.81E-45	1.685331417
19_55194508	ENSBTAG00000053829	21.56500796	6.62E-51	6.81E-45	1.310326314
19_55198792	ENSBTAG00000053829	20.91652042	3.04E-49	2.68E-43	1.248426165
19_55197663	ENSBTAG00000053829	20.68982154	1.17E-48	9.04E-43	1.259386092
19_55194812	ENSBTAG00000053829	20.62062476	1.77E-48	1.20E-42	1.28310037
5_74582502	ENSBTAG00000012192	20.60532269	1.94E-48	1.20E-42	2.687637277
19_55198745	ENSBTAG00000053829	20.54177124	2.84E-48	1.59E-42	1.266351832
26_18767780	ENSBTAG00000011137	20.44921316	4.95E-48	2.54E-42	0.934752774
8_112749253	ENSBTAG00000020495	19.31272823	4.89E-45	2.32E-39	0.638781045
10_26633128	ENSBTAG00000037452	19.02508237	2.87E-44	1.26E-38	1.28812778

18_14335628	ENSBTAG00000012041	18.96876953	4.06E-44	1.67E-38	0.404230385
25_3605301	ENSBTAG00000010015	-18.91517429	5.65E-44	2.18E-38	-1.00893595
7_110518080	ENSBTAG00000026909	18.51898258	6.61E-43	2.40E-37	1.191610493
24_33145636	ENSBTAG00000010042	18.3807056	1.56E-42	5.36E-37	0.550469506
3_99340266	ENSBTAG00000011976	18.28876149	2.78E-42	9.02E-37	1.393190424
19_55194080	ENSBTAG00000053829	18.25654411	3.40E-42	1.05E-36	1.292758013
4_12887299	ENSBTAG00000034645	18.08881686	9.72E-42	2.86E-36	3.018477421
25_38780	ENSBTAG00000053570	-18.00895282	1.61E-41	4.50E-36	-2.33156093
7_2653654	ENSBTAG00000007820	-17.65688772	1.48E-40	3.96E-35	-1.0031721
19_29461930	ENSBTAG00000037794	-17.55565781	2.80E-40	7.20E-35	-2.71989164
19_42132811	ENSBTAG00000025762	17.5000232	3.99E-40	9.83E-35	0.521286839
26_18682399	ENSBTAG00000011137	17.4763117	4.63E-40	1.10E-34	0.86669943
28_44155051	ENSBTAG00000018915	-17.20936789	2.53E-39	5.77E-34	-0.44314602
21_58671478	ENSBTAG00000031834	-17.19832792	2.71E-39	5.97E-34	-1.13642983
14_1096976	ENSBTAG00000016810	-17.1135416	4.65E-39	9.89E-34	-0.58193578

Supplementary Table 2. Trans-eQTL (Partial).

snps	gene	statistic	pvalue	FDR	beta
10_38403400	ENSBTAG00000001182	-32.1597	6.78E-75	4.87E-65	-4.12902
10_38403400	ENSBTAG00000019366	-30.6242	9.13E-72	3.28E-62	-3.93598
10_38403400	ENSBTAG00000013620	-28.8927	4.19E-68	1.00E-58	-5.78074
10_38403400	ENSBTAG00000006756	-28.4934	3.07E-67	5.52E-58	-3.62931
10_38403400	ENSBTAG00000015898	-28.2697	9.45E-67	1.36E-57	-5.38557
4_98334737	ENSBTAG00000037625	27.9629	4.45E-66	5.34E-57	1.979824
10_38403400	ENSBTAG00000010132	-27.8798	6.79E-66	6.97E-57	-5.81108
10_38403400	ENSBTAG00000016311	-27.2907	1.38E-64	1.24E-55	-5.08363
10_38403400	ENSBTAG00000003748	-27.2688	1.55E-64	1.24E-55	-3.46579
10_38403400	ENSBTAG00000012828	-27.24	1.80E-64	1.29E-55	-5.22685
10_38403400	ENSBTAG00000048843	-26.798	1.78E-63	1.16E-54	-5.08237
23_28366600	ENSBTAG00000048426	26.65867	3.68E-63	2.03E-54	3.301643
23_28402058	ENSBTAG00000048426	26.65867	3.68E-63	2.03E-54	3.301643
10_38403400	ENSBTAG00000021965	-26.5654	5.99E-63	3.07E-54	-4.97676
10_38403400	ENSBTAG00000000806	-26.1638	4.95E-62	2.37E-53	-4.37938

