UNIVERSIDADE FEDERAL DE SÃO CARLOS

CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA EVOLUTIVA E BIOLOGIA MOLECULAR

Carlos Eduardo Buss

Prospecção de Regiões Pleiotrópicas para Características Complexas Relevantes ao Melhoramento Bovino a partir de GWAS Bi-característica

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Genética Evolutiva e Biologia Molecular do Centro de Ciências Biológicas e da Saúde da Universidade Federal de São Carlos, como parte dos requisitos para obtenção do título de Doutor em Ciências com área de concentração em Genética Evolutiva.

Orientadora: Drª. Luciana Correia de Almeida Regitano
Co-Orientador: Drº. Gerson Barreto Mourão

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Centro de Ciências Biológicas e da Saúde
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Folha de Aprovação

Assinaturas dos membros da comissão examinadora que avaliou e aprovou a Defesa de Tese de Doutorado do candidato Carlos Eduardo Buss, realizada em 31/05/2019:

Profa. Dra. Luciana Correia de Almeida Regitano
EMBRAPA

p/ Prof. Dr. Gerson Barreto Mourão
USP

Prof. Dr. Marcos Roberto Chiaratti
UFSCar

Profa. Dra. Simone Cristina Méo Niciura
EMBRAPA

Profa. Dra. Priscila Silva Neubern de Oliveira
EMBRAPA

p/ Prof. Dr. Juliana Petrini
UNIFAL

DEDICATÓRIA

À minha amada Manu.

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RESUMO

A demanda por sistemas de produção de carne bovina sustentável direciona o desenvolvimento de novas ferramentas que visem reduzir os custos de produção e evitar perdas na qualidade da carne. O objetivo do presente estudo foi identificar possíveis interações entre qualidade de carne, conteúdo de Cálcio (Ca^{2+}) e eficiência alimentar, bem como investigar quais mecanismos biológicos estão subjacentes à modulação conjunta destes fenótipos em animais da raça Nelore. Para tanto, estimamos herdabilidade, correlações genéticas e GWAS Bi-característica. Nossos resultados indicaram que a seleção de animais eficientes (baixo CAR) não necessariamente deve gerar animais com menos deposição de gordura subcutânea, dada a ausência de correlação, podendo, entretanto aumentar a gordura intramuscular (GIM). Identificamos uma região localizada no cromossomo 20, próximos aos genes CD180 e MAST4, ambos relacionados à regulação do metabolismo lipídico. O GIM não apresentou correlação com outros depósitos de gordura em nossos dados, indicando que poderia ser selecionado separadamente dos demais para fins de melhoramento. Adicionalmente no segundo capítulo, analisamos a influência do Ca^{2+} na maciez da carne medida 14 dias após o abate (FC14), tendo estimado correlação negativa entre estes fenótipos, indicando que baixos índices de força de cisalhamento (mais macio) estão associados a altos índices de conteúdo de Ca^{2+}. Essas associações podem ser causadas pelo efeito das proteases dependentes de cálcio na maciez da carne. A partir de uma abordagem integrativa (*Transcriptome-wide association study* – TWAS), combinando GWAS bivariado e cis-eQTL, utilizando o método *Summary Mendelian Randomization* (SMR), identificamos dois novos genes candidatos (*DAP3* e *ALKBH3)*, reguladores da apoptose, essenciais para o processo de conversão de músculo em carne. Assim, as abordagens possibilitaram estimar a associação de fenótipos complexos relevantes ao melhoramento bovino, bem como indicar novos genes candidatos e vias metabólicas, decompondo parte da variância aditiva dessas características, contribuindo para o direcionamento de novas estratégias de seleção genômica.

ABSTRACT

The demand for sustainable beef production systems drives the development of new tools that aim to reduce the production costs and to avoid losses in meat quality. The objective of this study was to identify possible interactions among meat quality, calcium (Ca^{2+}) content and feed efficiency, as well as to investigate which biological mechanisms underlie Nelore's complex traits modulation. For this, we estimated heritability, genetic correlations, and bivariate Genome Wide Association Study (GWAS). Our results indicated that the efficient animal's selection (low RFI) may not necessarily generate animals with less subcutaneous fat deposition, but may increase intramuscular fat (IMF). We identified a region located on chromosome 20, close to the genes *CD180* and *MAST4*, both related to the regulation of lipid metabolism. IMF showed no correlation with other fat deposits in our data, indicating this trait could be selected independently from the others for breeding purposes. In the second chapter, we analyzed the influence of Ca^{2+} on meat tenderness measured at 14 days after slaughter (WSF14), and we estimated a negative correlation between these traits, which evidences a low shear force indices (tender beef) are associated with high Ca^{2+} content index. These associations may be the effect of calcium-dependent proteases on meat tenderness. From an integrative *Transcriptome-wide association study* (TWAS) approach, combining bivariate GWAS and cis-eQTL (SMR), we identified two candidate genes (*DAP3* and *ALKBH3*) regulators of apoptosis, essential for the muscle-to-meat conversion process. Thus, the approaches allowed to estimate the association of complex phenotypes relevant to bovine breeding, as well as to indicate new candidate genes and metabolic pathways, decomposing part of the additive variance of these traits, contributing toward new strategies on genomic selection.

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SUMMARY

CHAPTER I

1. INTRODUCTION

1.1. Cattle farming in Brazil and the world

Extensive beef cattle ranching[1] has relevance in the national economic scenario (Carvalho and Zen, 2017). According to the Brazilian Association of Meat Exporting Industries (ABIEC) in 2017, Brazilian cattle farming had about 221.81 million animals, representing 22% of the global herd and the largest commercial cattle herd in the world. The production from slaughtering reached 39.2 million heads, Brazilian consumers absorbed 79.1% of the production, and international markets, from more than 100 countries, bought the remaining 20.9% (ABIEC, 2018).

In the last three decades, cattle farming has leaped modernization sustained by technological advances in the entire production chain, mainly in the areas of food, genetics, management, and animal health (ABIEC, 2018). These investments resulted in increased birth rates, weight gain for animals, decreasing age at slaughter, and reduced mortality (Gomes et al., 2017).

In this period, the herd of cattle doubled in size. At the same time, the area of pasture remained without significant expansions, including some regions of the country reduced the spaces destined to cattle raising, to contribute to emphasize the gains in productivity (Gomes et al., 2017). In addition to productivity gains, another factor that gives Brazil a competitive advantage over the world scenario is the production cost of Brazilian cattle, which is one of the lowest in the world (ABIEC, 2014).

[1] Extensive beef cattle animals in Brazil use natural resources, especially on native pastures (BELLIDO *et al.*, 2001).

A most of success in productivity is due to the composition of the national herd of around 80% of Zebu breeds (Bos indicus), the majority belongs to Nellore composition, of proven adaptability in tropical environments (ABIEC, 2014). However, these animals, when slaughtered early, present a carcass with a more homogeneous distribution of subcutaneous fat and less intramuscular fat compared to Taurean animals (Luchiari Filho and Mourão, 2006).

The introduction of Zebu in Brazil had an essential role for the expansion and consolidation of the Brazilian herd, especially in the central region of the country. The genetic diversity of existing breeds allowed to select attributes of economic interest through crossbreeding, generating gains in rusticity, performance, and quality (Gomes et al., 2017).

These aspects conferred greater adaptability when compared to breeds of European origin (*Bos taurus*). Zebu breeds have higher heat tolerance, resistance to parasites, and fertility, resulting in higher productivity in tropical conditions. However, these animals present a carcass with a more homogeneous distribution of subcutaneous fat and less intramuscular fat compared to taurean animals (Luchiari Filho and Mourão, 2006).

The recognition of the cattle ranching importance in the national economy, and mainly due to the possibility of increasing gains in productivity and meat quality, motivated the development of studies on the genetic basis of complex traits relevant to animal breeding (Coutinho et al., 2010).

1.2. Genetic improvement strategies applied to Cattle farming

The animal breeding has been used successfully for decades for genetic selection for some traits, such as scrotal weight and diameter, using pedigree information to predict the genetic merit of each animal (Weller et al., 2017). However, most of this genetic progress in cattle generated didn't clarify about the influence of genes underlying genetic variation, the

number of genes involved and their broader properties, such as the magnitude of effects, frequencies, as well as possible epistatic interactions and pleiotropic (Hopkins et al., 2016).

Currently, the advances in sequencing techniques, one of the strategies adopted and widely used is the association between genomic regions and characteristics of economic interest in animal production. Loci mapping of quantitative traits (Quantitative Trait Loci, *QTLs*) offers an insight into the genetic architecture of quantitative traits (Coutinho et al., 2010).

The development of chips from thousands of SNPs (Single Nucleotide Polymorphisms) for taurines animals (*Bos taurus*) enabled the first studies of broad genome association (GWAS) in cattle (Utsonomiya et al., 2013).

These studies demonstrated several polymorphic SNPs for economic traits. Although, the same pattern was not repeated in Zebu animals (*Bos indicus*), mainly of the Nellore breed, due to the reduced variability for most of the SNPs represented on the chip (Utsonomiya et al., 2013). From the development of new genotyping products optimized for Zebu animals, the first univariate GWAS studies in Nelore were possible (Cesar et al., 2014; Santana et al., 2013; Tizioto et al., 2013; Utsonomiya et al., 2013; 2014).

Numerous significant associations with economic traits have been reported in Zebu animals, obtaining indications that several bovine chromosomes contain SNPs associated with meat quality (Tizioto et al., 2013, Cesar et al., 2014), mineral content (Tizioto et al., 2015).

1.3. Multivariate studies applied to bovine breeding

In general, the phenotypes of interest to animal breeding are complex traits formed throughout development by networks of multiple interactions between different factors, such as DNA, RNA, and proteins, as well as abiotic and biotic factors, which modulate the genotype-phenotype relationship. Theses elements determine the genetic architecture and the

connection of phenotypes, establishing the direction of phenotypic change in response to the selection of associated traits (Wolf, 2002).

These gene interactions attributed to the pleiotropic effects of genes, or the linkage disequilibrium, have relevance for genetic improvement by predicting the unintended selection action on other traits, which it will be indirectly selected due to the genetic correlations between phenotypes (Falconer and Mackay, 1996).

Positive genetic correlation can indicate a proportional increase between phenotypes directly related to productivity, generating productivity gains in both traits. This same principle can be applied to select attributes of meat quality, production, and feed efficiency simultaneously, according to the breeding program goals (Cabling et al., 2015).

On the other hand, the predictions of negative correlations between traits can evidence na inversely proportional relationship. A relevant insight to improvement which reveal the existence of an energy balance between production traits and basal metabolic functions, resulting in an energy restriction for production traits, or even an energy deduction of characteristics of interest. (Barendse et al., 2010, Guillaume and Otto, 2012, Welch et al., 2012).

Recently, multivariate GWAS approaches have been developed to study the influence of regions of the genome on correlated quantitative traits (Medina-Gomez et al., 2017), to predict the genetic variation underlying the correlations, indicating the magnitude of production traits associations (Welch et al., 2012). Multivariate methodologies offer advantages over univariate GWAS providing integrative biological vision in cases of pleiotropy (Galesloot et al., 2014).

GWAS studies have found thousands of genetic variants associated with traits of importance to animal breeding (Goddard et al., 2016). However, these variants individually correspond to a small fraction of the total heritability[2] associated with the target trait and

remain largely unknown (McCarthy and Hirschhorn, 2008). eQTL[3] studies can help to identify the influence that a variant may have on gene expression. In contrast, the extent to which this variant can modulate gene expression to influence complex traits remains a poorly understood topic of great interest in the genetics community (Veturi and Ritchie, 2018), especially in animal breeding (Cesar et al., 2018).

One way to address this issue is to conduct studies in which both gene expression and traits are available in the same set of individuals, combining characteristics from the GWAS and eQTLs to identify trait-gene relationships using the Transcriptome-Wide Association Study (TWAS) (Veturi and Ritchie, 2018). This a approach explore the relationship between a genetic variant and gene expression to identify new pleiotropic associations of gene characters, detecting the co-localization of expression signals in loci identified in GWAS, based on Summary-statistics Mendelian Randomization (SMR) (Zhu) et al. 2016).

The first studies on the integration of GWAS and eQTL identified new pleiotropic regions associated with complex human diseases, as well as corroborating the influence of genes previously reported in GWAS (Veturi and Ritchie, 2018, Zhu et al. 2016), which can be a potential tool for animal breeding purposes.

1.4. The importance of studies about feed efficiency and beef quality

The costs of feeding cattle can compromise about 70% of the total value of beef production. An alternative to improve the beef production chain is the selection of efficient animals, that is, with better performance in converting food ("input") into the beef ("output"), consuming fewer resources and resulting in savings in the final cost (Liu et al., 2000).

The genetic selection of feed efficiency has made significant progress in recent decades in breeding programs applied to poultry and pig farming, requiring greater exploitation in beef cattle.

There is a scarcity of studies due to the difficulty in measuring food consumption in extensive systems, culminating in the lack of scientific knowledge about the impact that the selection of efficient animals would cause on production traits (Arthur *et al.*, 2004).

In cattle, as well as in other mammals, changes in the destination of energetic substrates from food or tissue catabolism occur due to the action of physiological mechanisms related to the metabolic state, oxidative stress, and the number of nutrients available in the bloodstream. Unfavorable environmental stimuli, such as food scarcity or the presence of parasites, can trigger the activation of specific metabolic pathways that can selectively control the distribution and oxidation of energetic substrates in different tissues, based on the need and efficiency of tissue absorption (Beerda et al. , 2009).

The regulation of the energy balance in response to the food provided plays a critical role in feed efficiency and meat quality due to the complex nature of these characters (Herd and Arthur, 2009; Welch et al., 2012). Many mechanisms associated with meat quality, such as protein turn-over (Barendse et al., 2010) and patterns of fat deposition in different sources, can interfere with feed conversion (Guimarães et al., 2017).

Among the traits used to quantify food efficiency, Residual Feed Intake (RFI) is the most relevant measure, as it does not correlate with criteria such as carcass size and body growth rate. The variation in RFI represents the variability inherent in the basic metabolic processes that can determine the efficiency of converting energy from food into productivity (Archer et al., 1999b).

The consequences of associations between RFI and meat quality attributes can provide valuable information on the joint regulation of these traits. It is elucidates the biological mechanisms involved in energy balance and homeostasis, helping to adopt future genetic selection models that aim to reduce costs without losses in meat quality (Welch et al., 2012).

Using a bivariate approach with a panel of SNPs (Single Nucleotide Polymorphism), we perform Bivariate GWAS for beef quality and feed efficiency-related traits detailed in the second chapter. To do so, we decomposed the matrices of variances/covariance, estimated genetic correlations and heritabilities for the measured characters, as well as the effects of markers and estimation of haplotype blocks, to identify regions of QTLs that modulate biological functions common to both characteristics, as proposed by Zhou and Stephens (2012).

1.5. Impact of calcium concentration on beef tenderness

Beef is an excellent source of micronutrients essential to the human diet, but the consistency of the mineral content in beef is highly variable (Duan et al., 2009). Understanding the relationship between mineral concentrations and other traits in beef cattle can be advantageous for improving the nutritional value of beef as well as meat quality (Garmyn et al., 2011).

Among the organoleptic quality traits of beef, tenderness has a more significant influence on consumer satisfaction. This trait is mainly attributed to the proteolysis of myofibrils in the *post-mortem* (Alves and Mancio, 2006). The conversion of muscle to meat is a complex process in which the availability of calcium (Ca^{2+}) plays a central role in the activation of the proteolytic system of myofibrils in calpains and calpastatin (Hanna et al., 2008). Thus, the increase in the concentration of calcium in muscle and plasma is equivalent to the rise in the activity of proteases that cascade other proteolytic processes that promote tenderness (Zinn and Shen 1996).

Consequently, the variation in the Ca^{2+} content in beef can directly influence the standardization of tenderness in a herd (Tizioto et al., 2014). Given this perspective, genetic

selection strategies can be applied to improve the Ca^{2+} levels of the muscle (Tizioto et al., 2014).

Although the relationship between Ca^{2+} content and proteolytic proteins is well established, it is still necessary to have a greater understanding of the biological mechanisms that promote the uptake, the bioavailability of Ca^{2+} within the muscle cell. Besides the possible connections between the genotype, intermediate phenotype (expression), and final phenotype (tenderness) (Kemp et al., 2010).

In the third chapter, we explore this gap by investigating the biological mechanisms underlying the association between shear force measurements, taken after seven (WSF7) and after fourteen (WSF14) days of slaughter, and Ca^{2+} through the integration of information from eQTL and GWAS Bi -trait.

2. OBJECTIVES

2.1. MAIN OBJECTIVE

The present study aims to contribute to comprehension of network interactions between meat quality, mineral content, and feed efficiency, as well as to investigate which biological mechanisms are underlying the joint modulation of these phenotypes in Nellore animals.

2.2. SPECIFIC OBJECTIVES

2.2.1. Estimate SNP-based heritability for the twenty traits related to beef quality and feed efficiency.

2.2.2. Inferring genetic correlations among the phenotypes analyzed from bivariate analyzes.

2.2.3. Identify possible loci that explain part of the estimated genetic effect in correlation analyzes.

2.2.4. Estimate the haplotype blocks around significant SNPs in the analysis of GWAS Bi-trait.

2.2.5. Implement the annotation and analysis of functional enrichment of the QTL regions, to identify biological mechanisms underlying the modulation of traits relevant to bovine breeding.

2.2.6. Predict eQTL's mapping, detecting sites in the genome that control gene expression in cis.

2.2.7. Implement Summary Mendelian Randomization (SMR) and Heterogeneity in Dependent Instruments (HEIDI) methods to test the association between the level of gene expression, Ca content and softness, integrating data from eQTL and bi-trait GWAs.

REFERENCES

1. ABIEC – Associação Brasileira das Indústrias Exportadoras de Carnes. Perfil da Pecuária no Brasil. 2018. Disponível em: <http://abiec.siteoficial.ws/images/upload/sumario-pt-010217.pdf> Acesso em: 07/04/2019.

2. ABIEC – Associação Brasileira das Indústrias Exportadoras de Carnes. Rebanho Bovino de Corte. 2014. Disponível em:<http://www.abiec.com.br/img/newsletter/clipping-abiec-101213.pdf> Acesso em 19/05/2018.

3. Alves, D., Goes, R. H. de, & Mancio, A. (2006). MACIEZ DA CARNE BOVINA. *Ciência Animal Brasileira*, *6*(3), 135-149. Recuperado de https://www.revistas.ufg.br/vet/article/view/370

4. Archer, J.A. and S.A. Barwick. 1999. Economic analysis of net feed intake in industry breeding schemes. Proc Assoc Advmt Anim Breed Genet 13: 337–340.

5. Arthur, P.F.; Archer, J.A.; Herd, R.M. Feed intake and efficiency in beef cattle: overview of recent Australian research and challenges for the future. Australian Journal of Experimental Agriculture, v.44, p.361-369, 2004.

6. Barendse, W., Reverter, A., Bunch, R. J., Harrison, B. E., Barris, W., & Thomas, M. B. (2007). A validated whole-genome association study of efficient food conversion in cattle. *Genetics*, *176*(3), 1893–1905. https://doi.org/10.1534/genetics.107.072637

7. Beerda, B., W. Ouweltjes, L.B.J. ˇSebek, J.J. Windig, and R.F. Veerkamp. 2007. Effects of genotype by environment interactions on milk yield, energy balance, and protein balance. J Dairy Sci 90: 219–228.

8. Bellido, m, m.; Sánchez, m. E,; diaz, m. F. J.; Vega, l. A. R. De; Garcia, F. P. 2001. Sistemas extensivos de producción animal. Archivos de Zootecnia, v. 50, p. 466-489.

9. Cabling, M. M., Kang, H. S., Lopez, B. M., Jang, M., Kim, H. S., Nam, K. C., … Seo, K. S. (2015). Estimation of genetic associations between production and meat quality traits in Duroc pigs. Asian-Australasian Journal of Animal Sciences, 28, 1061–1065. https://doi.org/10.5713/ajas.14.0783

10. Carvalho T. B.; Zen S. De. A cadeia de Pecuária de Corte no Brasil: evolução e tendências. Revista iPecege 3 (1), 85-99.

11. Cesar, A. S. M., Regitano, L. C. A., Tullio, R. R., Lanna, D. P. D., Nassu, R. T., Mudadu M. A., Oliveira, P. S. N., Nascimento, M. L., Chaves, A. S., Maurício M Alencar, M. M., Sonstegard, T. 17 S., Garrick, D. J. G., Reecy, J. M., Coutinho, L. L. Genome-wide

association study for intramuscular fat deposition and composition in Nellore cattle. BMC Genetics 2014, v.15(1):39.

12. Coutinho, L. L., Jorge, E. C.; Rosário, M. F. do; Regitano, L. C. de A. 2010. A genômica na bovinocultura de corte. In: PIRES, A. V. (Ed.) Bovinocultura de corte. Piracicaba: Fealq. v.2 (1): 813-26.

13. Duan, Q., J. M. Reecy, R. Tait, J. P. Schoonmaker, D. C. Beitz, A. van Eenennam, R. Mateescu, and A. Garmyn. 2009. Phenotypic variation of mineral contents in beef. Fed. Am. Soc. Exp. Biol. J. 23:LB412.

14. Falconer, D.S.; Mackay, T.F.C. Introduction to quantitative genetics. 4.ed. Edinburgh : Longman Group Limited, 1996. 464p.

15. Goddard ME, Kemper KE, MacLeod IM, Chamberlain AJ, Hayes BJ. 2016 Genetics of complex traits: prediction of phenotype, identification of causal polymorphisms and genetic architecture. Proc. R. Soc. B 283: 20160569. http://dx.doi.org/10.1098/rspb.2016.0569

16. Galesloot TE, van Steen K, Kiemeney LALM, Janss LL, Vermeulen SH (2014) A Comparison of Multivariate Genome-Wide Association Methods. PLoS ONE 9(4): e95923. https://doi.org/10.1371/journal.pone.0095923

17. Garmyn AJ, Hilton GG, Mateescu RG, Morgan JB, Reecy JM, Tait RG, Beitz DC, Duan Q, Schoonmaker JP, Mayes MS, et al: Estimation of relationships between mineral concentration and fatty acid composition of longissimus muscle and beef palatability traits. J Anim Sci. 2011, 89 (9): 2849-2858. 10.2527/jas.2010-3497.