10_38403400	ENSBTAG00000039442	-25.78	3.80E-61	1.71E-52	-3.95858
10_38403400	ENSBTAG00000012124	-25.693	6.05E-61	2.56E-52	-4.25708
10_38403400	ENSBTAG00000014440	-25.4516	2.20E-60	8.80E-52	-5.32615
10_38403400	ENSBTAG00000016900	-25.4267	2.52E-60	9.53E-52	-4.86619
10_38403400	ENSBTAG00000009103	-25.3961	2.97E-60	1.05E-51	-4.98576
10_38403400	ENSBTAG00000011096	-25.3896	3.08E-60	1.05E-51	-4.23658
10_38403400	ENSBTAG00000016309	-25.1658	1.03E-59	3.36E-51	-4.82942
10_38403400	ENSBTAG00000007595	-25.0014	2.51E-59	7.83E-51	-4.50379
10_38403400	ENSBTAG00000002493	-24.8962	4.44E-59	1.33E-50	-3.69533
10_38403400	ENSBTAG00000018690	-24.855	5.56E-59	1.60E-50	-4.52536
10_38403400	ENSBTAG00000020381	-24.6935	1.34E-58	3.71E-50	-5.86173
10_38403400	ENSBTAG00000009106	-24.5855	2.42E-58	6.45E-50	-3.52226
8_97058010	ENSBTAG00000009976	24.56402	2.72E-58	6.99E-50	1.634044
10_38403400	ENSBTAG00000015732	-24.292	1.22E-57	3.01E-49	-4.66867

Supplementary Table 3. ATAC-Seq peaks.

Chr	Position	rsID	ATAC-Seq Peak	Phenotype	Genes
1	84024455	rs434168252	chr1:84020926 - 84024722	aC14	MCCC1
1	88360397	Novel SNP	chr1:88359511-88364618	bC14	PLCXD1
1	106728357	rs717901458	chr1:106725622-106728829	LC7	B3GALNT1
1	152698895	rs440039289	chr1:152698344-152699628	aC	OXNAD1
2	18418748	Novel SNP	chr2:18417446-18419199	LC7	UACA
2	124853535	rs211465419	chr2:124850143-124854843	umidade	Novel gene
2	135864739	rs517612671	chr2:135864630-135867264	LC7	FBXO42
3	58490048	rs134186717	chr3:58488688-58492002	PPC	ODF2L
3	117507829	Novel SNP	chr3:117507404-117509262	aC	KLHL30
4	44518567	rs479851387	chr4:44518269-44519383	aC14	PMPCB
4	98814425	rs208919780	chr4:98810298-98814639	aC	Novel gene
5	19399298	rs1116179345	chr5:19397507-19400500	aC	Novel gene
5	96875461	rs461770318	chr5:96874798-96876886	aC14	HEBP1
6	58620067	rs520953487	chr6:58619352-58621152	aC	Novel gene
6	116841998	rs379925554	chr6:116841843-116843867	aC	HAUS3

7	2748890	Novel SNP	chr7:2748091-2749647	agualivre	Novel gene
7	2748890	Novel SNP	chr7:2748091-2749647	agualivre	Novel gene
7	5883998	rs383227003	chr7:5879340-5884582	bC7	USE1
7	5883998	rs383227003	chr7:5879340-5884582	LC7	USE1
7	21873182	Novel SNP	chr7:21872462-21873956	aC	RAD50
7	40389504	rs378945649	chr7:40387828-40391845	aC	IFI47
7	80749220	rs454670226	chr7:80747915-80749802	LC7	DHFR
8	75740696	rs519074782	chr8:75740538-75746081	LC7	UBAP2
9	28470881	rs518458001	chr9:28467718-28471755	LC7	SMPDL3A
9	44840001	rs1114770936	chr9:44839401-44840708	LC7	DPP6
9	87151695	rs133900775,rs799001618	chr9:87151281-87155999	aC	Novel gene
10	21923481	rs381047402	chr10:21920310-21926607	aC	THTPA
10	35956049	rs722216579	chr10:35951875-35960663	aC	EIF2AK4
10	35956802	Novel SNP	chr10:35951875-35960663	aC	EIF2AK4
11	48758868	rs524843546	chr11:48758128-48760405	LC7	THNSL2
11	48842172	rs440716903	chr11:48839038-48845037	aC	ELMOD3
11	101477987	rs715454582	chr11:101474705-101478844	LC7	FAM78A