18. Gomes R.C., Feijó G.L.D. & Chiari L. 2017. Evolução e Qualidade da Pecuária Brasileira. Nota técnica Embrapa Gado de Corte.

19. Guimarães, André Lasmar, Mercadante, Maria Eugênia Zerlotti, Canesin, Roberta Carrilho, Branco, Renata Helena, Lima, Maria Lucia Pereira, & Cyrillo, Joslaine Noely dos Santos Gonçalves. (2017). Phenotypic association between feed efficiency and feeding behavior, growth and carcass traits in Senepol cattle. *Revista Brasileira de Zootecnia*, *46*(1), 47-55.

20. Hanna, R. A., Campbell, R. L., & Davies, P. L. (2008). Calcium-bound structure of calpain and its mechanism of inhibition by calpastatin. Nature, 456, 409–412.

21. Herd, R.M. and P.F. Arthur. 2009. Physiological basis for residual feed intake. J Anim Sci 87: E64–E71.

22. Kemp, C.M., Sensky, P.L., Bardsley, R.G., Buttery, P.J., and Parr, T. 2010. Tenderness—An enzymatic view. *Meat Sci.* 84:248–256.

23. Lee SI, Dudley AM, Drubin D, Silver PA, Krogan NJ, Pe'er D, Koller D (2009). "Learning a prior on regulatory potential from eQTL data". *PLoS Genetics*. **5** (1): e1000358. doi:10.1371/journal.pgen.1000358

24. Liu, M.F.; Goonewardene, L.A.; Bailey, D.R.C.; et al. A study in the variation of feed efficiency in station tested beef bulls. Canadian Journal of Animal Science, v.80, p.435-441, 2000.

25. Luchiari Filho A., Mourão G.B. 2006. Melhoramento, Raças e seus Cruzamentos na Pecuária de Corte Brasileira. Editora A. Luchiari Filho, São Paulo, v.1 (1):14.

26. McCarthy, M. I. & Hirschhorn, J. N. (2008). Genome-wide association studies: potential next steps on a genetic journey. *Hum. Mol. Genet.* **17** (R2). R156–R165.

27. Medina-Gomez C, et al. (2017) Bivariate genome-wide association meta-analysis of pediatric musculoskeletal traits reveals pleiotropic effects at the SREBF1/TOM1L2 locus. Nat Commun 8:121.

28. Oliveira, P. S. N., Cesar, A. S. M., do Nascimento, M. L., Chaves, A. S., Tizioto, P. C., Tullio, R. R., Regitano, L. C. A. (2014). Identification of genomic regions associated with feed efficiency in Nelore cattle. BMC Genetics, 15, 100. https://doi.org/10.1186/s12863-014-0100-0.

29. Page, B.T.; Casas, E.; Heaton, M.P.; Cullen, N.G.; Hyndman, D.L.; Morris, C.A.; Crawford, A.M.; Wheeler, T.L.; Koohmaraie, M.; Keele, J.W.; Smith, T.P.L. 2002. Evaluation of single-nucleotide polymorphisms in CAPN1 for association with meat tenderness in cattle. Journal of Animal Science, v.80 (12): 3077-3085.

30. Perotto, D.; Abrahão, J.J.S.; Cubas, A.C. Efeitos da raça e da heterozigose sobre características ponderais de bezerros Nelore e mestiços Red Angus x Nelore. Revista Brasileira de Zootecnia, Viçosa, v.28, n.3, p.504-511, 1999.

31. Przybylski, W., Hopkins, D. Meat Quality: Genetic and Environmental Factors, Chemical & Functional Properties of Food Components. Editora CRC Press, 2015. ISBN 1498766676, 9781498766678. Pág. 288.

32. Rosa, A. do N.; Variabilidade fenotípica e genética do peso adulto e da produtividade acumulada de matrizes em rebanhos de seleção da raça Nelore no Brasil. Ribeirão Preto:Departamento de Genética, FMRP-USP, 1999. Tese de Doutoramento.

33. Santana, M. H. A., Utsunomiya, T. Y., Haroldo HR., Neves H.H.R., Gomes R. C., Garcia, J. G., Fukumasu, H., Silva S. L., Oliveira, G. A. J., Alexandre1, P. A., Leme, P. R. Brassaloti, R. A., Coutinho, L.L., Lopes, T. G.,Meirelles F. V., Eler, P. J., Ferraz, J.B.S. 2013. Genome-wide association analysis of feed intake and residual feed intake in Nellore cattle. BMC Genetics, v.15 (1):21.

34. Tizioto PC, Taylor JF, Decker JE, Gromboni CF, Mudadu MA, Schnabel RD, Coutinho LL, Mourão GB, Oliveira PS, Souza MM. Detection of quantitative trait loci for mineral content of Nelore longissimus dorsi muscle. Genet Sel Evol. 2015;47(1):1.

35. Tizioto, P.C., Gromboni, C.F., de Araujo Nogueira, A.R., de Souza, M.M., de Alvarenga Mudadu, M., Tholon, P., Rosa, A.N., Tullio, R.R., Medeiros, S.R., and Nassu, R.T. 2014. Calcium and potassium content in beef: Influences on tenderness and associations with molecular markers in Nellore cattle. *Meat Sci.* 96:436–440.

36. Tizioto, P. C. ; Decker, J. E. ; Taylor, J. F. ; Schnabel, R. D. ; Mudadu, M. A. ; Silva, F. L. ; Mourao, G. B. ; Coutinho, L. L. ; Tholon, P. ; Sonstegard, T. ; Rosa, A.N. ; Alencar, M.M. ; Tullio, R.R. ; Medeiros, S.R. ; Nassu, R. T. ; Feijó, G.L.D. ; Silva, L.O.C. ; Torres, R.A.A. ; Siqueira, F. ; Higa, R. H. ; Regitano, L.C.A. 2013. A genome scan for meat quality traits in Nelore beef cattle. Physiological Genomics (Print), p. 1012-1020.

37. Toyama, B. H.; Hetzer, M. W. (2013). "Protein homeostasis: Live long, won't prosper". *Nature Reviews Molecular Cell Biology*. 14 (1): 55–61. doi:10.1038/nrm3496

38. Utsunomiya, Y.T.; Carmo, A.S.; Neves, H.H.de R.; Carvalheiro, R.; Mattos, M.C.; Zavarez, L.B.; Ito, P.K.R. K.; O'Brien, A.M.P.; Sölkner, J.; Porto-Neto, L.R.; Schenkel, F.S.; McEwan, J.; Cole, J.B.; da Silva, M.V.G.B.; Van Tassell, C.P.; Sonstegard, T.S.; Garcia, J.F. 2014. Genome-wide mapping of loci explaining variance in scrotal circumference in Nellore cattle. PLOS One, 9(2):88561.

39. Utsunomiya Y.T., Carmo A.S., Carvalheiro R., Neves H.H.R., Matos M.C., Zavarez L.B., Pérez O'Brien A.M., Sölkner J., McEwan J., Cole J.B., Van Tassell C.P., Schenkel F.S., Silva M.V.G.B., Porto Neto L.R., Sonstegard T.S., Garcia J.F. 2013a. Genome-wide association study for birth weight in Nellore cattle points to previously described orthologous genes affecting human and bovine height. BMC Genetics, v.14 (1): 52.

40. Veturi, Y., & Ritchie, M. D. How powerful are summary-based methods for identifying expression-trait associations under different genetic architectures? In:Pacific Symposium on Biocomputing, 23. Hawaii, USA (2018), pp. 228-239.

41. Welch, C. M., M. McGee, T. A. Kokta, and R. A. Hill. 2012. “Muscle and adipose tissue: Potencial roles in driving variantion in feed efficiency.” In *Feed efficiency in the beef industry*, edited by R. A.Hill, 175-198. Iowa: Wiley-Blackwell. Ames.

42. Weller J.I., Ezra E., Ron M. Invited review: A perspective on the future of genomic selection in dairy cattle (2017) *Journal of Dairy Science*, 100 (11) , pp. 8633-8644.

43. Wolf, J.B. (2002) The geometry of phenotypic evolution in developmental hyperspace. Proc Natl Acad Sci U S A 99(25): 15849-15851.

44. Zhu Z, Zhang F, Hu H, Bakshi A, Robinson MR, Powell JE, Montgomery GW, Goddard ME, Wray NR, Visscher PM & Yang J (2016) Integration of summary data from GWAS and eQTL studies predicts complex trait gene targets. *Nature Genetics*, 48:481-487.

45. Zinn, R.A., and Shen, Y. 1996. Interaction of dietary calcium and supplemental fat on digestive function and growth performance in feedlot steers. *J. Anim. Sci.* 74:2303–2309.

CHAPTER II

Bivariate GWAS reveals pleiotropic regions among feed efficiency and beef quality related-traits in Nelore cattle

Carlos Eduardo Buss[1]*, Gerson Barreto Mourão[2], Jason B. Wolf[4], Juliana Petrini[2], Juliana Afonso[1], Priscila N. de Oliveira[5], Polyana Cristine Tizioto[3], Aline S. M. Cesar[2], Gregori A. Rovadoski[2], Luiz Lehmann Coutinho[2], Luciana Correia de Almeida Regitano[5]

[1]*Department of Genetic and Evolution, Federal University of São Carlos, São Carlos, SP, BR. *bussxy@gmail.com,* [2]*Department of Animal Science, University of São Paulo/ESALQ, Av. Pádua Dias, Piracicaba, SP, BR,* [3]*NGS Soluções Genômicas.* [4]*Department of Biology & Biochemistry, University of Bath, Milner Centre for Evolution Bath, BA2 7AY, UK.* [5]*Embrapa Southeast Cattle, Rodovia Washington Luiz, Km 234, s/n, Fazenda Canchim, São Carlos, SP,BR.*

Abstract

Feed-efficient cattle selection is pointed out as one of the leading solutions for reducing the costs of beef production. However, it is still poorly applied in livestock due to the difficulty on measuring the phenotype at the industry level, as well as because there is little scientific knowledge about how feed efficiency can affect relevant traits, such as fatness and tenderness. Here we presented candidate biological mechanisms underlying the association between feed efficiency and meat quality. For experimental data obtained in half-sibling design, we analyzed the genetic correlations among traits and described thirteen pleiotropic Quantitative Trait Loci (QTL) explaining part of the phenotypic variations under a bi-trait GWAS model. Our results indicated that the efficient animal's selection (low RFI) may not necessarily generate animals with less subcutaneous fat deposition, but may increase intramuscular fat (IMF). We identified a region located on chromosome 20, close to *CD180* and *MAST4*, both related to the regulation of lipid metabolism. IMF showed no correlation with other fat deposits in our data, indicating that it could be selected separately from the others for breeding purposes. Our findings contributed to clarifying the joint modulation of complex phenotypes, indicating Quantitative Trait Loci (QTLs) for the bovine genetic improvement, directing new researches for the genomic selection.

Keywords: genome-wide, beef cattle, pleiotropy, feed intake, tenderness

1. Introduction

Beef quality and feed efficiency are highly-priority traits for the modern industry due to their impact on profitability (Hayes & Goddard, 2013; Liu *et al.*, 2000).

Selection for feed efficiency has made substantial progress in recent decades in breeding programs applied to poultry and pig farming, but beef cattle are still insufficiently explored (Arthur *et al.*, 2004). It occurs due to the difficulty in measuring food consumption in extensive systems, and the failure of scientific knowledge to establish the impact of efficient animal selection may cause on other traits associated with performance, such as reproductive rate, marbling, carcass finishing and meat tenderness (Welch *et al.*, 2012).

In general, these complex traits are produced from interactions among several factors such as DNA, RNA, and proteins, as well as abiotic and biotic factors, which modulate the genotype-phenotype relationships, establishing the direction of phenotypic change and response to the selection of associated traits (Wolf, 2002). Therefore, estimating these genetic correlations may reveal associations between phenotypes attributed to the pleiotropic effects of genes or linkage disequilibrium (Falconer & Mackay, 1996).

The genetic variation underlying the correlations between traits allow predicting the consequences of selection on other characteristics that will be indirectly selected (Guillaume & Otto 2012). Negative correlations described between feed efficiency and meat quality traits may reveal the existence of an energy balance between production traits and basal metabolic functions, resulting in an energy restriction for these traits (Welch *et al.*, 2012).

In mammals, alterations in the allocation of energetic substrates from food or tissue catabolism occur due to the action of physiological mechanisms related to the metabolic state, oxidative stress and the amount of nutrients available in the bloodstream (Herd & Arthur, 2009). Unfavorable environmental stimuli such as resource shortages or parasites loads could trigger the activation of specific metabolic pathways, which selectively control the

distribution and oxidation of energetic substrates in different tissues (Beerda *et al.,* 2007). The refining of an energy balance in response to the nutrients plays a critical role in feed efficiency and meat quality (Herd & Arthur, 2009; Barendse *et al.,* 2007).

In the present study, we performed a bivariate genome-wide association analysis (GWAS) for meat quality and feed efficiency related traits. For this, we decomposed the variance/covariance matrices, estimated genetic correlations and heritabilities, as well as the effects of the markers, to identify regions of QTLs associated with biological functions that modulate both traits.

Our findings can provide insights into the role of regulatory genes and biological processes relevant to animal breeding, which may improve the efficiency of genomic selection to increase beef industry profit.

2. Results

The phenotypic variances explained (PVE) by genotypes (genomic heritability) were estimated using Bayesian sparse linear mixed model (BSLMM) with Monte Carlo Markov Chain (MCMC) under a mixed animal model (Supplementary Table 1).

The relative growth rate (RGR; %/d), sum of the omega 3 (N3; mg), saturated fatty acid content (SFA; mg) and water holding capacity after seven days (WHC7; %) had high heritability (greater than 0.40). While most of the traits, dry matter intake (DMI; kg/d), average daily gain (ADG; kg/d), feed conversion (FC; feed intake (kg)/ weight gain (kg)), maintenance efficiency (ME; kg/kg), efficiency of gain (GE; kg/Mcal), feed efficiency (FE; average weight gain (kg)/average feed intake (kg)), total digestible nutrient (TDN; kg/d), rib eye muscle area (REA/cm^2), backfat thickness (BTF; mm), myofibrillar fragmentation index (MFI), Warner–Bratzler shear force after seven days (WSF7; maximum shear force - kg), pH after seven days (PH7; pH), intramuscular fat (IMF; mg)

and omega 6 content (N6; mg) had moderate heritability (from 0.20 to 0.40). However, live weight gain (LWG; kg/d) and residual feed intake (RFI; kg/d), showed low estimates of heritability (0.17 and 0.15, respectively) (Figure 1A). The standard errors (SE), genetic variances (Vg) and environmental variances (Ve) are available in Supplementary Table 1.

We applied a linear mixed model combining the fixed effects, random effects, and experimental errors to estimate the variance/covariance matrix, SNP-based genetic correlations, and bi-trait GWAS. Also, for the significant bi-trait GWAS, we fitted single-trait GWAS in the same dataset to compare the statistical significance with the bi-trait approach. Overall, bi-trait analyzes with significant tag SNPs showed high or moderate correlations between traits, as shown in Figure 1B (green squares). The positive correlations ranged from 0.30 to 0.87, and the negative correlation from -0.40 to -0.80. FC-PH7 was an exception that showed a low negative correlation ($r=-0.12 \pm 0.4509$) (Table 1).

Table 1. SNP-based genetic correlations and respective standard errors.

Bivariate analyses*	Genetic correlations	Standard errors (SE)
IMF-REA	-0.75	0.1097
WSF7-REA	-0.69	0.2554
IMF-RFI	-0.80	0.0004
RFI-ME	0.28	0.0005
IMF-WHC7	-0.41	0.2975
IMF-ME	-0.55	0.0401
PH7-TDN	-0.75	0.3495
WSF7-TDN	-0.76	0.0047
IMF-ADG	0.30	0.0686
IMF-MFI	-0.40	0.1325
ADG-FC	-0.59	0.0500
WSF7-GE	0.56	0.0009
PH7-GE	-0.66	0.0599
N3-RGR	0.87	0.0008
FC-PH7	-0.12	0.4509

* intramuscular fat (IMF; mg), rib eye muscle area (REA;cm^2), Warner–Bratzler Shear Force after seven days (WSF7; kg), residual feed intake (RFI; kg/d), maintenance efficiency (ME; kg/kg), water holding capacity after seven days (WHC7; %), pH after seven days (PH7; pH), total digestive Nutrients (TDN; Kg/Kg), average daily gain (ADG; kg/d), efficiency of gain (GE; kg/Mcal), sum of the omega 3 (N-3; mg), Relative Growth Rate (RGR; %/d), feed conversion (FC; kg/kg).

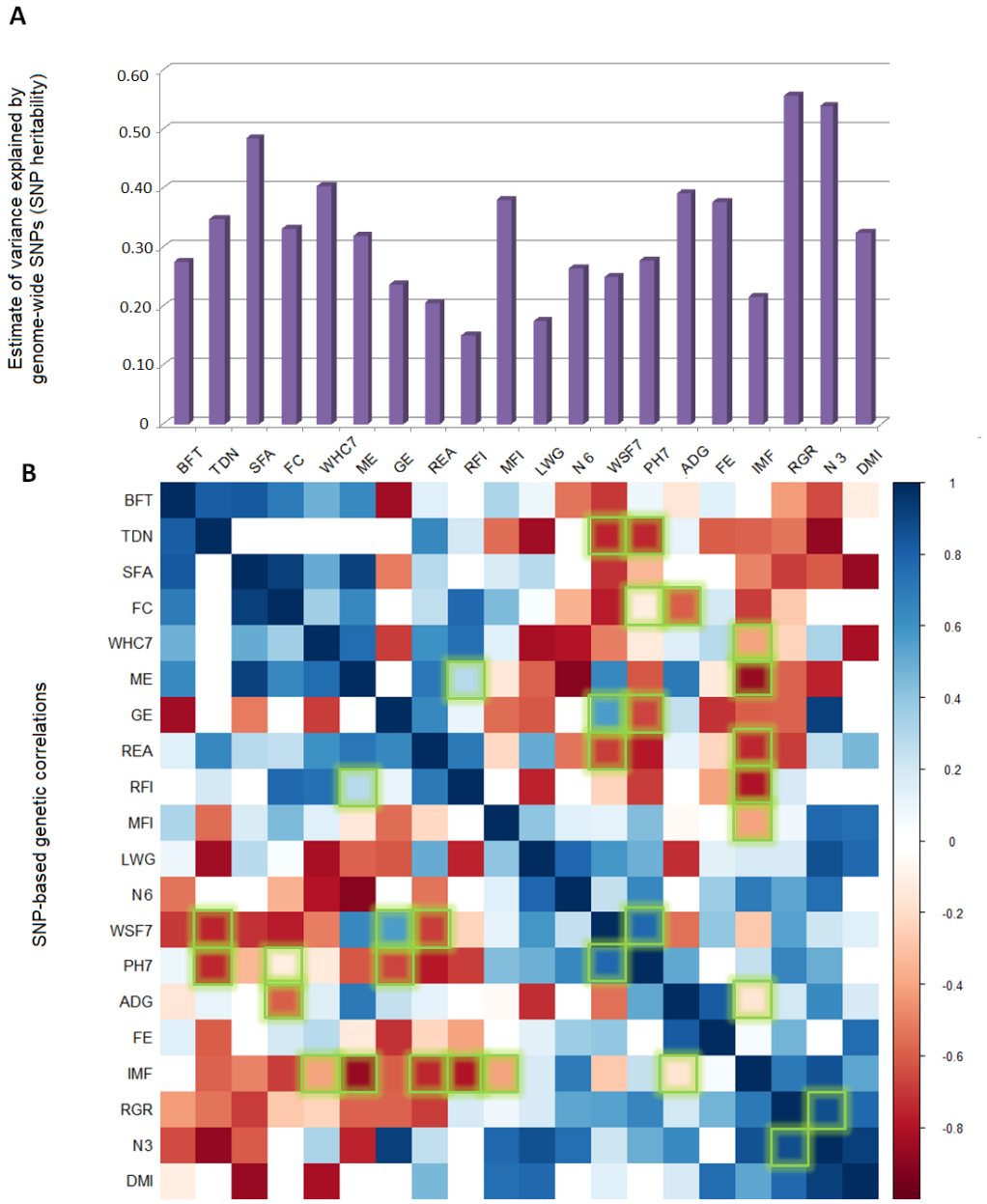

Figure 1.A. Barplot showing the estimates of phenotypic variances explained (PVE) by genotypes (chip heritability): backfat thickness (BTF; mm), total digestive Nutrients (TDN; Kg/Kg), saturated fatty acid content (SFA; mg), feed conversion (FC; kg/kg), water holding capacity after seven days (WHC7; %), maintenance efficiency (ME; kg/kg), efficiency of gain (GE; kg/Mcal), rib eye muscle area (REA;cm^2), residual feed intake (RFI; kg/d), myofibrillar fragmentation index (MFI), dry matter intake (DMI; kg/d), omega 6 content (N-6; mg), Warner–Bratzler Shear Force after seven days (WSF7; kg), pH after seven days (PH7; pH), average daily gain (ADG; kg/d), feed efficiency (FE; kg/kg), intramuscular fat (IMF; mg), Relative Growth Rate (RGR; %/d), sum of the omega 3 (N-3; mg) and live weight gain (LWG; kg/d). **B.** Heatmap of the SNP-based genetic correlations between twenty traits analysed. The sidebar indicates the correlation values of the colour spectrum. The green squares show significant tag SNPs identified in Bi-trait GWAS.

The Manhattan and Q–Q plots (Figure 2) show fifteen overlapping bivariate analyses of which significant tag SNPs were below the Bonferroni threshold (adjusted p-value $1.00x10^{-6}$).

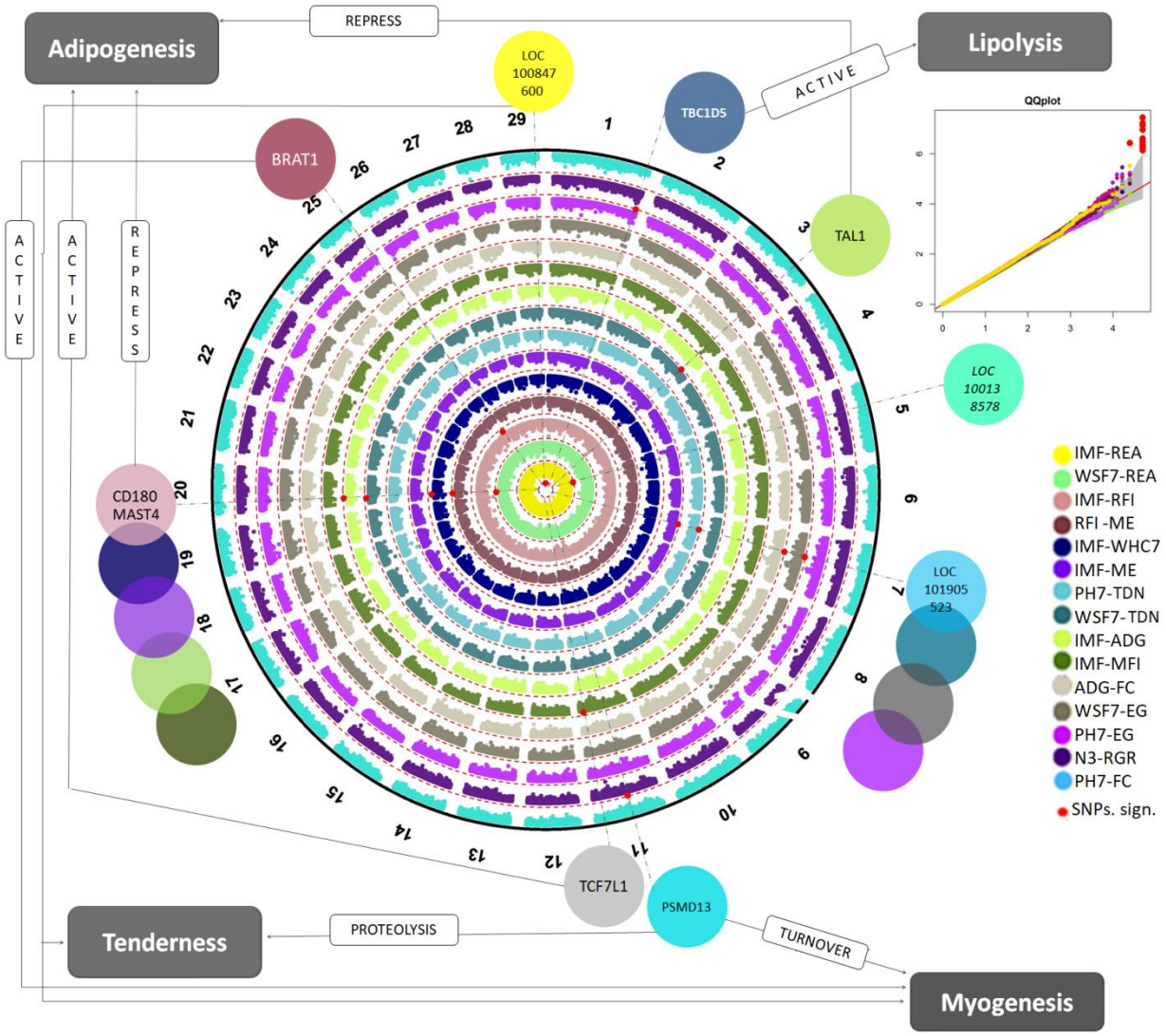

Figure 2. Manhattan plot and Q-Q plot for Bi-trait GWAS results for all autosomes. Each circle superimposed corresponds to one Manhattan plot with significant tag SNPs (red dots); colors indicate the respective bivariate analysis. The smaller circles correspond to genes and/or regulatory regions nearby the variant. The gray squares in the corners show the biological functions of the genes and/or regulatory regions nearby the variant. In the upper right corner, there is the Q-Q plot of the same analyses.

We identified one marker (rs41621597; p-value < $8.39x10^{-7}$) on an intronic region of the TBC1 Domain Family Member 5 (*TBC1D5*) in a 10.10 kb LD block on BTA1, associated with N3 and RGR, which explained 0.46% of the additive variance effect for N3 and 3.78% for RGR (Table 1). Functional enrichment analyzes show that this region is related to the endosomal transport process (GO:1990126) (Table 3).

Among the phenotypes analyzed, the IMF stood out for showing genomic regions associated with six other traits (Table 2). Two tag SNPs showed associations with IMF-ADG. The tag SNP rs133408775 (P-value < $3.73x10^{-7}$), located in an intronic region of the TAL bHLH Transcription Factor 1, Erythroid Differentiation Factor 1 (*TAL1*) gene on BTA3, which belongs to 30.79 kb LD block, explaining 5.53% of the variance observed for IMF and 0.03% for ADG. The haplotype that surrounds tag SNP rs133408775, containing only *TAL1* as annotated gene, is involved in biosynthesis processes (GO: 0009058), Cellular processes (GO: 0009987) and in nitrogen metabolism (GO: 0006807) (Table 3).

The tag SNP rs42243764 (p-value < $3.73x10^{-7}$) located in an intronic region of the microtube-associated serine/threonine kinase (*MAST4*) gene on BTA20, explained 5.47% and 0.52% of the observed variance for IMF and ADG, respectively (Table 2).

In the same region (12-13Mb on BTA20), we also identified tag SNPs associated with IMF and two feed efficiency related traits (RFI; ME), IMF and meat quality-related traits (MFI; WHC7). The highest tag SNP effect was 4.95% of the additive variance of IMF for rs42242748, associated with IMF-MFI (p-value < $6.26x10^{-07}$), located at the intronic region of the MAST4 (Figure 1). This region is composed of small LD blocks that contain genes related mainly with cell growth (GO: 0007010) and organization of the cytoskeleton (GO: 0007010) (Table 3).

Table 2. Regions associated with meat quality and feed efficiency-related traits identified by bivariate GWAS analyzes.

SNP	Chr	Trait[A]		AF[B]	β[C]		Add[D]		Bi-trait *p-value*	Single-trait *p-value*[E]		Candidate Gene/ Location[F] (Kb)
		T1[G]	T2[H]		T1	T2	T1	T2		T1	T2	
rs41621597	1	N3	RGR	0.460(T/C)	-0.01	2.52	0.46%	3.78%	8.63E-07	1.95E-01	2.11E-06	*TBC1D5* INTRON1
rs133408775	3	ADG	IMF	0.211(G/A)	0.03	2.38	0.03%	5.35%	3.73E-07	6.89E-01	2.81E-06	*TAL1* INTRON 2
rs136439872	5	REA	WSF7	0.491(T/C)	-0.45	0.02	2.34%	3.04%	7.24E-07	3.08E-06	5.96E-04	*LOC100138578, INTRON 1*
rs134052644	7	WSF7	TDN	0.114(C/T)	-0.04	0.04	3.32%	4.49%	4.39E-07	2.14E-01	3.42E-03	*LOC100850697*, U 111.592kb
		WSF7	GE	0.114(C/T)	-0.04	0.08	3.52%	4.79%	2.92E-07	2.14E-01	3.39E-08*	
		PH7	TDN	0.114(C/T)	-2.27	0.05	1.55%	5.30%	9.25E-07	1.48E-03	3.42E-03	
		PH7	GE	0.114(C/T)	-2.38	0.09	1.69%	5.58%	5.28E-07	1.48E-03	3.39E-08*	
rs29021673	11	PH7	FC	0.127(C/T)	0.42	-0.04	2.94%	2.90%	1.09E-07	1.75E-04	9.72E-05	*PSMD3* U UTR
rs134260782	11	ADG	FC	0.084(T/C)	2.9	0.18	5.52%	0.25%	5.34E-07	9.83E-07*	4.86E-01	*TCF7L1* INTRON 1
rs109492201	20	RFI	IMF	0.075(G/A)	-0.02	3.4	2.19%	4.67%	4.67E-07	7.79E-04	9.83E-07*	*CD180* U 173.66
rs42243802	20	WHC7	IMF	0.075(A/G)	-0.10	3.4	1.46%	4.93%	4.94E-07	6.89E-03	9.83E-07*	*MAST4* INTRON 3
rs109657177	20	IMF	ME	0.074(T/C)	3.53	-0.02	5.17%	3.08%	3.57E-08	9.83E-07*	6.20E-05	*MAST4* INTRON 5
rs42243764	20	ADG	IMF	0.078(A/G)	0.16	3.66	0.52%	5.47%	3.37E-07	6.89E-01	9.83E-07*	*MAST4* INTRON 2
rs42242748	20	IMF	MFI	0.074(T/C)	3.41	-0.06	4.95%	2.45%	6.26E-07	3.02E-01	9.83E-07*	*MAST4* INTRON 2
rs109847518	25	RFI	ME	0.066(T/C)	0.02	-0.02	2.11%	1.17%	7.20E-08	1.17E-03	7.05E-03	*BRAT1* INTRON 4
rs132812787	29	REA	IMF	0.442(G/A)	-0.60	1.04	4.16%	1.54%	6.95E-07	3.08E-06	1.36E-01	*LOC100847600* INTRON 1

[A] Phenotypes analized in bi-trait GWAS.[B] Allele frequency of the minor allele (second SNP).[C] Effect of allele substitution of the trait1 and trait2,respectively.[D]Proportion of the additive variance explained by SNP of the trait1 and trait2, respectively. [E] Univariate p-values of the trait1 and trait2, respectively.[F] Candidate or nearest gene and respective location. U correspond that SNP is upstream of a gene and D downstream. [G]Trait 1.[H]Trait 2. [*] Univariates p-values for log likelihood test statistic (LRT) for significant SNP less than Bonferroni threshold (1.00×10^{-6}) bolded.

The SNP rs134052644, in a 73.41 kb LD block on BTA7, corresponding to an intergenic region 111,592 kb upstream of *LOC100850697*, showed association in four bi-trait analyses: WBSF7-DMDN (p-value <$4.39x10^{-07}$), WBSF7-GE (p-value <$2.92x10^{-07}$), PH7-TDN (p-value <$9.25x10^{-07}$), and PH7-GE (p-value <$5.28x10^{-07}$) (Table 2).

In BTA11, we identified two significant markers. First, rs29021673 (p-value <$1.09x10^{-07}$) associated with PH7-FC, explained 2.94% of the additive variance effect for PH7 and 2.90% for FC. This SNP is in a 128.51kb block, surrounding the *PSMD3* (*proteasome subunit 26S, not ATPase3*), *LOC789596*, *ODF3* (*Outer Dense Fiber Of Sperm Tails 3*) , *RIC8A* (*RIC8 Guanine Nucleotide Exchange Factor A*), *SCGB1C1* (*Secretoglobin Family 1C Member 1*), *SIRT3* (*Sirtuin 3*), *NLRP6* (*NLR Family Pyrin Domain Containing 6*), *BET1L* (*Bet1 Golgi Vesicular Membrane Trafficking Protein-Like*), *LOC107131188*, *LOC511919* , *COX8B* (*Cytochrome C Oxidase Subunit 8B*), *IFITM5* (*Interferon Induced Transmembrane Protein 5*), *IFITM2* (*Interferon Induced Transmembrane Protein 5*). The functional enrichment analyzes showed that this region is related to the catabolic process (GO:0009056), proteolysis (GO:0006508) and structuring of complex proteins (GO:0006461). Second, rs134260782 (p-value < $5.34x10^{-07}$), located in a 41.59 kb block on *the transcription factor 7 like 1* (*TCF7L1*) associated with transcription regulation processes (GO:0006357, GO:0006366; GO:0006351) (Table 3), was significantly associated with ADG-FC, which explained 5.52% of the additive variance effect for ADG and 0.25% for FC.

The marker rs109847518, located in a 37.35 kb block on BTA25 associated and (p-value < $7.20x10^{-08}$) with RFI-ME, explained 2.11% of the additive variance effect for RFI and 1.17% for ME (Table 2). This LD region surrounded *BRCA1 Associated ATM Activator 1* (*BRAT1*), *IQ Motif Containing E* (*IQCE*) and *Archaelysin Family Metallopeptidase 1* (*AMZ1*) and showed the enrichment term for cytosines synthesis process (GO:0001816) (Table 3).

The tag SNP rs132812787, located on 81.34 kb block, surrounding LOC100847600, *INCENP* (*Inner Centromere Protein*), *LOC107131970*, and *LOC107131971* at BTA29, showed association with IMF-REA, reaching a p-value < $6.95x10^{-07}$ and explaining 1.54% of the additive variance for IMF and 4.16% for REA (Table 2). This gene encodes a ncRNA of yet unknown function, probably associated with expression modulation. However, the QTL region where it is located has relevante for tenderness and fat content in other bovine populations, as shown in the QTLdb annotation (Table 3).

Table 3. QTLs annotation and enrichment analysis from gene ontology terms.

Chr	Tag SNP[a]	Location Tag SNPs[B]	Block[C]	Annotate Genes[D]	GO Terms [E]	Biological Process	Bi-trait[H]	Bovine QTLs[G]
1	rs41621597	*TBC1D5* INTRON 1	10.10kb	*TBC1D5*	GO:1990126	Endosomal transport	RGR	Daily Weight Gain Maintenance Efficiency
3	rs133408775	*TAL1* INTRON 2	30.79kb	*TAL1*	GO:0009058 GO:0009987 GO:0006807	Biosynthesis process Cellular process Nitrogen metabolic compound	ADG-IMF	*
5	rs136439872	*LOC100138578* INTRON 1	17.61kb	*LOC100138578*	*	*	REA-WSF7	*
7	rs134052644	*LOC101905523* U 171.661kb	73.41kb	*LOC101905523*	*	*	WSF7-TDN WSF7-EG PH7 -TDN PH7-EG	Cold Tolerance (temperature regulation)
11	rs29021673	*PSMD3* Região UTR	128.51kb	*PSMD13, LOC789596, ODF3, RIC8A, SCGB1C1,SIRT3, NLRP6, BET1L, LOC107131188, LOC511919, COX8B, IFITM5, IFITM2*	GO:0009056 GO:0006508 GO:0006461	Catabolic process Proteolysis Structuring of complex Proteins	PH7-FC	[F]*Net Merit*
11	rs134260782	*TCF7L1* INTRON 1	41.59 kb	*TCF7L1*	GO:0006357 GO:0006366 GO:0006351	Transcription regulation via RNA Promoter Polymerase II Transcription via RNA Polymerase II Transcription, DNA-dependent	ADG-FC	Potassium content in milk
20	rs42242748	MAST4	5.45 kb				MFI-IMF WHC-IMF	

		INTRON 3						
20	rs42243802	MAST4 INTRON 3	1.01 kb	*MAST4*	GO:0007010 GO:0006996 GO:0071840	Cell growth Development of the nervous system Tissue development	ME-IMF ADG-IMF	Percentage of fat in milk
20	rs109657177	MAST4 INTRON 1	19.99 kb					
20	rs42243764	MAST4 INTRON 3	5.30 kb					
20	rs109492201	CD180 U 173.66	0.93 kb	*CD180*	GO:0007010 GO:0006996 GO:0071840	Organization of the cytoskeleton Organization of organelles Biosynthesis of cellularComponents	IMF-RFI	
25	rs109847518	*BRAT1* INTRON 4	37.35 kb	*IQCE,AMZ1* *BRAT1*	GO:0001816	Cytosines synthesis	RFI-ME	*
29	rs132812787	*LOC100847600* INTRON 1	81.34 kb	*LOC100847600* *INCENP* *LOC107131970* *LOC107131971*	GO:0007067 GO:0051301 GO:0000280	Mitotic nuclear division Cell division Nuclear division	REA-IMF	Shear force Oleic acid content Stearic acid content

* Missing or non-significant data (FDR <0.05). [A] Significant markers in the GWAS analyzes. [B] Location of the associated markers in the GWAS analyzes for the nearest gene. [C] Size of haplotype blocks around significant SNP tags in kilobases (kb). [D] Annotated genes. [E] Genes annotated up to a maximum of 10 kb around estimated blocks. [F] Net Merit is a genetic index that simplifies the selection process of bulls based on their genetic merit of a combination of economically important characteristics such as milk, fat, protein, health and physical fitness. [G] Annotation of overlapping QTLs identified in other bovine studies (QTLdb). [H] Traits associated with the respective region.

3. Discussion

In designing beef cattle genetic improvement, a primary challenge is to consider the overall sustainability of the production system, which needs to balance the attributes of feed efficiency, growth and meat quality (Ahola & Hill, 2012).

In the present paper, we described the pleiotropic effects of genomic regions over feed efficiency and beef quality related traits. Although the results are relative to a small Nelore sample, they shed light on the biological mechanisms that might be involved in phenotypic correlations between these traits.

Residual feed Intake (RFI) is considered the most relevant measure of feed efficiency because it is independent of the size and rate of body growth. RFI variation represents the variability of essential metabolic processes that can determine the production efficiency (Liu *et al.,* 2000). Our bi-trait GWAS results corroborate that RFI is independent of average daily weight gain (ADG) and relative growth rate (RGR) (Supplementary Table 2), indicating that the selection of animals with low RFI indexes (more efficient) would not exhibit consequences for body growth.

The estimate of positive genetic correlation (0.28 ± 0.0005) between RFI and Maintenance Efficiency (ME) indicates that these traits vary proportionally (Figure 1B). The bi-trait GWAS analysis revealed an association between RFI-ME and a LD block spanning *BRAT1*, *IQCE,* and *AMZ1* genes (Table 3).

BRAT1 is a candidate to minimize oxidative stress and promote muscle growth (So & Ouchi, 2013; 2014). The transient inactivation of *BRAT1* increases the reactive oxygen species (ROS), generating mitochondrial malfunction, consequently decreasing ATP synthesis (So & Ouchi, 2014). The oxidative stress has been implicated in determining variation at RFI in this population (De Oliveira *et al.,* 2014 and Tizioto *et al.,* 2016). Besides, *BRAT1* promotes the signaling of rapamycin (*mTOR*), which in turn fosters hypertrophy (So & Ouchi,

2014). These pathways regulate protein synthesis, resulting in the formation of a higher number of myofibrils, leading to hyperplasia due to increased myocyte protein volume (So & Ouchi, 2014).

In the comparison between feed efficiency and fat deposition, we observed that RFI is independent of subcutaneous fat and fatty acid content. However it showed a negative correlation (-0.80 ± 0.0004) with IMF, indicating selection applied for a low RFI (efficient animals) could be accompanied by an increase in IMF, the later showing an independent distribution from other measures related to body fat deposition. Thus, as found in previous studies (Herd & Arthur, 2009, Exton *et al.,* 2010, Moraes *et al.,* 2017), our results reaffirm the need to understand the metabolic differences between intramuscular fat and other fat deposits (Hocquette *et al.,* 2010).

In addition to the association between IMF-RFI, we identified a region located on chromosome 20, close to genes *CD180* and *MAST4*, which also was associated with IMF-ADG, IMF-MFI, IMF-WHC7, and IMF-ME. The *CD180* gene belongs to complex with Lymphocyte antigen 86 (*LY86*), modulating the innate immune response of bovines (Casas *et al.,* (2011). *CD180/LY86* knockdown enhances chronic inflammation of adipose tissue induced by the food supply, suggesting this complex promotes a crucial regulator of obesity and insulin resistance in humans (Söhle *et al.,* 2012). Similarly, transient inactivation of *MAST4* increases fat accumulation (Nagai *et al.,* 2013).

When we analyzed IMF-ADG, we observed a low positive genetic correlation (0.30 ± 0.0686), indicating that the traits will respond in the same direction (Santana et al., 2012). Associated with these traits, we found an intronic region of TAL1 (*rs133408775*), an important transcription factor of adipogenesis in cattle. *TAL1* is responsible for modulating the co-expression of genes associated with feed efficiency in Angus cattle, such as *zinc finger E-box binding homeobox 2* (*ZEB2*), *Myogenic Differentiation 1* (*MYOD1*), *Zinc finger X-*

chromosomal protein (*ZFX*), *Iroquois homeobox 3* (*IRX3*), *zinc finger and SCAN domain containing 21* (*ZSCAN21*) and *zinc finger protein 35* (*ZNF35*) (Weber *et al.,* 2012).

In the bi-trait GWAS analysis for ADG-FC, we identified a marker that explained 5.52% of the additive variance for ADG and 0.25% for FC, located in the intronic region of the transcription factor *TCF7L1* on the chromosome 11. This gene acts on the activation of adipogenesis from the fibroblasts confluency, a mechanism necessary to induce differentiation in adipocytes, mediating changes in the structural proteins that regulate cell differentiation. The process induces the activation of Peroxisome Proliferator-Activated Receptor Gamma (*PPARG*), a gene considered the "central regulator of adipogenesis," essential for both pre-adipocyte determination and terminal differentiation (Cristancho *et al.,* 2011). Studies with this same Nelore cattle population indicated that the regulation of lipid metabolism via *PPARG* is targeted by microRNAs associated with intramuscular fat (Oliveira *et al.,* 2018) and feed efficiency (De Oliveira *et al.,* 2018).

In the comparison between N3 and RGR, we found a high positive correlation (0.87 ± 0.0008), suggesting that these traits would respond proportionally under artificial selection. We also identified an intronic region in *TBC1D5* that explained 0.46% of the additive variance for N3 and 3.78% for RGR. Recent findings have shown that this gene regulates lysosomal-mediated lipid degradation in adipocytes (Jia *et al.,* 2016, Lizaso *et al.,* 2013).