13	30919197	rs455630968	chr13:30917557-30920430	bC7	PTER
13	30919197	rs455630968	chr13:30917557-30920430	LC7	PTER
13	54734787	rs526304612	chr13:54730972-54735896	umidade	Novel gene
14	77391683	rs1115722715	chr14:77390485-77392282	aC	Novel gene
15	42994593	rs480639475	chr15:42993516-42995178	aC	TMEM41B
15	46384601	Novel SNP	chr15:46382957-46385075	agualivre	Novel gene
15	46384601	Novel SNP	chr15:46382957-46385075	CRA	Novel gene
15	53759536	Novel SNP	chr15:53757425-53760877	PPC	UCP2
16	79316484	rs456863959	chr16:79315683-79319914	LC7	DDX59
16	80249491	Novel SNP	chr16:80249259-80250581	LC7	Novel gene
17	51757137	Novel SNP	chr17:51756644-51758221	agualivre	ABCB9
17	51757137	Novel SNP	chr17:51756644-51758221	CRA	ABCB9
17	62326108	rs517813981	chr17:62322490-62327183	LC7	BICDL1
17	72636708	rs517521198	chr17:72633991-72639539	aC	UFD1
18	10244561	rs517831705	chr18:10238099-10252100	umidade	OSGIN1
18	49996146	rs518241587	chr18:49995020-49997009	aC	SHKBP1

18	49996660	rs41884156	chr18:49995020-49997009	aC	EID2
18	53694398	rs720834421	chr18:53691093-53694530	agualivre	CCDC8
18	53694398	rs720834421	chr18:53691093-53694530	CRA	CCDC8
19	19651292	rs207773543	chr19:19649938-19653193	aC	NLK
19	20428850	rs723506371	chr19:20428145-20429824	agualivre	ALDOC
19	20428850	rs723506371	chr19:20428145-20429824	CRA	ALDOC
19	51027599	rs523204098	chr19:51020493-51028401	aC	P4HB
19	51155251	Novel SNP	chr19:51152984-51156542	aC	CCDC57
19	55670007	rs522968671	chr19:55668156-55671033	LC7	TRIM47
19	63113169	rs715816337	chr19:63112263-63118975	LC7	CACNG4
19	63128156	rs470995266	chr19:63126986-63129968	LC7	CACNG4
19	63128551	rs519113615	chr19:63126986-63129968	LC7	CACNG4
21	22782727	rs521112302	chr21:22780925-22783619	LC7	FSD2
21	22841828	rs722291877	chr21:22838128-22843806	LC7	BOLA-DQB
21	22841828	rs722291877	chr21:22838128-22843806	LC7	BLM
21	22841828	rs722291877	chr21:22838128-22843806	bC14	BOLA-DQB
21	27709470	rs719705851	chr21:27706431-27709635	LC	FBXW8

21	27709470	rs719705851	chr21:27706431-27709635	aC	FBXW8
21	58535011	rs524543720	chr21:58533308-58538592	agualivre	Novel gene
21	58535011	rs524543720	chr21:58533308-58538592	CRA	CCDC197
21	58754317	rs385985642	chr21:58754047-58755971	LC7	Novel gene
22	14817206	rs1114418646	chr22:14812411-14819370	LC7	SEC22C
22	14826110	rs515929286	chr22:14820319-14828438	LC7	Novel gene
23	7858920	Novel SNP	chr23:7858485-7870283	aC	Novel gene
23	7861222	rs715025256	chr23:7858485-7870283	LC7	Novel gene
23	17742332	rs719100822	chr23:17741797-17745806	agualivre14	POLH
23	17742332	rs719100822	chr23:17741797-17745806	CRA14	POLH
23	24948132	rs378187242	chr23:24947280-24948695	CRA	GSTA4
23	24948132	rs378187242	chr23:24947280-24948695	agualivre	GSTA4
23	28551191	Novel SNP	chr23:28550601-28553111	LC7	JSP.1
23	28551309	Novel SNP	chr23:28550601-28553111	aC	BoLA
23	28551861	Novel SNP	chr23:28550601-28553111	agualivre	Novel gene
23	28551861	Novel SNP	chr23:28550601-28553111	CRA	JSP.1

23	28551891	Novel SNP	chr23:28550601-28553111	aC	JSP.1
23	31731831	rs378022190	chr23:31731104-31732078	aC	HFE
23	50964983	rs1115267361	chr23:50963599-50965672	LC7	SERPINB9
24	21006330	rs718794157	chr24:21005875-21006536	aC	ELP2
24	22131338	rs523724742	chr24:22131128-22132680	LC7	FBXW8
24	22132289	rs386030514	chr24:22131128-22132680	LC7	FBXW8
25	118439	rs451382204	chr25:116607-123414	aC	NPRL3
25	126832	rs208104252	chr25:125862-127824	aC	SNRNP25
25	1500147	rs720785477	chr25:1496486-1502667	aC	LMF1
25	2581925	rs723762847	chr25:2580809-2588747	umidade	Novel gene
25	7650895	rs209538840	chr25:7650786-7654072	LC7	TMEM186
25	34128566	rs714082568	chr25:34127103-34129176	aC	RFC2
25	38065368	rs207519251	chr25:38064303-38065833	aC	EIF2AK1
25	40625884	rs716576302	chr25:40625306-40626840	aC	BRAT1
26	44097695	rs110783329	chr26:44097475-44098947	bC14	LHPP
27	15711640	rs522608835	chr27:15710918-15711769	aC	C27H4orf47
28	18070960	rs42339246	chr28:18063918-18072748	aC	CABCOCO1

29	41144922	rs378624329	chr29:41143955-41151543	aC	DAGLA

Supplementary Table 4. Phenotypes information (Partial).

Chr	Position	rsID	Genes regulated	Ensembl ID	Gene consequences	Type	FDR	Phenotype	Beta
5	74582984	rs470096482	Novel gene	ENSBTAG00000012192	-	cis	2.24E-22	LC	0.3031
5	74721305	rs1115576439	Novel gene	ENSBTAG00000012192	-	cis	7.84E-21	LC	0.3051
5	73927646	rs111022834	Novel gene	ENSBTAG00000012192	-	cis	2.98E-15	LC	0.283
13	12524316	rs135210062	ECHDC3	ENSBTAG00000012377	downstream_gene_variant	cis	1.93E-09	LC	0.2567
18	55618655	rs458777604	PPP1R15A	ENSBTAG00000001294	upstream_gene_variant	cis	0.000000246	LC	0.2622
18	55618655	rs458777604	KASH5	ENSBTAG00000008614	-	cis	0.000000246	LC	0.2622
18	55618655	rs458777604	PIH1D1	ENSBTAG00000016526	synonymous_variant	cis	0.000000246	LC	0.2622

23	27873900	rs463632686	JSP.1	ENSBTAG00000020116	3_prime_UTR_variant	cis	0.0000186	LC	-0.263
5	74721305	rs1115576439	Novel gene	ENSBTAG00000037799	missense_variant	cis	7.84E-21	LC	0.3051
5	73927646	rs111022834	Novel gene	ENSBTAG00000037799	missense_variant	cis	2.98E-15	LC	0.283
18	55618655	rs458777604	SLC6A16	ENSBTAG00000030543	synonymous_variant	cis	0.000000246	LC	0.2622
5	73927646	rs111022834	APOL3	ENSBTAG00000040244	-	cis	2.98E-15	LC	0.283
5	74721305	rs1115576439	APOL3	ENSBTAG00000040244	-	cis	7.84E-21	LC	0.3051
18	55618655	rs458777604	ALDH16A1	ENSBTAG00000000330	missense_variant	cis	0.000000246	LC	0.2622
13	12524316	rs135210062	PROSER2	ENSBTAG00000039571	intron_variant	cis	1.93E-09	LC	0.2567
5	74721305	rs1115576439	APOL3	ENSBTAG00000000667	3_prime_UTR_variant	cis	7.84E-21	LC	0.3051
23	27873900	rs463632686	GNL1	ENSBTAG00000025516	missense_variant	cis	0.0000186	LC	-0.263