Several studies have associated N3 to lipolysis process in cattle (Elis *et al.,* 2016). The recent elucidation of the mechanisms of action of *TBC1D5* corroborates the relationship between N3 and mechanisms of autophagic lipolysis (Jia *et al.,* 2016, Lizaso *et al.,* 2013).

TBC1D5 also acts on the autophagic glucose uptake machinery of cells in situations of oxidative stress when there is an increase in energy demand (Roy *et al.,* 2017). During livestock production, animals are exposed to external factors, such as climatic variations, parasite exposure, and management stress. These agents can promote oxidative stress by

mobilizing an additional portion of the energy initially allocated for growth and carcass finishing under optimum conditions. Consequently, physiological responses to stress increase the metabolic rate and energy consumption, promoting tissue catabolism (Russel *et al.,* 2016). The pathways that act to balance oxidative stress are relevant for maintaining the relative growth rate at acceptable levels and should be better studied in breeding programs (Welch *et al.,* 2012).

Both muscle growth and meat tenderness are dependent on the control of protein degradation (turnover) through enzymatic mechanisms related to cathepsins, calpain, caspases, and the ubiquitin-proteasome system (Bilodeau *et al.,* 2016). These processes represent a high energy cost, which can lead to variations in feed efficiency (Richardson *et al.,* 2004).

Previous univariate GWAS with the same population studied have reported the relevance of proteolytic systems in modulating economically important traits. Tizioto *et al.* (2013) identified the association between meat tenderness and QTLs of small effect spanning the *calpain* and *calpastatin* genes, while, De Oliveira *et al.* (2014) found associations of ubiquitin-*specific peptidase 21* (*USP21*) and *ubiquitin-fold modifier conjugating enzyme 1* (*UFC1*) genes, proteolytic systems which control the energy balance, with feed efficiency traits.

Our results indicated an association between a gene *Proteasome 26S subunit, non-ATPase, 13* (*PSMD13*) (ubiquitin-proteasome system) and PH7-FC. This region explained 2.94% of the additive variance of PH7 and 2.90% of FC, evidencing a protein turnover by the ubiquitin-proteasome system associated with FC, corroborating Barendse *et al.,* (2007) in a study with different bovine breeds. The ubiquitin-proteasome system plays an essential role in *postmortem* proteolytic activity, being substantially active up to seven days after death (at pH below 6) (Lamare *et al.,* 2002). Although there is evidence about QTL regions jointly

modulating the PH7-FC, the correlation between these phenotypes was low and associated with a high standard error (0.12 ± 0.4509) in the present study.

We found WSF7 and Residual Feed Intake (RFI) as independent (Supplementary Table 2), indicating that the selection of efficient animals does not necessarily lead to correlated response with tenderness related traits. Corroborating this information, Gomes *et al.* (2012) did not observe differences in meat quality parameters between divergent RFI Nelore.

Regarding beef quality traits, the estimates of the correlation between WSF7 and REA showed negative value, indicating that their artificial selection responses would be opposite (-0.69 ± 0.2554). This antagonism would be favorable for improvement since it allows for jointly selecting low WSF7 (softer) and larger loin eye area. Part of the additive variance of these traits (2.34% for AOL and 3.04% for FC7) was explained by the association with the SNP rs136439872, located in an intronic region of *LOC100138578* (unknown function).

Another region close to *LOC100850697* was associated with WSF7-TDN, WSF7-GE, PH7-TDN, and PH7-GE. Although this putative gene has an unknown function, it overlaps a QTL region related to the regulation of body temperature on chromosome 7 (Table 3).

On the other hand, IMF-REA presented a moderate negative correlation (-0.75 ± 0.1097), indicating an inversely proportional response between muscle allometry and intramuscular fat deposition. Both traits are accessed through non-invasive ultra-sound evaluation, but while IMF can only be precisely measured under high energy diets, REA can measured in grazing animals. In this condition, selection for REA, may induce an unintentional selection. Also, we located on block formed by *LOC100847600, INCENP*, *LOC107131970,* and *LOC107131971* on chromosome 29 associated with IMF-REA. This region explained 1.54% of the additive variance of IMF and 4.16% of REA. This gene encodes an ncRNA of yet unknown function, probably associated with expression modulation

(Peschansky & Wahlestedt, 2013). However, the QTL region where it is located has relevance for tenderness and fat content in other bovine populations, as shown in the QTLdb annotation (Table 3).

The genomic regions identified here shed light to the genetic mechanism underlying joint modulation of complex phenotypes of relevance to animal breeding.

4. Conclusions

By analyzing bi-trait GWAS, we suggested pleiotropic genomic regions for feed efficiency and beef quality traits. Although pleiotropic loci explained only a small proportion of the genetic variance for each trait, the identification of QTLs effect on two traits may explain part of the genetic correlations between them. Moreover, we indicate new candidate genes and metabolic pathways that may explain part of the additive variance of these traits, contributing to the direction of new strategies research on genomic selection.

5. Material and methods

5.1. Animal and phenotype collection

Animals were managed following standard procedure from Institutional Animal Care and Use Ethics Committee Guidelines (Brazilian Agricultural Research Corporation; EMBRAPA, Brazil - approval code CEUA 01/2013).

We used a total of 353 Nellore steers derived from 34 unrelated sires. The sires were selected to represent the main genealogies present in the Brazilian population. Animals were produced by artificial insemination and raised under similar nutrition and handling conditions in feedlot until slaughter at an average age of 25 months. The number of animals (N) used for GWAS was slightly different among traits due to missing data. The relationship among individuals was demonstrated graphically from the genetic relationship matrix based in markers (VanRaden diagram), calculated from the package GAPIT-R (Supplementary Figure 7).

The phenotypes for BFT (mm), REA (cm2), MFI, WSF7 (kg), WHC7 (%) and PH7(pH) were measured from 2.54 cm thick steaks harvested as a cross-section of the *longissimus dorsi* muscle between the 11th and 13th ribs collected at slaughter. Briefly, samples were used to measure BFT, REA, and MFI at 24 h *postmortem* and measurements of WSF7, PH7, and WHC7 were conducted after seven days. Muscle pH also was measured at three locations across the steak using a Testo pH measuring instrument model 230 (Testo, Lenzkirch, Germany). We determined WHC7 by a compression technique, of the 0.2 kg meat sample at a force of 10 kg for 5 min. This measure was estimated as the difference between the weight of the sample before and after compression. After these analyses, to measure WSF7, the steaks were weighted and cooked at 170°C until the temperature at the center of each sample reached 70°C. We obtained the WSF7 measures with the texture analyzer TA - XT2i coupled to a Warner-Bratzler blade with 1.016 mm thickness (Hamm, 1986).

Muscle samples (~100 g) were lyophilized to measure Intramuscular Fat (IMF, mg), Sum of Fatty Acids (SFA, mg), Sum of omega-3 (N-3, mg) and Sum of omega-6 (N-6, mg). We obtained the IMF measures using an Ankom XT20 extractor. Fatty acids were identified by comparison of the retention time of methyl esters and quantified by normalizing the area under the curve of methyl esters using Chromquest 4.1 software (Thermo Electron, Italy), described in details by Cesar *et al.*. (2014).

Individual dry matter intake (DMI, kg/d) was obtained by the difference between offer and refusal, and average daily gain (ADG, kg/d) was estimated by regression of body weight (BW) on days of feed, using PROC REG (SAS, 2010). Feed conversion ratio (FCR, kg/kg) was computed as the ratio of DMI to ADG (kg/d), where the inverse of this ratio was represented by feed efficiency (FE, kg/kg). Residual feed intake (RFI, kg/d) was computed as the residuals from the regression of DMI on midtest, BW and ADG using mixed models. The efficiency of gain (EG, kg/Mcal) was obtained by dividing ADG by metabolizable energy

intake (Mcal/d). To calculate the relative growth rate (RGR, %/d) the equation was: RGR = 100*(log BW final – log BW initial)/days of the experiment. Maintenance Efficiency (ME, kg/kg) was computed using the NRC 1996 maintenance requirement equations (Zinn & Shen, 1998). The feed efficiency related traits were described in details in de Oliveira *et al.,* (2014) and Nascimento *et al.,* (2016).

5.2.Genotype data and Quality Control

All 34 sires and 353 half-siblings were genotyped with the high-density SNP panel BeadChip Illumina BovineHD770K. DNA sample collection and the SNP chip genotyping were previously described by Tizioto *et al.* (2013).

The quality control filters used were: minor allele frequency (5%), call rate for sample SNPs (95%) and Hardy-Weinberg equilibrium (0.005), resulting in 428,884 SNPs. All autosomal SNPs were pruned using the indep-pairwise option, with a window size of 50 SNPs, a step of five SNPs, and an R2 threshold of 0.2, resulting in 49,839 tag SNPs. We performed both analyzes using PLINK software v.1.0744 (Purcell *et al.,* 2007).

5.3. Statistical analyses

We applied a linear mixed model to estimate the Variance/Covariance matrices, combining the fixed effects, random effects, and experimental errors, to control false positives. The fit of the model from these parameters allowed the estimation of variance/covariance matrix, correlation, univariate and, bivariate GWAS by the GEMMA software v0.94[11] through the Restricted Maximum Likelihood (REML) method.

The bi-trait model can be represented as:

$$\mathbf{Y} = \mathbf{W\alpha} + \mathbf{x\beta}^{\boldsymbol{T}} + \mathbf{U} + \mathbf{E}$$

$$G \sim MN_{n\times d}(0, K, V_g),\ E \sim MN_{n\times d}(0, I_{n\times n}, V_e)$$

Where **Y** is an n by d matrix of two phenotypes for n individuals; **W** = (w1, · · ·, wc) is an n×c matrix of covariates (fixed effects composed of birth, feedlot sites, breeding season, slaughter group) including a column of 1s; **α** is a c by d matrix of the corresponding coefficients including the intercept; **x** is an n-vector of marker genotypes; **β** is a d vector of marker effect sizes for the d phenotypes; ^{T} means the matrix is transposed; **U** is an n by d matrix of random effects; **E** is an n by d matrix of errors; K is a known n by n relatedness matrix, $I_{n\times n}$ is an n by n identity matrix, Vg is a d by d symmetric matrix of genetic variance component, Ve is a d by d symmetric matrix of environmental variance component and $MN_{n\times d}$(0, V1, V2) denotes the n×d matrix normal distribution with mean 0, row covariance matrix V1 (n by n), and column covariance matrix V2 (d by d) (Zhou, 2016).

Univariate GWAS model is similar to the above model except with a reduced dimension (Zhou, 2014). Associations between genotype and phenotypes were obtained by testing the null hypothesis ***H0***: **β** = 0 (marker effect sizes for all phenotypes are zero). For each SNP in turn, it was obtained the restricted maximum likelihood estimate (REML) of genetic variance and environmental variance components and corresponding p-value (Zhou, 2014). The significance of phenotype-genotype of the univariate and bivariate association was assessed using a Bonferroni threshold at **α** = 0.05 (Devlin & Roeder, 1999). The Manhattan circus plot and quantile-quantile (QQ) overlapping plot depicting -log^{10} observed P-values were generated using the CMplot R-package.

We calculated the genomic heritability fitted the Markov chain Monte Carlo (MCMC) approach implemented in the software GEMMA software v0.94 (Zhou, 2014).

5.4. Haplotype and linkage disequilibrium analysis

To further characterize candidate regions that affect traits, we performed linkage disequilibrium (LD) analyses and inferred the haplotype blocks.

We performed the haplotype blocks analysis according to Confidence Interval by Gabriel et al. (2002). This method takes as a criterion the LD existing between adjacent markers from the LD coefficient called D' calculated from the frequencies of the pairs of SNPs. D' values can range from 0 to 1, the closer to zero the less evidence of binding imbalance between the marker pairs, and the closer to one, the stronger the LD. A SNPs pair is rated as strong LD when D' values are within a range of 0.70 to 0.98. D' values outside these thresholds were considered non-informative. When 95% of the pairs of adjacent SNPs are in strong LD, we categorized as a haplotype block. These analyzes were performed using Haploview v4.2 software (Barret *et al.*, 2005).

5.5. Annotation and functional enrichment analysis

The functional enrichment analyzes were developed from the PANTHER online databases (Huaiyu *et al.*, 2010) on the significant SNPs and searched for candidate genes in the blocks based on Bos taurus genome (UMD 3.1). The statistical over-representation test of PANTHER was used to obtain the association of the gene ontology (biological processes). Subsequently, the search for regions of overlapping QTLs identified in other bovine publications available in the Cattle QTLdb database [http://www.animalgenome.org/QTLdb] (Hu et al., 2016) was performed. The annotation included 10-kb up- and downstream to the LD blocks around the SNP.

5. References

1. Aloha JK, Hill RA. Input Factors Affecting Profitability: A changing paradigm and a challenging time. 2012: Feed Efficiency in the Beef Industry, First Edition. John Wiley & Sons.
2. Arthur, P.F.; Archer, J.A.; Herd, R.M. Feed intake and efficiency in beef cattle: overview of recent Australian research and challenges for the future. Australian Journal of Experimental Agriculture, v.44, p.361-369, 2004.
3. Barendse, W., Reverter, A., Bunch, R. J., Harrison, B. E., Barris, W., & Thomas, M. B. (2007). A validated whole-genome association study of efficient food conversion in cattle. *Genetics*, *176*(3), 1893–1905. https://doi.org/10.1534/genetics.107.072637
4. Barrett, J. C., Fry, B., Maller, J., & Daly, M. J. (2005). Haploview: Analysis and visualization of LD and haplotype maps. *Bioinformatics*, *21*(2), 263–265. https://doi.org/10.1093/bioinformatics/bth457
5. Beerda, B., W. Ouweltjes, L.B.J. ˇSebek, J.J. Windig, and R.F. Veerkamp. 2007. Effects of genotype by environment interactions on milk yield, energy balance, and protein balance. J Dairy Sci 90: 219–228.
6. Bilodeau, P. A., Coyne, E. S., & Wing, S. S. (2016). The ubiquitin proteasome system in atrophying skeletal muscle: roles and regulation. *American Journal of Physiology - Cell Physiology*, *311*(3), C392–C403. https://doi.org/10.1152/ajpcell.00125.2016
7. Casas, E., Garcia, M. D., Wells, J. E., & Smith, T. P. L. (2011). Association of single nucleotide polymorphisms in the ANKRA2 and CD180 genes with bovine respiratory disease and presence of Mycobacterium aviumsubsp. paratuberculosis. *Animal Genetics*, *42*(6), 571–577. https://doi.org/10.1111/j.1365-2052.2011.02189.x
8. Cesar, A. S., Regitano, L. C., Mourão, G. B., Tullio, R. R., Lanna, D. P., Nassu, R. T., Coutinho, L. L. (2014). Genome-wide association study for intramuscular fat deposition and composition in Nellore cattle. *BMC Genetics*, *15*(1), 39. https://doi.org/10.1186/1471-2156-15-39
9. Cristancho, A. G., Schupp, M., Lefterova, M. I., Cao, S., Cohen, D. M., Chen, C. S., Lazar, M. A. (2011). Repressor transcription factor 7-like 1 promotes adipogenic competency in precursor cells. *Proceedings of the National Academy of Sciences*, *108*(39), 16271–16276. https://doi.org/10.1073/pnas.1109409108
10. De Oliveira, P., Coutinho, L. L., Tizioto, P. C., Cesar, A., de Oliveira, G. B., Diniz, W., De Lima, A. O., Reecy, J. M., Mourão, G. B., Zerlotini, A., … Regitano, L.

(2018). An integrative transcriptome analysis indicates regulatory mRNA-miRNA networks for residual feed intake in Nelore cattle. *Scientific reports*, *8*(1), 17072. doi:10.1038/s41598-018-35315-5

11. De Oliveira, P. S. N., Cesar, A. S. M., do Nascimento, M. L., Chaves, A. S., Tizioto, P. C., Tullio, R. R., Regitano, L. C. A. (2014). Identification of genomic regions associated with feed efficiency in Nelore cattle. *BMC Genetics*, *15*, 100. https://doi.org/10.1186/s12863-014-0100-0
12. Devlin, B., & Roeder, K. (1999). Genomic control for association studies. *Biometrics*, *55*(4), 997–1004. https://doi.org/10.1111/j.0006-341X.1999.00997.x
13. Exton, S. C., R. M. Herd, and P. F. Arthur. 2004. Identifying bulls superior for net feed intake, intramuscular fat and subcutaneous fat. Anim. Prod. Aust. 25:57–60.
14. Elis, S., Desmarchais, A., Freret, S., Maillard, V., Labas, V., Cognié, J., … Uzbekova, S. (2016). Effect of a long-chain n-3 polyunsaturated fatty acid–enriched diet on adipose tissue lipid profiles and gene expression in Holstein dairy cows. *Journal of Dairy Science*, *99*(12), 10109–10127. https://doi.org/10.3168/jds.2016-11052.
15. Falconer, D.S.; Mackay, T.F.C. Introduction to quantitative genetics. 4.ed. Edinburgh : Longman Group Limited, 1996. 464p.
16. Gabriel S.B., Schaffner S.F., Nguyen H., Moore J.M., Roy J., Blumenstiel B.. The structure of haplotype blocks in the human genome. Science 2002; 296:2225–9. doi: 10.1126/science.1069424 PMID:12029063
17. Gomes, R.C.; Sainz, R.D.; Silva, S.L.; César, M.C.; Bonin, M.N.; Leme, P.R. 2012. Feedlot performance, feed efficiency reranking, carcass traits, body composition, energy requirements, meat quality and calpain system activity in Nellore steers with low and high residual feed intake. Livestock Science 150:265-273.
18. Guillaume, F., & Otto, S. P. (2012). Gene functional trade-offs and the evolution of pleiotropy. *Genetics*, *192*(4), 1389–1409. https://doi.org/10.1534/genetics.112.143214
19. Jia, D., Zhang, J. S., Li, F., Wang, J., Deng, Z., White, M. A. Rosen, M. K. (2016). Structural and mechanistic insights into regulation of the retromer coat by TBC1d5. *Nature Communications*, *7*. https://doi.org/10.1038/ncomms13305
20. Hamm R. Functional properties of the myofibrillar system and their measurement. In: Muscle as Food, edited by Bechtel PJ. Orlando: Academic Press, 1986, p. 135–199.

21. Hayes B.J., Lewin H.A., Goddard M.E. The future of livestock breeding: genomic selection for efficiency, reduced emissions intensity, and adaptation. Trends Genet 2013, 29:206-214.
22. Herd, R.M. and P.F. Arthur. 2009. Physiological basis for residual feed intake. J Anim Sci 87: E64–E71.
23. Hocquette J. F., Gondret F., Baza E. M., dale F., Jurie C., Pethick D. W. Intramuscular fat content in meat-producing animals: Development genetic and nutritional control and identification of putative markers. Animal. 2010;4:303–319. doi: 10.1017/S1751731109991091.
24. Huaiyu Mi, Qing Dong, Anushya Muruganujan, Pascale Gaudet, Suzanna Lewis and Paul D. Thomas 2010. PANTHER version 7: improved phylogenetic trees, orthologs and collaboration with the Gene Ontology Consortium. *Nucl. Acids Res.*. 38: D204-D210.
25. Hu, Z. L., Park, C. A., & Reecy, J. M. (2016). Developmental progress and current status of the Animal QTLdb. *Nucleic Acids Research*, *44*(D1), D827–D833. https://doi.org/10.1093/nar/gkv1233
26. Lamare, M., Taylor, R. G., Farout, L., Briand, Y., & Briand, M. 2002. Changes in proteasome activity during postmortem aging of bovine muscle. Meat Science, 61: 199-204.
27. Gomes, R.C.; Sainz, R.D.; Silva, S.L.; César, M.C.; Bonin, M.N.; Leme, P.R. 2012.
28. Lizaso, A., Tan, K. T., & Lee, Y. H. (2013). β-adrenergic receptor-stimulated lipolysis requires the RAB7-mediated autolysosomal lipid degradation. *Autophagy*, *9*(8), 1228–1243. https://doi.org/10.4161/auto.24893
29. Liu, M.F.; Goonewardene, L.A.; Bailey, D.R.C.; et al. A study in the variation of feed efficiency in station tested beef bulls. Canadian Journal of Animal Science, v.80, p.435-441, 2000.
30. Moraes, G. F. de, Abreu, L. R. A., Ferreira, I. C., & Pereira, I. G. (2017). Genetic analysis of residual feed intake adjusted for fat and carcass and performance traits in a Nellore herd. *Ciência Rural*, *47*(2). https://doi.org/10.1590/0103-8478cr20151505
31. Nagai, Y., Watanabe, Y., & Takatsu, K. (2013). The TLR family protein RP105/MD-1 complex: A new player in obesity and adipose tissue inflammation. *Adipocyte*, *2*(2), 61–66. https://doi.org/http://dx.doi.org/10.4161/adip.22929