21	31251508	rs378611140	ACSBG1	ENSBTAG00000006231	downstream_gene_variant	cis	0.026622189	LC	0.2171
1	150728955	rs43282862	PIK3R4	ENSBTAG00000020787	upstream_gene_variant	cis	0.035482126	LC	0.219
23	28527535	rs483164965	Novel gene	ENSBTAG00000005146	downstream_gene_variant	cis	0.0000526	LC14	0.3308
18	26018121	rs378218317	CFAP20	ENSBTAG00000013874	3_prime_UTR_variant	cis	0.000431807	LC14	-0.2271
5	74578997	rs516000565	Novel gene	ENSBTAG00000012192	-	cis	1.28E-32	LC7	0.2634
5	74578810	rs523974202	Novel gene	ENSBTAG00000012192	-	cis	1.09E-31	LC7	0.2437
5	75448230	rs211170554	Novel gene	ENSBTAG00000012192	-	cis	4.1E-21	LC7	0.1911
22	14734342	rs715902009	Novel gene	ENSBTAG00000048844	non_coding_transcript_exon_variant	cis	1.19E-17	LC7	-0.1898

22	14828620	rs109266169	Novel gene	ENSBTAG00000048844	non_coding_transcript_exon_variant	cis	5.12E-16	LC7	-0.1844
22	14826110	rs515929286	Novel gene	ENSBTAG00000048844	non_coding_transcript_exon_variant	cis	4.9E-15	LC7	-0.194
22	14792156	rs109758294	Novel gene	ENSBTAG00000048844	non_coding_transcript_exon_variant	cis	6.18E-15	LC7	-0.1788
22	14875322	rs207956360	Novel gene	ENSBTAG00000048844	non_coding_transcript_exon_variant	cis	8.39E-15	LC7	-0.1747

Supplementary Table 5. Functional enrichment of color genes (Partial).

Category	Term	Count	%	PValue
INTERPRO	IPR011162:MHC classes I/II-like antigen recognition protein	19	4.139434	8.79E-14
INTERPRO	IPR011161:MHC class I-like antigen recognition	13	2.832244	2.40E-09
INTERPRO	IPR003597:Immunoglobulin C1-set	15	3.267974	6.49E-08
SMART	SM00407:IGc1	15	3.267974	6.88E-08
INTERPRO	IPR003006:Immunoglobulin/major histocompatibility complex, conserved site	13	2.832244	7.70E-08
INTERPRO	IPR001039:MHC class I, alpha chain, alpha1/alpha2	8	1.742919	2.15E-07
KEGG_PATHWAY	bta05330:Allograft rejection	10	2.178649	2.80E-06
KEGG_PATHWAY	bta04940:Type I diabetes mellitus	10	2.178649	3.77E-06
KEGG_PATHWAY	bta05416:Viral myocarditis	11	2.396514	4.86E-06
KEGG_PATHWAY	bta05332:Graft-versus-host disease	10	2.178649	8.58E-06
GOTERM_BP_DIRECT	GO:0002476~antigen processing and presentation of	6	1.30719	1.90E-05

	endogenous peptide antigen via MHC class Ib			
KEGG_PATHWAY	bta05320:Autoimmune thyroid disease	10	2.178649	2.01E-05
UP_SEQ_FEATURE	DOMAIN:GST N-terminal	7	1.525054	2.59E-05
GOTERM_CC_DIRECT	GO:0009897~external side of plasma membrane	22	4.793028	2.67E-05
GOTERM_BP_DIRECT	GO:0006890~retrograde vesicle-mediated transport, Golgi to ER	8	1.742919	4.14E-05
INTERPRO	IPR004045:Glutathione S-transferase, N-terminal	7	1.525054	4.49E-05
INTERPRO	IPR010987:Glutathione S-transferase, C-terminal-like	7	1.525054	5.30E-05
GOTERM_CC_DIRECT	GO:0005739~mitochondrion	39	8.496732	5.48E-05
KEGG_PATHWAY	bta04514:Cell adhesion molecules	14	3.050109	6.25E-05
KEGG_PATHWAY	bta04145:Phagosome	14	3.050109	7.07E-05
UP_SEQ_FEATURE	REGION:Disordered	266	57.95207	7.73E-05
UP_SEQ_FEATURE	DOMAIN:GST C-terminal	7	1.525054	8.35E-05

UP_KW_MOLECULAR_FUNCTION	KW-0808~Transferase	44	9.586057	8.83E-05
UP_KW_CELLULAR_COMPONENT	KW-0963~Cytoplasm	75	16.33987	9.57E-05
GOTERM_BP_DIRECT	GO:0006749~glutathione metabolic process	8	1.742919	9.83E-05
UP_KW_PTM	KW-0007~Acetylation	55	11.98257	1.09E-04
KEGG_PATHWAY	bta04612:Antigen processing and presentation	10	2.178649	1.20E-04
UP_KW_DOMAIN	KW-0853~WD repeat	12	2.614379	1.55E-04
UP_KW_CELLULAR_COMPONENT	KW-0496~Mitochondrion	33	7.189542	1.55E-04

Supplementary Table 6. Functional enrichment of WHC genes (partial)

Category	Term	Count	%	PValue
GOTERM_BP_DIRECT	GO:0002476~antigen processing and presentation of endogenous peptide antigen via MHC class Ib	5	5.208333	6.06E-07
INTERPRO	IPR001039:MHC class I, alpha chain, alpha1/alpha2	5	5.208333	2.24E-06
INTERPRO	IPR003006:Immunoglobulin/major histocompatibility complex, conserved site	6	6.25	2.28E-05
INTERPRO	IPR011162:MHC classes I/II-like antigen recognition protein	6	6.25	3.00E-05
SMART	SM00407:IGc1	6	6.25	3.61E-05
KEGG_PATHWAY	bta05330:Allograft rejection	5	5.208333	1.05E-04
INTERPRO	IPR003597:Immunoglobulin C1-set	6	6.25	1.12E-04
INTERPRO	IPR011161:MHC class I-like antigen recognition	5	5.208333	1.19E-04
KEGG_PATHWAY	bta04940:Type I diabetes mellitus	5	5.208333	1.20E-04
KEGG_PATHWAY	bta05332:Graft-versus-host disease	5	5.208333	1.75E-04
KEGG_PATHWAY	bta05320:Autoimmune thyroid disease	5	5.208333	2.60E-04
KEGG_PATHWAY	bta05416:Viral myocarditis	5	5.208333	3.36E-04
KEGG_PATHWAY	bta05169:Epstein-Barr virus infection	7	7.291667	4.86E-04
KEGG_PATHWAY	bta04612:Antigen processing and presentation	5	5.208333	6.09E-04

KEGG_PATHWAY	bta04145:Phagosome	6	6.25	8.32E-04
GOTERM_BP_DIRECT	GO:0019882~antigen processing and presentation	4	4.166667	8.92E-04
INTERPRO	IPR010579:MHC class I, alpha chain, C-terminal	3	3.125	0.001161
INTERPRO	IPR008405:Apolipoprotein L	3	3.125	0.001161
UP_SEQ_FEATURE	DOMAIN:Immunoglobulin C1-set	3	3.125	0.001931
GOTERM_CC_DIRECT	GO:0071556~integral component of lumenal side of endoplasmic reticulum membrane	3	3.125	0.00218
GOTERM_BP_DIRECT	GO:0042157~lipoprotein metabolic process	3	3.125	0.004176
UP_KW_MOLECULAR_FUNCTION	KW-0808~Transferase	11	11.45833	0.004364
KEGG_PATHWAY	bta04514:Cell adhesion molecules	5	5.208333	0.006103
GOTERM_BP_DIRECT	GO:0006955~immune response	6	6.25	0.006349
UP_KW_MOLECULAR_FUNCTION	KW-0456~Lyase	4	4.166667	0.007123
KEGG_PATHWAY	bta05168:Herpes simplex virus 1 infection	7	7.291667	0.008303
GOTERM_CC_DIRECT	GO:0030670~phagocytic vesicle membrane	3	3.125	0.00858
GOTERM_BP_DIRECT	GO:0006096~glycolytic process	3	3.125	0.008715
GOTERM_CC_DIRECT	GO:0005654~nucleoplasm	17	17.70833	0.008962