32. Nascimento, M. L., Souza, A. R. D. L., Chaves, A. S., Cesar, A. S. M., Tullio, R. R., Medeiros, S. R., … Lanna, D. P. D. (2016). Feed efficiency indexes and their relationships with carcass, non-carcass and meat quality traits in Nellore steers. *Meat Science*, *116*, 78–85. https://doi.org/10.1016/j.meatsci.2016.01.012
33. Oliveira, G. B. *et al*. Integrative analysis of microRNAs and mRNAs revealed regulation of composition and metabolism in Nelore cattle. *BMC Genomics*, https://doi.org/10.1186/s12864-018-4514-3 (2018).
34. Peschansky VJ, Wahlestedt C. Non-coding RNAs as direct and indirect modulators of epigenetic regulation. *Epigenetics*. 2013;9(1):3-12.
35. Purcell, S., Neale, B., Todd-Brown, K., Thomas, L., Ferreira, M. A. R., Bender, D., … Sham, P. C. (2007). PLINK: A Tool Set for Whole-Genome Association and Population-Based Linkage Analyses. *The American Journal of Human Genetics*, *81*(3), 559–575. https://doi.org/10.1086/519795
36. Richardson, E.C.; Herd,; R.M. Archer J.A. et al. Metabolic differences in Angus steers divergently selected for residual feed intake. *Australian Journal of Experimental Agriculture*, v.44, p.441-452, 2004.
37. Roy, S., Leidal, A. M., Ye, J., Ronen, S. M., & Debnath, J. (2017). Autophagy-Dependent Shuttling of TBC1D5 Controls Plasma Membrane Translocation of GLUT1 and Glucose Uptake. *Molecular Cell*, *67*(1), 84–95.e5. https://doi.org/10.1016/j.molcel.2017.05.020
38. Russell, J. R., Minton, N. O., Sexten, W. J., Kerley, M. S., & Hansen, S. L. (2016). Influence of feed efficiency classification on diet digestibility and growth performance of beef steers. *Journal of Animal Science*, *94*(4), 1610–1619. https://doi.org/10.2527/jas2015-9949
39. SAS Institute Inc: Base SAS® 9.2 Procedures Guide. Cary, NC: SAS Institute Inc.; 2010.
40. So, E. Y., & Ouchi, T. (2013). The Potential Role of BRCA1-Associated ATM Activator-1 (BRAT1) in Regulation of mTOR. *Journal of Cancer Biology & Research*, *1*(1), 1–3.
41. So, E. Y., & Ouchi, T. (2014). BRAT1 deficiency causes increased glucose metabolism and mitochondrial malfunction. *BMC Cancer*, *14*(1), 1–13. https://doi.org/10.1186/1471-2407-14-548

42. Söhle, J., Machuy, N., Smailbegovic, E., Holtzmann, U., Grönniger, E., Wenck, H., … Winnefeld, M. (2012). Identification of new genes involved in human adipogenesis and fat storage. *PLoS ONE*, *7*(2). https://doi.org/10.1371/journal.pone.0031193
43. Tizioto P. C., Coutinho L. L., Oliveira P. S., Cesar A. S., Diniz W. J., Lima A. O., Rocha M. I., Decker J. E., Schnabel R. D., Mourão G. B., L., Regitano, L. C. A.. Gene expression differences in longissimus muscle of Nelore steers genetically divergent for residual feed intake. *Scientific Reports*. 2016; 6:39493.
44. Tizioto, P. C., Decker, J. E., Taylor, J. F., Schnabel, R. D., Mudadu, M. A., Silva, F. L., Regitano, L. C. A. (2013). Genome scan for meat quality traits in Nelore beef cattle. *Physiological Genomics*, *45*(21), 1012–1020. https://doi.org/10.1152/physiolgenomics.00066.2013
45. Weber, K. L., Welly, B. T., Van Eenennaam, A. L., Young, A. E., Port-Neto, L. R., Reverter, A., & Rincon, G. (2016). Identification of Gene networks for residual feed intake in Angus cattle using genomic prediction and RNA-seq. *PLoS ONE*,*11*(3), 1–19. https://doi.org/10.1371/journal.pone.0152274
46. Welch, C. M., M. McGee, T. A. Kokta, and R. A. Hill. 2012. “Muscle and adipose tissue: Potencial roles in driving variantion in feed efficiency.” In *Feed efficiency in the beef industry,* edited by R. A.Hill, 175-198. Iowa: Wiley-Blackwell. Ames.
47. Wolf J. B., (2002) The geometry of phenotypic evolution in developmental hyperspace. Proc Natl Acad Sci U S A 99(25): 15849-15851.
48. Zinn A, Shen YA: An evaluation of ruminal degradable intake protein and metabolizable amino acid requirements of feedlot calves. J Anim Sci 1998.
49. Zhou, X. (2014). GEMMA User Manual, 1–27.
50. Zhou, X., & Stephens, M. (2012). Genome-wide efficient mixed model analysis for association studies. *Nature Genetics*, *44*(7), 821–824.https://doi.org/10.1038/ng.2310.Genome-wide

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Authors Contribution

C.E.B, G.B.M, J.B.W, L.L.C., and L.C.A.R designed the experiments and analysis. C.E.B, performed the experiments and analysis. J.B.W and G.B.M., statistical analyzes support. J.P., and G.A.R, bioinformatics technical support. C.E.B., J.P., J.A., P.C.T., A.S.M.C., interpreted the results. C.E.B., and L.C.A.R. drafted the manuscripts.

Supplementary Material

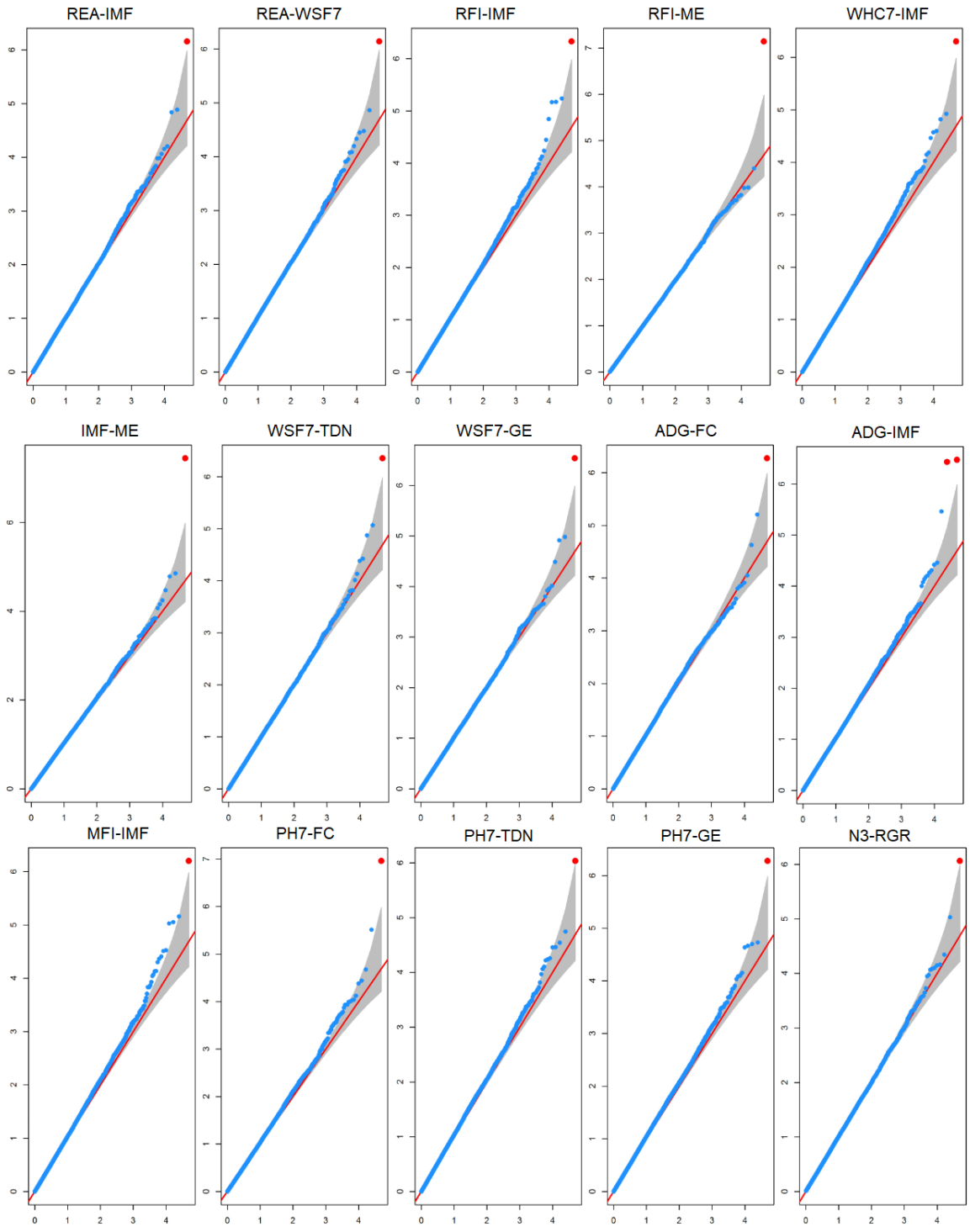
Supplementary Table 1. Proportion of phenotypic variance explained (PVE) by genotypes (Chip heritability) and respective standard errors (SE), genetic variances (Vg) and environmental variances (Ve).

Traits*	PVE	SE	Vg	Ve
BFT	0.2753	0.1164	0.5201	0.4799
TDN	0.3479	0.1540	0.6148	0.3852
SFA	0.4848	0.1257	0.7284	0.2716
FC	0.3316	0.1328	0.5878	0.4122
WHC7	0.4040	0.1357	0.6594	0.3406
ME	0.3197	0.1203	0.5724	0.4276
GE	0.2372	0.1482	0.4714	0.5286
REA	0.2055	0.1284	0.4264	0.5736
RFI	0.1507	0.1041	0.3370	0.6630
MFI	0.3804	0.1429	0.6365	0.3635
DMI	0.3247	0.1092	0.5782	0.4218
N6	0.2644	0.1235	0.5072	0.4928
WSF7	0.2500	0.1251	0.2743	0.7257
PH7	0.2778	0.1235	0.5072	0.4928
ADG	0.3917	0.1334	0.6470	0.3530
FE	0.3768	0.1290	0.6327	0.3673
IMF	0.2161	0.1261	0.4401	0.5599
RGR	0.5573	0.1295	0.7821	0.2179
N3	0.5394	0.1294	0.7695	0.2305
DMLW	0.1753	0.1407	0.3787	0.6213

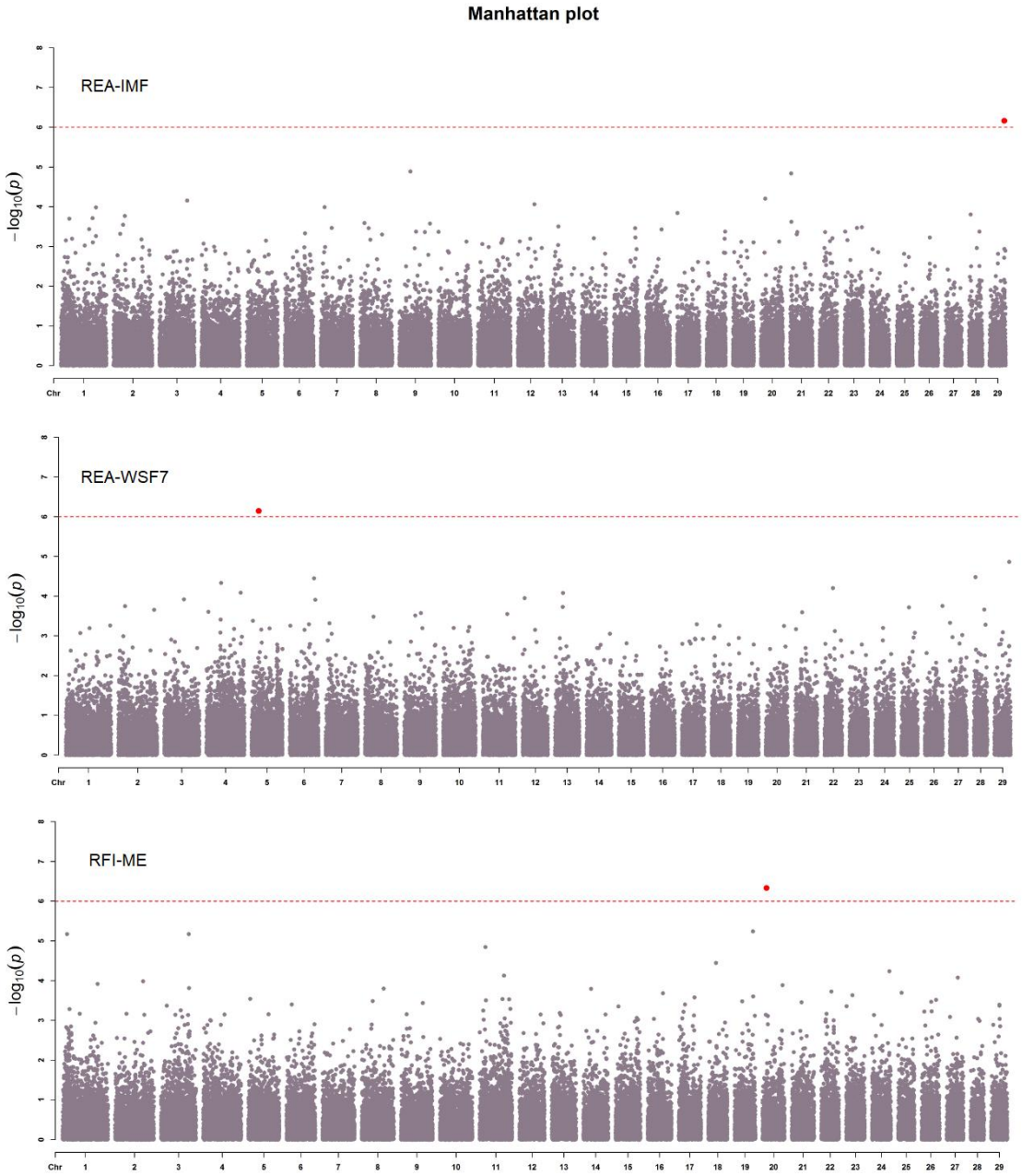
*Traits: Backfat thickness (BTF; mm), total digestive Nutrients (TDN; Kg/Kg), saturated fat acid content (SFA; mg), feed conversion (FC; kg/kg), water holding capacity after seven days (WHC7/%), maintenance efficiency (ME; kg/kg), efficiency of gain (GE; kg/Mcal), rib eye muscle area (REA/cm^2), residual feed intake (RFI; kg/d), myofibrillar fragmentation index (MFI), dry matter intake (DMI; kg/d), omega 6 content (N-6; mg), Warner–Bratzler Shear Force after seven days (WSF7; kg), pH after seven days (PH7; pH), average daily gain (ADG; kg/d), feed efficiency (FE; kg/kg), intramuscular fat (IMF; mg), Relative Growth Rate (RGR; %/d), sum of the omega 3 (N-3; mg) and live weight gain LWG; kg/d).

Supplementary Table 2. SNP-based genetic correlations between twenty traits analysed.

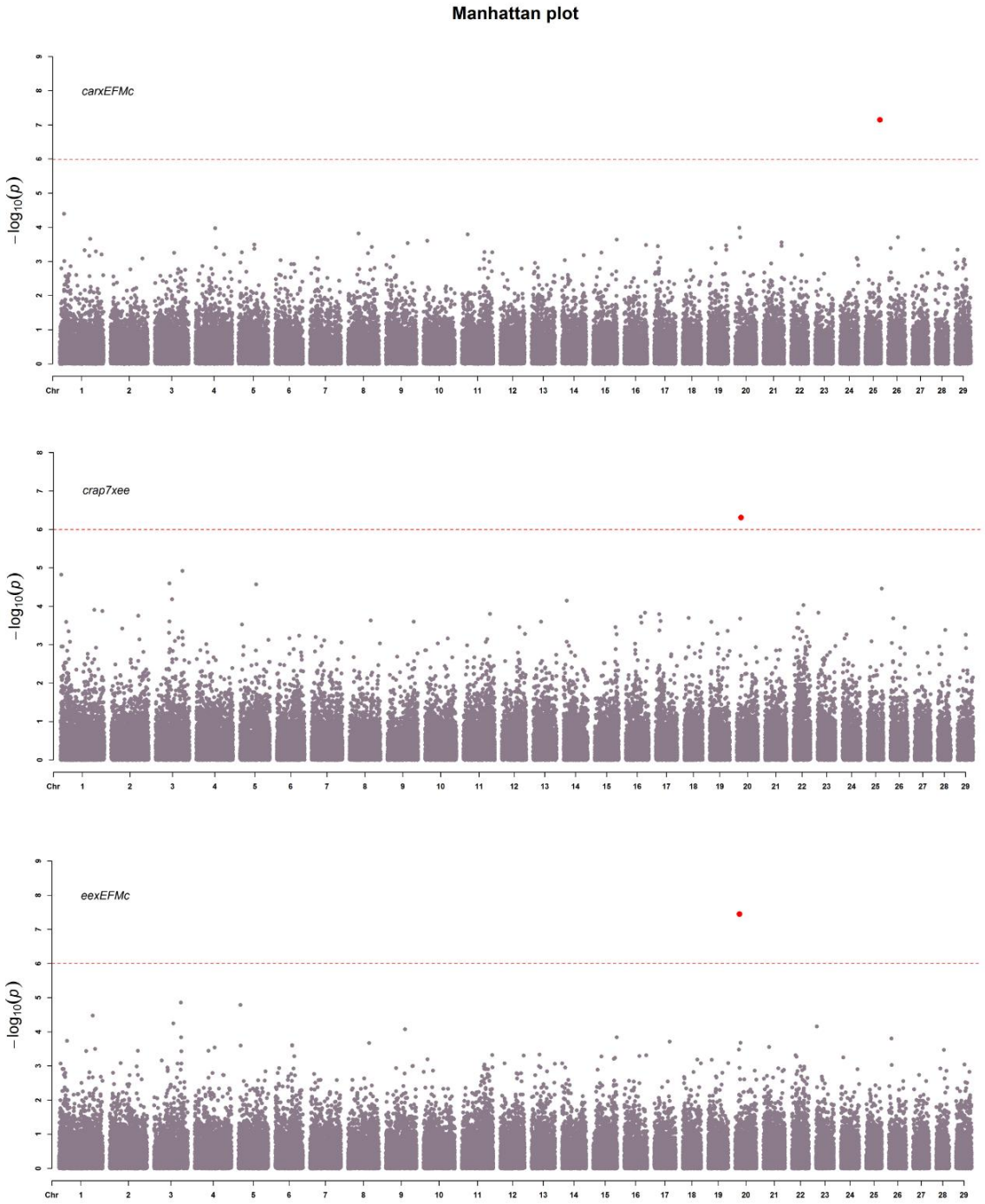
	REA	WSF7	PH7	WHC7	ADG	RFI	FE	DMI	BFT	MFI	IMF	SFA	N3	N6	FC	LWG	TND	GE	ME	RGR
REA	1																			
WSF7	-0.68863	1																		
PH7	-0.78326	0.77371	1																	
WHC7	0.60276	-0.50419	-0.13172	1																
ADG	0.12319	-0.54023	0.51980	0.16811	1															
RFI	0.70416	-0.24012	-0.69001	0.74795	0.05881	1														
FE	-0.22276	0.39095	0.02300	0.28909	0.82185	-0.40154	1													
DMI	0.50011	0.58901	0.48960	-0.82052	-0.72271	-0.75784	0.13637	1												
BFT	0.14505	-0.69802	0.09603	0.48551	-0.15584	0.12000	0.14761	0.09368	1											
MFI	-0.21565	0.12133	0.43024	0.14794	-0.04066	-0.01687	0.00301	0.40781	0.31011	1										
IMF	-0.74978	-0.28072	0.24000	-0.40924	0.308432	-0.80074	0.05431	0.18548	0.00923	-0.40579	1									
SFA	0.28965	-0.72021	-0.33268	0.50859	0.01046	0.00023	0.01299	0.27754	0.83766	0.17543	-0.4985	1								
N3	0.25368	0.21023	0.49818	0.32527	0.63012	0.00032	-0.00210	0.86614	-0.64502	0.77493	0.85822	-0.6101	1							
N6	-0.53279	0.24032	0.64189	-0.79375	0.00211	-0.00210	0.36877	0.78002	-0.53043	0.14216	0.69663	-0.00021	0.75451	1						
FC	0.25540	-0.77689	-0.11717	0.35001	-0.59301	0.77228	0.19602	0.04205	0.69138	0.44232	-0.68763	0.92487	0.00012	-0.3567	1					
LWG	0.45001	0.15089	0.03211	-0.82433	0.18915	-0.02201	0.76158	0.77745	-0.11513	0.75011	0.52100	-0.86741	0.92211	-0.0002	-0.00332	1				
TND	0.64455	-0.76065	-0.74926	0.0001	0.11333	0.19001	-0.59554	-0.84237	0.81111	-0.55734	-0.58678	0.00003	-0.88011	0.01002	-0.00042	-0.01100	1			
GE	0.64594	0.56032	-0.66012	-0.6901	0.24409	0.10012	-0.71924	-0.62001	-0.85001	-0.55538	-0.59581	-0.52001	0.92121	0.00023	-0.00011	-0.00031	-0.03412	1		
ME	0.71188	0.64023	-0.61864	0.76131	0.70482	0.28011	-0.13074	-0.58719	0.63022	-0.14034	-0.55246	0.91131	-0.75577	-0.9077	0.64712	0.00021	0.00234	0.00021	1	
RGR	-0.68863	0.54083	0.65192	-0.23545	0.20939	0.18001	0.47486	0.18148	-0.42832	0.08411	0.70208	-0.68513	0.87001	0.52237	-0.27932	0.77111	-0.53045	-0.58252	-0.58029	1



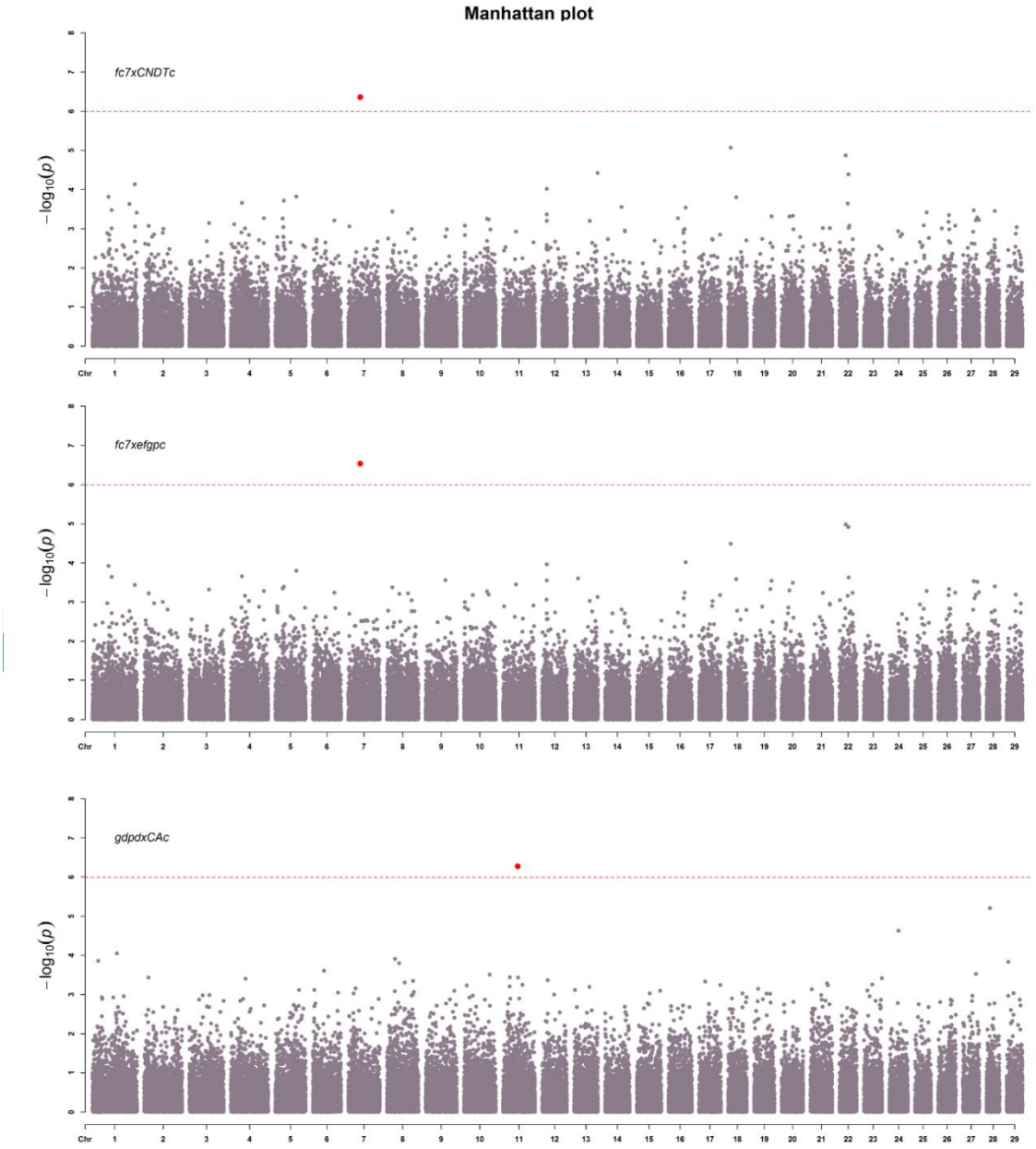
Supplementary Figure 1. Q-Q plots show fifteen overlapping bivariate analyses of which significant tag SNPs were below the Bonferroni threshold (adjusted p-value 1.00×10^{-6}).



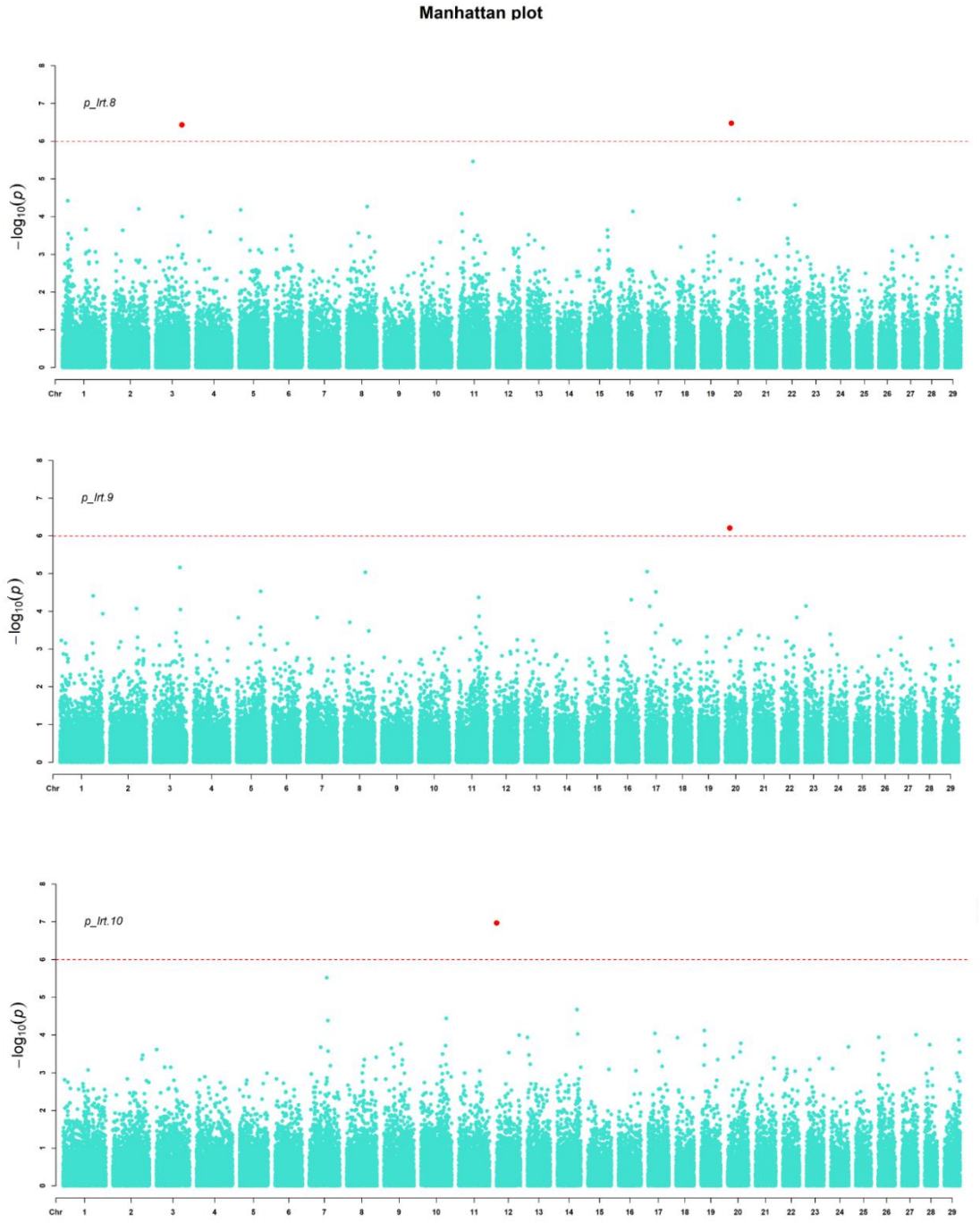
Supplementary Figure 2. Manhattan plots show bivariate analyses (REA-IMF, REA-WSF7 and RFI-ME) of which significant tag SNPs were below the Bonferroni threshold (adjusted p-value 1.00×10^{-6}).



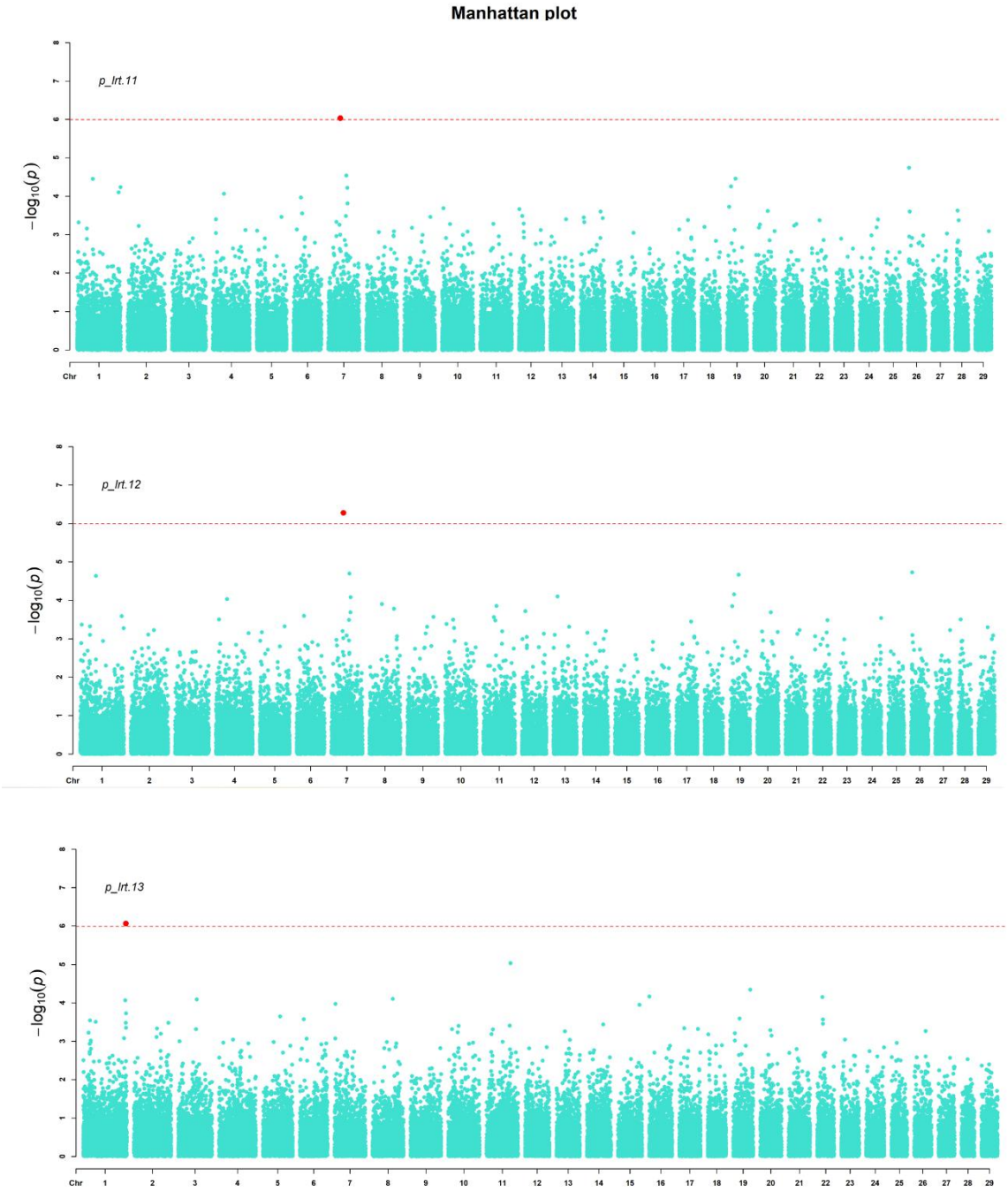
Supplementary Figure 3. Manhattan plots show bivariate analyses (RFI-ME, WHC7-IMF and IMF-ME) of which significant tag SNPs were below the Bonferroni threshold (adjusted p-value 1.00×10^{-6}).



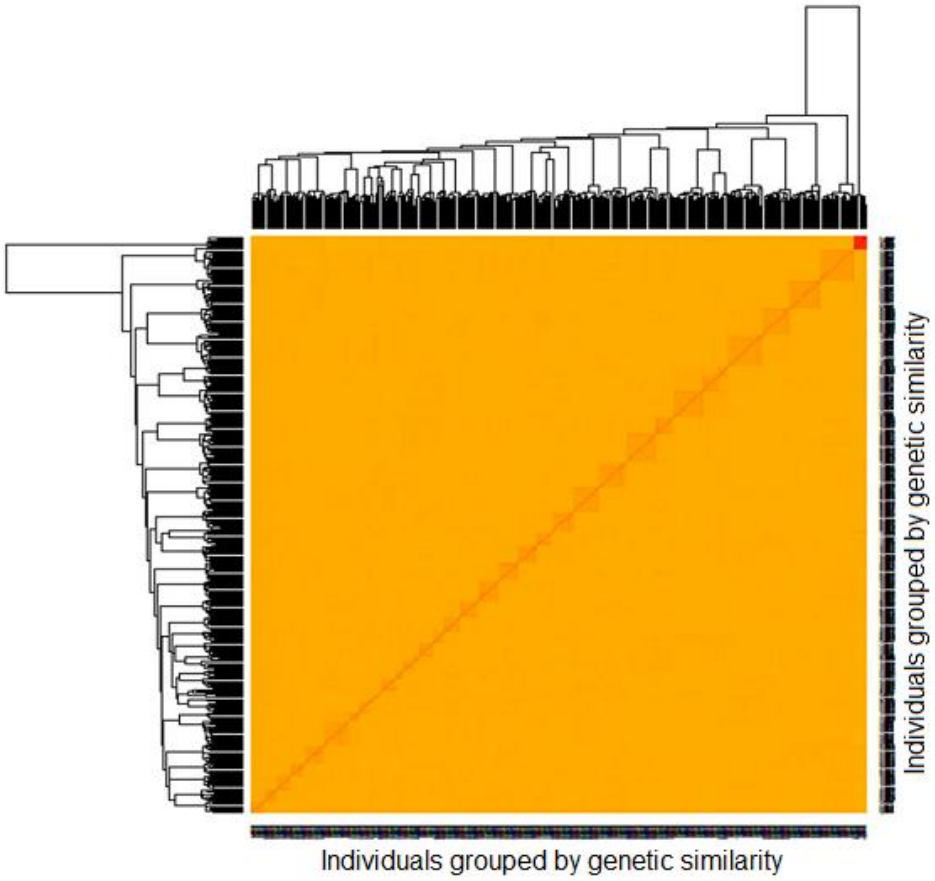
Supplementary Figure 4. Manhattan plots bivariate analyses (WSF7-TDN, WSF7-EG and ADG-FC) of which significant tag SNPs were below the Bonferroni threshold (adjusted p-value $1.00x10^{-6}$).



Supplementary Figure 5. Manhattan plots bivariate analyses (ADG-IMF, PH7-FC, MFI-IMF) of which significant tag SNPs were below the Bonferroni threshold (adjusted p-value $1.00x10^{-6}$).



Supplementary Figure 6. Manhattan plots bivariate analyses (PH7-TDN, PH7-GE e N3-RGR) of which significant tag SNPs were below the Bonferroni threshold (adjusted p-value $1.00x10^{-6}$).



Supplementary Figure 7. Relatedness matrix based on SNPs. Both axes, show the 34 bulls and 353 half-siblings grouped by genetic similarity. The squares arranged evenly on the diagonal of the graph shows how families are structured. The spectrum of colors tending to red indicates greater genetic similarity between individuals.

CHAPTER III

Integration of Bivariate GWAS and cis-eQTL for the prospection of regions associated with calcium content and beef tenderness

Carlos Eduardo Buss and Luciana Correia de Almeida Regitano

ABSTRACT

The beef tenderness is promoted mainly by the action of proteolytic systems dependent on Calcium (Ca^{2+}). The Ca^{2+} content variation may influence the standardization of the herd tenderness, depreciating of the product's price. However, it is still necessary to understand how genes and metabolic pathways influence tenderness through Ca^{2+} uptake in the postmortem. Thus, the objective of this work was to investigate the biological mechanisms underlying the association between shear force after seven (FC7) and fourteen days of slaughter (FC14) and Ca^{2+} content through the integration of genotypes, gene expression, and phenotypes. Therefore, we estimate heritabilities, genetic correlations, and bivariate genomic association study (GWAS). In total, we identified 35 QTL (Quantitative Trait Loci) regions associated with FC14- Ca^{2+}, of which 7 were reported in other studies on the shear force in cattle. We also highlighted a probable association with the *CAPN1* gene (p-value = $7.78x10^{-6}$), one of the main genes responsible for the proteolysis of myofibrils in the postmortem period. In view of the complexity of the chromosomal regions associated with FC14-Ca^{2}+, consisting of many genes, we applied the integrative approach (Transcriptome-Wide Studies - TWAS) to increase the chances of finding the causal mutations. These analyses revealed two candidate genes *DAP3* (pSMR=$1.60x10^{-2}$) and *ALKBH3* (pSMR=$1.03x10^{-2}$), which are of great importance in the regulation of programmed cell death, essential at the beginning of the process of converting muscle into the meat. Our study has contributed to clarifying mechanisms underlying the regulation of the beef tenderness, indicating QTLs of relevance for bovine genetic improvement to future selection program.

1. INTRODUCTION

Meat tenderness is the main organoleptic characteristic responsible for consumer satisfaction (Koohmaraie, 1996). The beef texture results from sarcomere contraction, connective tissues composition, and myofibrillar degradation (Koohmaraie et al., 2002). The postmortem period culminates in numerous molecular alterations that cause proteolysis of myofibrils, disrupting the integrity of muscle cells (Geesink and Koohmaraie, 2006).

Several enzyme systems generate myofibrils proteolysis. However, various studies suggest that the tendering process is promoted in the most cases by the action of the calpain enzyme system. These proteases are commonly found in muscle tissue in three isoforms (μ- and *m-calpain* and *calpain 3*), along with their respective specific endogenous inhibitor enzyme, called calpastatin (Geesink and Koohmaraie, 2006, Goll et al., 2003).

The calpain activities are regulated by the Ca^{2+} availability in the cytosol of muscle cells (Bhat et al., 2018). Thus, the increased of the Ca^{2+} concentration in muscle and plasma is equivalent to increased activity of proteases that cascade activates other proteolytic processes that promote tenderness (Zinn and Shen 1996).

Consequently, the Ca^{2+} variation in beef can directly influence the standardization of the beef tenderness of a herd (Tizioto et al., 2014). In this scenario, genomic selection strategies can be applied to improve muscle Ca^{2+} levels (Tizioto et al., 2014). However, further understanding is still needed to provide tools to improve the genomic selection of the genes and biological mechanisms that underlie and promote Ca^{2+} uptake within the muscle cell (Kemp et al., 2010).

In the last decade, analysis of GWAS has leapfrogged significant advances in understanding the modulation of meat quality traits, including the identification of hundreds and dozens of loci associated with Ca^{2+} content and tenderness, respectively (Http:

//www.animalgenome.org/QTLdb; Hu et al., 2016). However, the functional genes responsible for most of these associations remain poorly understood. This limitation may be due to the complexity of linkage disequilibrium (LD) between markers and causal mutations that influence production traits (McCarthy and Hirschhorn, 2008).

Recent methodological advances and increased computational power have provided the opportunity to analyze phenotype interactions more clearly and to identify functional genes as well their regulatory elements, from meta-analyses of GWAS data and specific tissue expression quantitative trait loci (eQTL) (Veturi and Ritchie, 2018).

The approach that combines these strategies is based on the concept of Summary Mendelian Randomization (SMR). This approach assumes that if the level of expression of a gene is influenced by a genetic variant (eQTL), there will be differences in the levels of gene expression between individuals of different genotypes with variance explained by the markers selected by GWAS (e.g., AA, Aa, and aa). Thus, the gene expression level may display and evidence a causative effect on the phenotype of interest would constitute potential markers for selection (Zhu et al., 2016).

In the present study, we investigated the biological mechanisms underlying the association between shear force, measured after seven (WSF7) and fourteen (WSF14) days of slaughter, and the muscle concentration of Ca^{2+}, through the integration of genotypes, gene expression, and phenotypes. Therefore, we decompose variance/covariance matrices, estimate genetic correlations and heritabilities, as well as marker effects, identifying regions of QTLs that modulate biological functions common to both traits.

Subsequently, we predicted cis-eqtl and integrated them with the information obtained from our bivariate GWAS results.

2. MATERIALS AND METHODS

2.1. Animal collection and traits

2.1.1. Experimental animals

The animals were reared and slaughtered according to the standard procedures of the Institutional Animal Use and Ethics Committee Guidelines (Brazilian Agricultural Research Corporation; EMBRAPA, Brazil - CEUA 01/2013).

We used a total of 361 Nelore steers derived from 24 bulls. The selection of the bulls was carried out from a total number of 616 Nelore bulls. These bulls belonged to the genealogies representative of the main lineages that make up the Nelore breed in Brazil, of frequent commercial use.

The reference families were formed with at least two and at most twenty females per bull. The animals were reproduced by artificial insemination and kept under similar conditions of management and nutrition, finished in confinement until slaughter, with an average age of 25 months. The number of animals (N) differed among the traits due to the availability of data.

2.1.2 Traits

Traits were obtained from previous studies, as described in Tizioto et al., (2013) and Tizioto et al., (2015).

2.1.2.1 Shear force quantification

Measurements of shear force were obtained from steaks 2.54 cm thick, in cross-section of the Longissimus dorsi muscle between the 11th and 13th ribs. The samples were matured at

2 ° C in a cold chamber manufactured by McQuay-Heatcraft of Brazil Ltda for 7 (WSF7-kg) and 14 days (WSF14-kg) after slaughter respectively, from the TA-XT2i texture analyzer coupled to a 1.016 mm thick Warner-Bratzler blade (Tizioto et al., 2013).

2.1.2.2 Ca2+ content determination

Mineral phenotypes were measured as described by Tizioto et al., (2015). A cross-section of the Longissimus dorsi muscle was lyophilized; homogenized and 100 mg were sampled for chemical analysis.

Analytical reagents and ultrapure water (Milli-Q system, Millipore, Billerica, MA, USA) were used to standardize the reactions. Samples were digested using 2 mL of sub-boiled concentrated HNO3, 2 mL ofH2O2(30% w / w) and 6.0 mL of ultrapure water.

The Vista Pro-CCD ICP-OES spectrometer determined the mineral content with radial vision (Varian, Mulgrave, Australia). The excitation wavelengths were chosen to minimize spectral interference and produce the highest emission intensity for each element. The experiment was carried out in triplicate.

2.2. Genotypic data and quality control

All 24 bulls and 361 half-siblings were genotyped with the BeadChip Illumina BovineHD770K high-density SNP panel. The frozen semen samples obtained from Brazilian artificial insemination plants were used to extract DNA from the bulls. For the progenies, blood samples were collected.

We measured the DNA concentration by spectrophotometry, followed by integrity inspection by agarose gel electrophoresis. All animals were genotyped using the Illumina BovineHD BeadChip. Assembly of the genotype library was performed based on the bovine

reference genome (ARS-UCD1.2) from Illumina Genome Studio software, as described by Tizioto et al., (2013).

Quality control criteria for markers elimination were considered (call rate - CR) below 95%, Minor Allele Frequency (MAF), whose rare allele frequency was less than 5% and Hardy-Weinberg equilibrium deviation (0.005), remaining a total of 405.400 SNPs were considered informative.

From this initial set of markers, SNPs tags we selected with the procedure called LD pruning, to define a central subset of independent and representative markers. A Pearson correlation coefficient filter (0.2) was applied to exclude highly correlated variants. We used the indep-pairwise function, which tested the correlations between genotypic pairs along the autosomal chromosomes in 50 SNP windows and 5 SNP intervals between each window, ultimately resulting in 49.330 SNP tags. Both quality control and redundant SNP exclusion analysis were performed using PLINK v.1.07 software (Purcell et al., 2007).

2.3 Statistical analyses

A linear mixed model was applied to estimate the Variance/Covariance matrices, combining the fixed effects, random effects, and experimental errors. In the present study, we considered as fixed effects the contemporary groups (composed by birth, feedlot sites, breeding season, slaughter group) and age of the animal at slaughter as covariate (Tizioto et al., 2013).

Random effects were calculated from the familial component, from the genetic variance components and the cumulative effect of all SNPs captured by a relationship matrix (kinship) between individuals. These factors were used to correct variations resulting from genetic heterogeneity among the animals of the population.

The adjustment of the model from these parameters allowed the estimation of variance/covariance matrix, correlation and bivariate, and univariate GWAS. The analyses were performed by GEMMA v0.94 software (Zhou, 2014) using the Restricted Maximum Likelihood (REML) method.

The bi-trait model can be represented as:

$$\mathbf{Y = \alpha W + x\beta + U + E}$$

Where Y is a matrix of phenotypes, α is a fixed effect incidence matrix; W is a matrix of fixed effects (group of contemporaries and the age of the animal at slaughter); x is a vector of marker genotypes; β is a marker effect size vector for phenotypes and intercept for each phenotype; U is a random-effects matrix, defined by the component of genetic variance and genetic relationship; and E is a residual matrix, defined by the residual variance component and the identity matrix (Zhou and Stephens, 2012).

Associations between genotype and phenotypes were obtained by testing the null hypothesis H0: $\beta = 0$, ie, that the proportion of marker effects for all phenotypes is zero.

For each SNP, the estimated restricted maximum likelihood (REML) of the genetic variance and the components of the corresponding environmental variance were given a p-value (Zhou and Stephens, 2012). The significance of the bivariate phenotype-genotype association was assessed using Bonferroni-corrected thresholds and multiple tests (FDR) at $\alpha = 0.05$ (Devlin and Roeder, 1999).

The graphical representations Manhattan plot and QQ plot (Quantil-Quantil), representing the observed P-values -log10 were generated using the CMplot package in the R environment.

The genomic heritability was estimated using GEMMA v0.94 software (Zhou and Stephens, 2012).

2.4. Annotation and analysis of functional enrichment

We investigated the biological mechanisms underlying the relevant SNPs, based on the interpretation of the gene functions of the associated regions. To track genes near markers, we use the Variant Effect Predictor (VEP) tool provided by the Ensembl [http://www.ensembl.org/info/docs/tools/vep/index.html] database, aligned with the genome of Bos taurus reference ARS-UCD1.2. The list of genes located near the significant SNP was mapped, allowing a maximum distance of 1MB from the associated marker.

The biological functions of these genes and their possible relationships with traits were explored using the information available for Bos taurus. Functional enrichment analyses were developed from PANTHER online databases (Huaiyu et al., 2010).

The statistical over-representation test of PANTHER was used to obtain the association of the gene ontology (biological processes). Subsequently, we searched regions overlapping QTLs identified in other cattle publications available in the Cattle QTLdb database [http://www.animalgenome.org/QTLdb] (Hu et al., 2016).

2.5. Expression Analysis: RNA Extraction and Sequencing

RNA extraction and sequencing analyzes are from previous work, described in detail in Cesar et. al., (2018).

Briefly, total RNA was extracted from 100 mg muscle of 198 newly slaughtered animals using the TRIzol reagent (Life Technologies, Carlsbad, CA). A total of 2 μg of total RNA from each sample was used for library preparation according to the protocol described in TruSeq RNA Sample Preparation Kit v2 guide (Illumina, San Diego, CA).

Average sizes of libraries were estimated using Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA) and quantitated using quantitative PCR with the KAPA Biosystems

Kit for library quantification (KAPA Biosystems, Foster City, CA, USA). Sequencing analyzes were performed at the Center for Genomic Analysis of ESALQ, Piracicaba, São Paulo, Brazil.

Quality control was estimated using FASTQC software 0.10.1 [https://www.bioinformatics.babraham.ac.uk/projects/fastqc/].

The RNA-Seq by Expectation (RSEM) approach was used to estimate the number of fragments originating from each gene in each replicated library in order to minimize differences in total counts read through the samples.

Subsequently, the samples were adjusted for sequencing reaction effect (slide and run in which the sample was sequenced - batch effect) to remove possible technical errors inherent to the data. Followingthis procedure the sequences were aligned using the Bos taurus reference genome ARS-UCD1.2 (Diniz et al., 2019).

2.6. Identification of eQTL regions

The associations between genetic variation of genotype and gene expression (RNA-Seq) were estimated from the Matrix eQTL package in R environment (Shabalin, 2012). Contemporary groups (including places of birth and confinement, vintage, slaughter group) were included in the model as fixed effects.

The total of previously selected SNPs tags were used to test for association with variation in gene expression, markers were defined within the 1 Mb gene limit, and were characterized as cis-eQTLs (local variants), according to the protocol described by Cesar et al. ., (2018).

The Matrix eQTL estimated the association between each marker and the transcript count of each gene, assuming the effect of the genotype as additive and the location of the snp-gene, performing a separate test for each pair (marker and gene).

Subsequently, the eQTLs were corrected for multiple tests with FDR corresponding to $\alpha = 0.05$ (Shabalin, 2012). The slope effect size based on the effect of the markers was also provided by Matrix eQTL (Shabalin, 2012).

2.7. Data integration of GWAS and eQTLs through Summary Data-Based Mendelian Randomization (SMR)

The SMR approach was recently developed (Zhu et. Al., 2016) to identify the association between gene expression and the SNP tag in complex LD pattern regions identified in GWAS. In short, the SMR considers the genetic variant (in our case SNP - Z tag) as the instrumental variable to test whether the effect on the phenotype of interest (Y) is mediated by the variation of expression, in which case the SNP tag would be considered a. cis-eQTL (cis-eQTL - X) (VanderWeele et al., 2015).

In this way, we test whether the effect of Z on Y is mediated by X (Z -> X -> Y). The estimate of the instrumental variable of the effect of X on Y (bXY) can be expressed as bXY = bZY / bZX, where bZY is the size of the effect of Z on Y and bZX is the size of the effect of Z on X. Thus, we use this approach to testing whether the magnitude of the effect of a SNP tag (Z) on characteristics (Y) identified from GWAS is mediated by the level of expression of a gene (X) affected by eQTL (Boef et al., 2015).

To test whether SMR results may reflect binding imbalance, we used the dependent instrument heterogeneity test (HEIDI) proposed by Zhu et al.(2016), which considers the

pattern of associations using all SNPs that are significantly associated with gene expression (eQTLs) in the cis region (Zhu et al., 2016).

We used FDR (0.05) as a correction for multiple tests based on 2005 cis-eQTL found by Matrix eQTL (Shabalin, 2012) that presented functional registers in ensembl ("probes") by annotation made with biomaRt (Durinck et al. 2009).

3. RESULTS

3.1. Heritability

Tenderness measurements FC7 and FC14 showed moderate heritability of 0.25 and 0.20, respectively. Calcium ($Ca^{2+)}$ content presented moderate-high heritability (0.46) (Table 1).

Table 1. The proportion of phenotypic variance explained by genotypes (SNP-based heritability, PVE) and respective standard errors (SE), genetic variances (Vg) and environmental variances (Ve).

Características*	PVE (%)	SE	Vg (%)	Ve (%)
Ca^{2+}	0.4618	0.1289	0.6876	0.3123
WSF7	0.2500	0.1251	0.2743	0.7257
WSF14	0.2064	0.1409	0.4910	0.5089

* Calcium (Ca^{2+}) content, Shear force after seven days of slaughter (WSF7), Shear force after 14 days after slaughter (WSF14).

Genetic Correlations

Ca^{+} content showed negative correlations with WSF7 and WSF14 softness measurements, respectively -0.3683 and -0.7221. WSF7-WSF14 showed a positive correlation of 0.6818 (Table 2).

Table 2. SNPs-based genetic correlation between Ca^{+} content and tenderness measurements.

Traços*	Ca^{2+}	FC7	FC14
Ca	1		
FC7	-0.3683	1	
FC14	-0.7221	0.6818	1

* Calcium (Ca^{2+}) content, Shear force after seven days of slaughter (WSF7), Shear force after 14 days after slaughter (WSF14).

3.2. Bi-trait GWAS

3.2.1. Identifications of candidate loci associated with Calcium and tenderness.

Bi-trait GWAS analysis between Ca^{2+} and WSFC14 by mixed linear model (REML) identified 35 loci after correction for FDR (0.05), distributed in 22 distinct chromosomes (Figure 1). The sum total of additive variance explained by significant SNPs tag was 0.50% for Ca^{2+} and 0.29% for FC14 (Table 3). The markers that individually explained most of the additive variance (top 3) for FC14 were rs42672508 (0.036385 - chr: 26), rs134287398 (0.025425 chr: 11), rs133303384 (0.021801 chr: 19). For Ca^{2+} were rs110429843 (0.039419, chr: 29), rs42730307 (0.028199, chr: 8) and rs132693107 (0.01264, chr: 4) (Table 3).

Of the 35 QTL regions associated with Ca-WSF14 (Table 4), seven were previously reported in studies on shear force and one on calcium content [http://www.animalgenome.org/QTLdb] (Hu et al., 2016). We highlighted the association between WSF14-Ca^{2+} and rs137177538, located on chromosome 29 near the main tenderness-associated gene CAPN1 (p-value = 7.78x10-6) (Table 3).

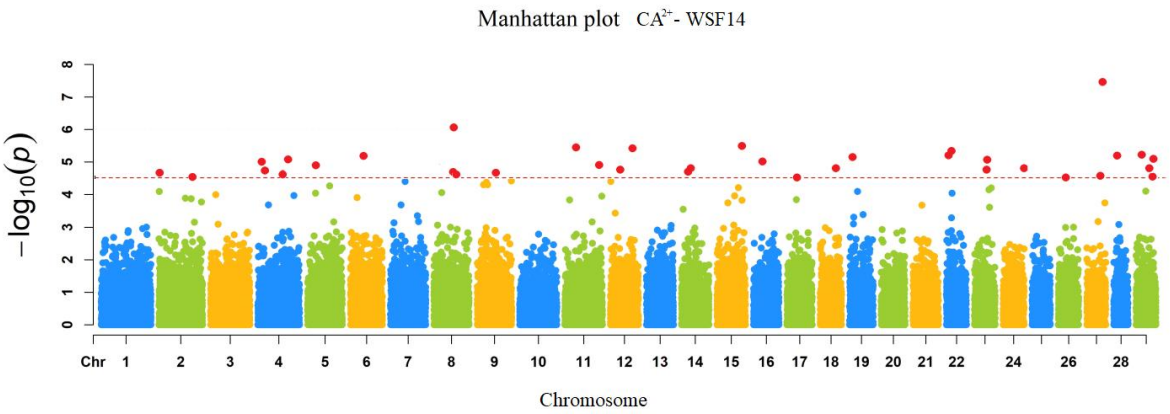


Figure 1. Manhattan plot shows the results of the bi-characteristic GWAS analysis between Ca^{2+}-WSF14 for all autosomal chromosomes. Red dots indicate FDR-corrected significant SNPs tags (0.05); the dashed line indicates the FDR threshold.

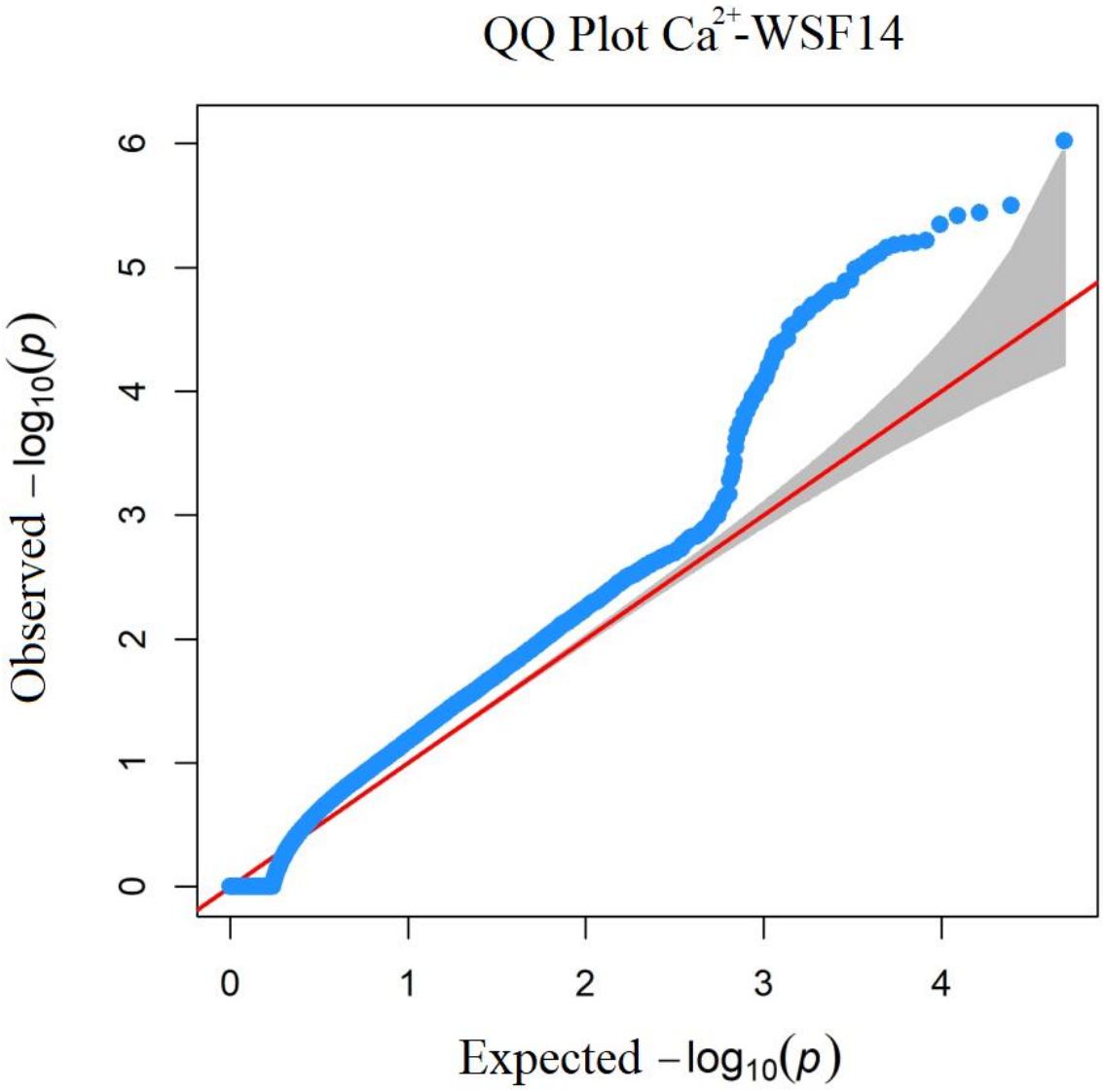


Figure 2. QQ-plot shows the expected distribution based on the χ2 distribution of the marker association tests (X-axis) compared to the observed values (Y-axis).

3.3. Functional Annotation and Enrichment Analysis

We identified essential pathways related to the regulation of muscle metabolism, apoptosis, calcium uptake, among others from the annotations and analysis of functional enrichment of significant regions, summarized in Table 4.

Table 3. Genomic regions identified by bi-trait GWAS associated with meat tenderness and feed efficiency.

Chr	SNP	Freq	Var(%) Ca	Var(%) WSF14	P-valor	FDR	GENE	POSITION
2	rs110920727	0.045	1.88E-05	0.018148	2.12E-05	0.038727	-	intergenic varian
2	rs110706175	0.398	0.007436	0.007162	3.05E-05	0.04286	FN1	upstream (<100k
4	rs132693107	0.279	0.01264	0.014994	8.26E-06	0.033705	MKLN1	upstream (<100k
4	rs43707427	0.2	0.006998	0.020873	1.03E-05	0.033705	FZD1	upstream (<5k)
4	rs110962076	0.252	0.01032	0.014459	1.89E-05	0.038149	COL28A1	intronic variant
4	rs135914321	0.086	0.004808	0.010315	2.29E-05	0.039537	-	intergenic varian
5	rs110316965	0.513	0.006522	0.008024	1.29E-05	0.037492	-	intergenic varian
6	rs109514011	0.455	0.017557	0.003171	6.54E-06	0.033705	RF00156	downstream (<100
8	rs42730307	0.048	0.028199	0.000212	8.51E-07	0.033705	-	intergenic varian
8	rs135577507	0.486	0.014508	0.005901	1.98E-05	0.038149	FRMPD1	intronic variant
8	rs136126550	0.077	0.015078	0.00372	2.37E-05	0.039537	-	intergenic varian
9	rs134725847	0.493	0.015792	0.005299	2.41E-05	0.039537	-	intergenic varian
11	rs134287398	0.478	0.002562	0.025425	3.60E-06	0.033705	MTA3	intronic variant
11	rs110646630	0.33	0.021203	0.000626	1.27E-05	0.037492	FNBP1	intronic variant
12	rs109459993	0.142	0.022956	0.002821	3.80E-06	0.033705	SOX21	upstream (<100k
12	rs132840848	0.45	0.000812	0.021242	1.71E-05	0.038149	FOXO1	downstream (<100
14	rs41724652	0.264	0.020341	0.002678	1.60E-05	0.037586	-	upstream (<100k
14	rs135357966	0.301	0.01962	3.32E-05	2.02E-05	0.038149	FAM91A1	intronic variant
15	**rs110393452***	**0.242**	**0.010142**	**0.011878**	**3.17E-06**	**0.033705**	**C15H11orf96**	**downstream (<25k**
16	rs110071828	0.491	0.019512	0.001382	9.74E-06	0.033705	TGFB2	intronic variant
17	rs109754663	0.189	0.01468	0.000654	2.93E-05	0.042381	PCDH10	upstream (<100k
18	rs109763092	0.487	0.016258	0.005867	1.55E-05	0.037586	PDCD2L	downstream (< 5K
19	rs133303384	0.075	0.005746	0.021801	6.99E-06	0.033705	UBA2	upstream (< 5KB
22	rs110909409	0.186	0.009557	0.019283	4.51E-06	0.033705	HLF	upstream (< 5KB
22	rs41988164	0.324	0.019283	0.001365	6.31E-06	0.033705	-	intergenic varian
23	rs136414328	0.288	0.015072	0.003311	9.04E-06	0.033705	-	intergenic varian
23	rs110921188	0.486	0.018381	0.000709	1.79E-05	0.038149	-	upstream (< 5KB

24	rs42690851	0.079	0.018719	0.00091	1.53E-05	0.037586	SERPINB7	downstream (< 25
26	rs42672508	0.342	0.000281	0.036385	2.78E-05	0.042381	-	upstream (<100k
27	rs134979095	0.423	0.015594	0.004587	2.69E-05	0.042381	FFAR4	intronic variant
28	rs133691060	0.057	0.019283	0.001365	6.37E-06	0.033705	-	intergenic variar
29	rs137786555	0.498	0.014837	0.010773	6.06E-06	0.033705	DLG2	intronic variant
29	rs137177538	0.484	0.021808	1.61E-05	7.78E-06	0.033705	CDC42BPG	3'UTR variant
29	rs110429843	0.117	0.039419	0.00562	1.55E-05	0.037586	KCNJ1	intronic variant
29	rs109331524	0.26	0.017881	0.002254	2.89E-05	0.042381	LGALS12	upstream (<100

Following: chromosome, SNP, allelic frequency, Ca^{2+} genetic variance percentage, WSF14 additive genetic variance percentage, p-value, correction for FDR tests (0.05), gene closest to the marker. Marker position to nearest gene. *In bold cis-eQTL identified in integration analyzes (SMR).

Table 4. Functional Annotation and Enrichment Analysis.

Chr	Tag SNP[a]	Location Tag SNPs[B]	Annotated Genes[D]	GO Terms [E]	Biological Process	Bovine QTL[G]
2	rs110920727	2:3252983	*RF00413*	-	-	shear force - tenderness intramuscular fat content amount of muscle iron internal fat meat fatty acid content Meat flavor intensity
2	rs110706175	2:103508281	*ABCA12,FN1,MREG, ATIC,RF00156, bta-mir-2285l*	GO:0007160 GO:0031589 GO:0051650	Cell Matrix Adhesion Adhesion Cell Substrate Vesicle Formation	muscle iron content average daily weight gain
4	rs132693107	4:94951441	*MKLN1,PODXL,TSGA13,MIR29A, bta-mir-29b-1,RF00396,KLF14*	-	-	milk somatic cell count
4	rs43707427	4:8641977	*CDK14*	-	-	meat tenderness average daily weight gain carcass muscle-bone ratio
4	rs110962076	4:15491229	*ASNS,C1GALT1,RPA3,MIOS, TAC1,COL28A1, RF00001,RF00026,RF00322, UMAD1*	GO:0044249 GO:0009058 GO:0007204	Cell Biosynthesis Process Biosynthetic process Up-regulation of calcium concentration	body weight carcass weight
4	rs135914321	4:75303008	*TNS3*	GO:0006915 GO:0007155 GO:0002376	Apoptosis Cell adhesion Imune system	milk protein content
5	rs110316965	5:16917109	*ENSBTAT00000027699 ENSBTAT00000063977*	-	-	fertility trait net merit milk protein percentage
6	rs109514011	6:49052583	*RF00001 RF00156 RF00019*	-	-	residual feed intake longissimus thoracic muscle area fatty acid content of meat cis-9-C14 back fat thickness internal fat

8	rs42730307	8:56787499	*lncRNA*	-	-	intramuscular fat content body weight
8	rs135577507	8:61741522	*ZBTB5,POLR1E, DCAF10,SLC25A51 EXOSC3, ZCCHC7, SHB, TOMM5, TRMT10B RF00026, bta-mir-2472, bta-mir-2474, bta-mir-2473, FRMPD1, GRHPR, FRMPD1 FBXO10, FRMPD1*	GO:0000959	Mitochondrial Transcription	body weight internal fat fertility trait
8	rs136126550	8:67701240	*LZTS1* *ATP6V1B2* *SLC18A1*	GO:0015931 GO:0001505 GO:0099504	Nucleotide base transport Neurotransmitter Regulation Synaptic vesicle cycle	Shear force - meat tenderness
9	rs134725847	9:56369632	*EPHA7*	GO:0007411 GO:0007169	Protein tyrosine kinase signaling pathway transmembrane receptor Axon Orientation	average daily weight gain
11	rs134287398	11:24901537	*COX7A2L, OXER1, HAAO, PKDCC, MTA3, KCNG3, RF00026*	GO:0016575 GO:0006464	Histone deacetylation Protein Modification Process	milk somatic cell count
11	rs110646630	11:100300518	*TOR1B, PRRX2, GPR107, HMCN2, NCS1, ASB6, NTMT1, PTGES, C11H9orf50, TOR1A, C11H9orf78, USP20, FNBP1, ASS1*	GO:0006693 GO:0043484 GO:0006749	Prostaglandin Metabolic Process Splicing Regulation Glutathione Metabolic Process	-
12	rs109459993	12:69239506	*TGDS,GPR180,SOX21, DCT, RF00004,RF00001,*	GO:0048066 GO:0044550 GO:0019438	Pigmentation Development biosynthetic process of metabolites secondary biosynthetic process of compounds aromatic	Iron content in muscle
12	rs132840848	12:22039988	*VPS36, SLC25A15, NEK5, THSD1, NEK3, MRPS31, CKAP2, RF00056, RF00096, FOXO1*	GO:0043328 GO:0043162 GO:0007039	protein transport to the vacuole involved in the protein catabolic process ubiquitin-dependent	subcutaneous fat thickness milk yield Methane production temperature regulation body
14	rs41724652	14:21352631	*RB1CC1,OPRK1,ST18,NPBWR1 PCMTD1,RF00100, RF00004,RF00026*	GO:0007218	Metabolic pathways of neuropeptidase.	thoracic muscle area response to parasitic infection body weight intramuscular fat content
14	rs135357966	14:16111828	*FAM91A1,ANXA13,FAM83A, WDYHV1,C14H8orf76,ZHX1, FBXO32,KLHL38,FER1L6, TRMT12,TMEM65,ATAD2, RF00100*	GO:0099041 GO:0014894 GO:0014870	vesicles of the golgi regulation muscle adaptation regulation response to muscle inactivity regulation	response to infection parasitic body weight skeletal carcass weight muscle calcium content
15	rs110393452	15:74054213	*HSD17B12,ALKBH3,ACCSL, TTC17,EXT2,MIR129-2, bta-mir-670,ACCS,ALX4, RF00026,C15H11orf96*	GO:0006778 GO:0006725 GO:0006520	metabolic process of porphyrin-containing metabolic process of aromatic compounds metabolic process of amino acids	average daily weight gain

16	*rs110071828*	*16:21813943*	*TGFB2,RRP15,RF00026,* *TGFB2,SPATA17*	GO:0007179 GO:0031323 GO:0043408	beta factor receptor signaling pathway growth regulation of cellular metabolic process cascade regulation from MAPK	shear force - meat tenderness saturated fatty acid meat content carcass weight thoracic muscle area intramuscular fat content
17	*rs109754663*	*17:25123924*	*PCDH10*	GO:0007155	Cell adhesion	meat fatty acid content C18: 3 (n-3) residual feed intake
18	*rs109763092*	*18:44837608*	*LSM14A,PDCD2L,UBA2,* *GPI,WTIP,KIAA0355,* *RF00026,RF00026,RF00003,* *RF00026*	GO:0060968 GO:0001666	gene expression regulation hypoxia response	residual feed intake body weight
19	*rs133303384*	*19:5487036*	*MMD,HLF,TOM1L1,* *COX11,STXBP4,RF00026*	-	-	average daily weight gain subcutaneous fat thickness
22	*rs110909409*	*22:9523719*	*ARPP21,bta-mir-128-2,* *RF00026*	-	-	body weight semen volume
22	*rs41988164*	*22:3614290*	*RBMS3*	-	-	subcutaneous fat thickness Muscle Creatine Content
23	*rs136414328*	*23:29865231*	*TRIM27,ZSCAN12,ZNF311,* *OR5V1,RF00017,ZSCAN12,* *GPX5,OR14J1,RF00026*	-	-	muscle carcass percentage
23	*rs110921188*	*23:30902420*	*ZNF165,ZKSCAN4,ZKSCAN8,ZSCAN16,* *HIST1H2AG,ZNF184,POM121L2,HIST1H2AG,* *HIST1H2BJ,NKAPL,HIST1H2BB,ZNF391,* *OR2B6,PRSS16,ZSCAN9,HIST1H2BN,* *bta-mir-2379,ZNF389,ZKSCAN8,* *PGBD1,ZNF165,ZSCAN26,bta-mir-7857-1*	GO:0006334 GO:0071840 GO:0022607	nucleosome assembly organization of cellular components or biogenesis cellular components assembly	muscle carcass percentage intramuscular fat content
24	*rs42690851*	*24:62025047*	*SERPINB10,SERPINB5,SERPINB13,* *KDSR,VPS4B,BCL2,SERPINB2,* *SERPINB12,SERPINB7,RF00026,* *SERPINB5,VPS4B*	GO:2001242 GO:0044267 GO:0044260	apoptotic signaling pathway regulation protein metabolic process macromolecules metabolic process	Fatty acid content of meat cis-9-C1
26	*rs42672508*	*26:14889689*	*FFAR4,RBP4,PDE6C,FRA10AC1,* *LGI1,CEP55,EXOC6,CYP26A1,* *MYOF,SLC35G1,LGI1,CYP26C1,* *ASMTL,SLC25A6,RF00001*	GO:0006696	ergosterol biosynthetic process	Shear force - meat tenderness
27	rs134979095	27:35204537	*ADAM18,IDO2,IDO1,TCIM,* *ZMAT4,ADAM3A,* *bta-mir-2284q,RF00026*	GO:0009063 GO:0044270 GO:0009108	catabolic process of amino acids catabolic nitrogen process coenzyme biosynthetic process	-

28	rs133691060	28:11372308	*RF00026*, *RF00619*	-	-	body weight amount of muscle zinc carcass weight intramuscular fat content back fat thickness
29	rs137786555	29:11879807	*DLG2*	-	-	scrotal circumference thoracic muscle area intramuscular fat content carcass weight
29	rs137177538	29:43059346	*NRXN2,RASGRP2,PYGM,SF1,MAP4K2,MEN1, ARL2,SNX15,SAC3D1,TIGD3,TM7SF2,ZNHIT2, MRPL49,SYVN1,SPDYC,ATG2A,PPP2R5B, GPHA2,NAALADL1,KCNK4,TRMT112,PRDX5, CAPN1,CDC42BPG,POLA2,BATF2,BAD, ZFPL1,VPS51,RPS6KA4,SLC22A12,SLC22A11, FAU,DPF2,GPR137,ESRRA,bta-mir-192, bta-mir-194-2,CDC42EP2,CDCA5,MAJIN, bta-mir-2407,CDC42BPG,SLC22A12,GPR137, RASGRP2,CCDC88B,SLC22A11,DPF2,SLC22A11, EHD1,TMEM262,SNX15,ZFPL1,SF1,TM7SF2*	GO:0007096	Mitosis regulation	Shear force - meat tenderness Meat succulence
29	rs110429843	29:32223534	*KCNJ1,ETS1,FLI1, ARHGAP32,BARX2,KCNJ5*	GO:0098657	Endocytosis	Body weight subcutaneous fat thickness
29	rs109331524	29:41714651	*LGALS12,CHRM1,SLC22A6,SLC22A8, SLC22A10,SLC22A10,SPINDOC,RTN3, C29H11orf95,SLC22A9,PLA2G16, ATL3,HRASLS5,MARK2*	-	-	Shear force - meat tenderness Meat succulence Creatine content in muscle

3.4. eQTL Analyses

The total of 2005 cis-eQTLs in autosomal chromosomes were identified and annotated, 56% of which were located in intronic regions, 24% in intergenic regions, 8% in gene upstream, 8% in gene downstream, 1% in 3 'UTR regions, 1% synonymous variants and 1% non-coding transcripts.

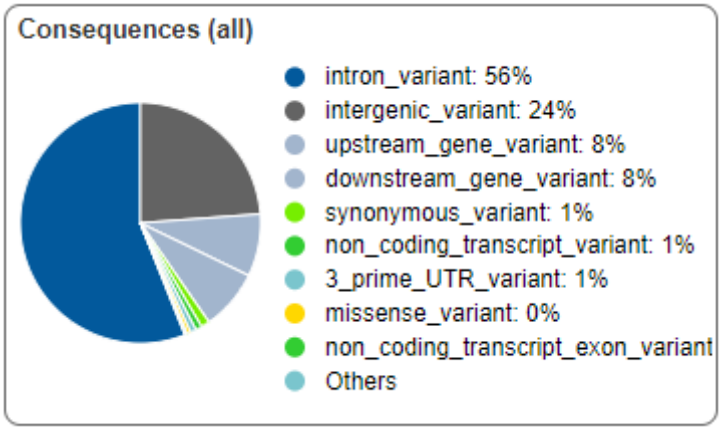


Figure 3. Variant Effect Predict (VEP) annotation of the cis-eQTLS identified in the present study.

3.5. Integration between GWAS and eQTL through Summary data-based Mendelian randomization (SMR)

Given the complexity of the chromosomal regions associated with FC14-Ca^{2+}, made up of numerous nearby genes (Table 4), we used the SMR approach to prioritize genes underlying the markers identified in the Bivariate GWAS.

Two cis-eQTLs (rs110393452 and rs134069047) ranked Top GWAS ($<9x^{10-4}$) were identified, with rs110393452 being the top 2 (FDR 0.05; 3.17 x^{10-6}) (Table 3).

Results indicated that the *alpha-ketoglutarate-dependent dioxygenase ALKB homolog 3* (*ALKBH3*) gene, located 35.35 KB from cis-eQTL rs110393452, is the gene most likely to

contribute to the association with FC14-Ca2 + (pSMR = 1.03 x10-02) in the genomic context shown in Figure 4A.

Similarly occurred with the *Death Associated Protein 3* (*DAP3*) gene located on chromosome 3 at 243KB of rs134069047 (pSMR = 1.60x10-02) (Figure 4B). The other information about the SMR analyzes for these regions are summarized in Table 5.

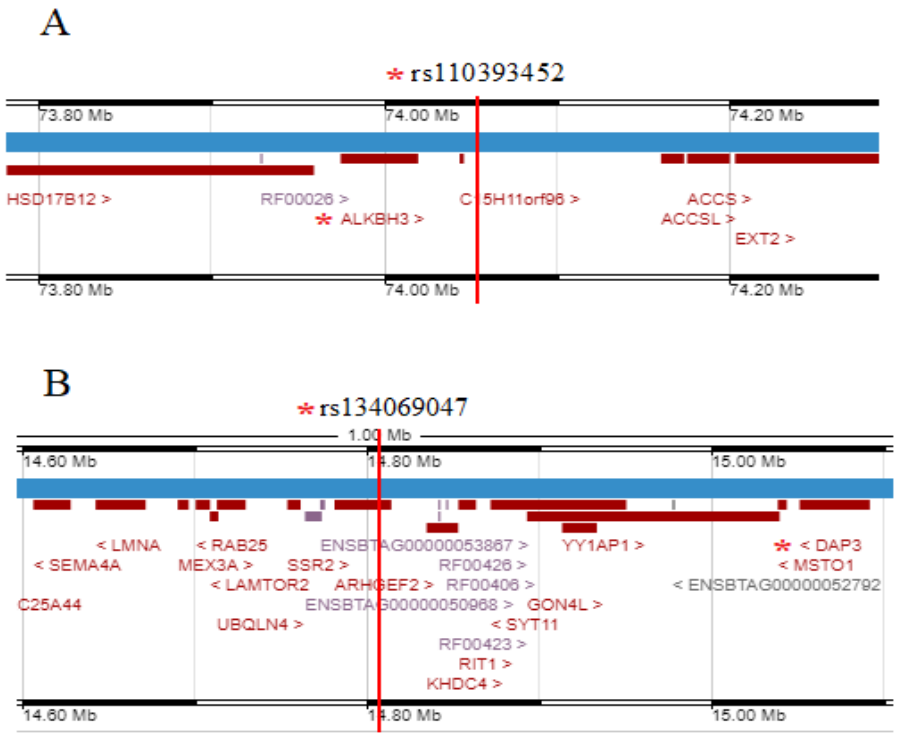


Figure 4. FC14-Ca2 + genomic regions constituted by
by numerous proximal genes. **4A.** Cis-eQTL rs110393452 and candidate gene *ALKH3*. **4B.** Cys-eQTL rs134069047 and candidate gene *DAP3*.

Table 5. Summary statistics of GWAS, eQTL, and SMR for the *DAP3* and *ALKBH3* genes.

Chr[1]	Gene[2]	Cis-eQTL[3]	se_GWAS[4]	p_GWAS[5]	se_eQTL[6]	p_eQTL[7]	se_SMR[8]	p_SMR[9]
3	*DAP3*	rs134069047	0.054618	1.01E-04	0.022429	2.15E-03	1.2804	1.60E-02
15	*ALKBH3*	rs110393452	0.041721	3.17E-06*	0.036588	2.13E-03	0.674707	1.03E-02

[1]Chromossome, [2]Gene associated, [3]cis-eQTL identificated no GWAS, [4]standard error GWAS, [5]p-value GWAS, [6] standard error eQTL, [7]p-value eQTL, [8] standard error SMR, [9]p-value SMR., *FDR (0.05).

To distinguish whether *DAP3* and *ALKBH3* are under the effects of linkage disequilibrium or pleiotropy/casualty (if there is only one causal variant), we performed the heterogeneity in dependent instruments - HEIDI test (0.05) (Zhu et al. , 2016).

Both genes did not pass the HEIDI test (0.05), indicating that these regions are under the influence of linkage disequilibrium (LD).

4. Discussion

The negative correlation for FC7-Ca^{2+} (-0.36) and FC14-Ca^{2+} (-0.72) indicates the responses an artificial selection would be the opposite. These estimates of the responses could be favorable to the improvement since low shear force rates (tender) are associated with high levels of Ca^{2+} content. A possible biological role for these associations is the effect of the calcium-dependent protease on meat tenderness (Koohmaraie, 1996).

Among the regions identified by GWAS Bi-trait, we highlight the association between FC14-Ca2 + and *CAPN1* (p-value = 7.78x10-6) (Table 3). Corroborating the studies carried out by Tizioto et al., (2013) in the same population of Nellore, in which they indicated an association between *CAPN1* and Ca2 + content.

The *CAPN1* gene is located on chromosome 29 and encodes μ-calpain, a protease-activated by Ca2 +, acting on the degradation of myofibrillar proteins in the post-mortem.

Several studies have shown significant associations between *CAPN1* and shear force, suggesting that this gene may be the main responsible for the variation in meat tenderness (Koohmaraie, 1994; Koohmaraie, 1996; Casas et al., 2005; Casas et al., 2006; Curi et al., 2009, Braz et al., 2018).

Also, GWAS revealed six other regions of QTL associated with shear force in other previous studies and one associated with calcium content [http://www.animalgenome.org/QTLdb] (Hu et al., 2016). Additionally, it indicated new regions jointly associated with FC14 and Ca2 + (Table 4).

Given the complexity of the associated regions in GWAS, consisting of numerous adjacent genes (Table 4), we apply the SMR approach, which integrates data from GWAS and eQTL to identify the possible genes that contribute to the modulation of the phenotype from expression (Zhu et al., 2016).

Our results from SMR analyzes revealed the association of tenderness and calcium content with two apoptotic genes *DAP3* (pSMR = 1.60^{x10-2}) and *ALKBH3* (pSMR = 1.03^{x10-2}) (Figure 4).

Most molecular studies of meat tenderness have been dedicated to elucidating the action of calcium-dependent peptidases, such as calpains and cathepsins. However, it is widely accepted that tenderness is a process generated by multiple proteolytic systems, in which the programmed cell death regulation pathways are the first stage in converting muscle to meat (Ouali et al., 2006 and Keppeler et al ., 2019).

DAP3 is responsible for triggering the initial stages of apoptosis. This gene stimulates conformational modification (heterodimerization) from *Pro-caspase 8* to *Caspase 8* (Wazir et al., 2015).

Once active, these caspases trigger the activity of effector caspases such as caspases 3, 6, and 7, capable of cleaving specific substrates, promoting an ordered cascade of proteolytic events that culminate in apoptosis (Wazir et al., 2015).

In contrast, *ALKBH3* is related mainly to fibroblast death and steroid metabolism (Nay et. Al., 2012). Although it has less known mechanisms, *ALKBH3* was associated with the longissimus dorsi area in a study with an independent Nelore population (Silva et al., 2017).

The results presented can contribute to the understanding of the Ca^{2+} dependent proteolytic mechanisms underlying the regulation of meat tenderness during the post-mortem period. This study corroborates the influence of *CAPN1* on tenderness, as well as indicating new candidate genes *DAP3* and *ALKBH3* through an innovative methodology that prioritizes candidates based on the interaction of GWAS and eQTL data.

5. REFERENCES

1. Bhat, Z.F., Morton, J.D., Mason, S.L., Bekhit, A.E.-Din.A. Role of Calpain System in Meat Tenderness: A Review, *Food Science and Human Wellness* (2018).https://doi.org/10.1016/j.fshw.2018.08.002

2. Boef AG, Dekkers OM, le Cessie S. Mendelian randomization studies: a review of the approaches used and the quality of reporting. Int J Epidemiol. 2015;44(2):496–511. doi: 10.1093/ije/dyv071.

3. Braz, C. U., Taylor, J. F., Decker, J. E., Bresolin, T., Espigolan, R., Garcia, D. A., … de Oliveira, H. N. (2018). Polymorphism analysis in genes associated with meat tenderness in Nelore cattle. *Meta Gene,* 18, 73–78.doi:10.1016/j.mgene.2018.08.002

4. Casas, E., White, S.N., Riley, D.G., Smith, T.P., Brenneman, R.A., Olson, T.A., Johnson, D.D., Coleman, S.W., Bennett, G.L., Chase Jr., C.C., (2005). Assessment of single nucleotide polymorphisms in genes residing on chromosomes 14 and 29 for association with carcass composition traits in Bos indicus cattle. *J. Anim. Sci.* 83, 13–19.

5. Casas, E., White, S.N., Wheeler, T.L., Shackelford, S.D., Koohmaraie, M., Riley, D.G., Chase Jr., C.C., Johnson, D.D., Smith, T.P. (2006). Effects of calpastatin and microcalpain markers in beef cattle on tenderness traits. *J. Anim. Sci.* 84, 520–525.

6. Cesar A. S. M., Regitano L. C. A., Reecy J. M., Poleti M. D., Oliveira P. S. N., De Oliveira G. B., et al. (2018). Identification of putative regulatory regions and transcription factors associated with intramuscular fat content traits. *BMC Genomics* 19:499. 10.1186/s12864-018-4871-y

7. Curi, R.A., Chardulo, L.A.L., Mason, M.C., Arrigoni, M.D.B., Silveira, A.C., De Oliveira, H.N., (2009). Effect of single nucleotide polymorphisms of CAPN1 and CAST genes on meat traits in Nellore beef cattle (Bos indicus) and in their crosses with Bos taurus. *Anim. Genet.* 40, 456–462.

8. Diniz, W., J., Mazzoni G., L. L. Coutinho., Banerjee P., Geistlinger, L. Cesar A S. M., Bertolini, F. Afonso,J., De Oliveira,P. S. N. Tizioto, P. C. Kadarmideen. H. N. and Regitano, L. C. A. Detection of Co-expressed Pathway Modules Associated With Mineral Concentration and Meat Quality in Nelore Cattle. 2019. Front. Genet., 13 March 2019 | https://doi.org/10.3389/fgene.2019.00210

9. Durinck, S., Bullard, J., Spellman, P. T., & Dudoit, S. (2009). GenomeGraphs: integrated genomic data visualization with R. BMC Bioinformatics, 10(1). doi:10.1186/1471-2105-10-2

10. Geesink, G.H., Kuchay, S., Chishti, A.H., Koohmaraie, M. (2006). μ-Calpain is essential for postmortem proteolysis of muscle proteins. *J. Anim. Sci.* 84, 2834-2840.

11. Goll, D.E., Thompson, V.F., Li, H.Q., Wei, W., Cong, J.Y. (2003). The calpain system. *Physiol. Rev*. 83, 731-801.

12. Hu, Z. L., Park, C. A., & Reecy, J. M. (2016). Developmental progress and current status of the Animal QTLdb. *Nucleic Acids Research*, *44*(D1), D827–D833. https://doi.org/10.1093/nar/gkv1233

13. Huaiyu Mi, Qing Dong, Anushya Muruganujan, Pascale Gaudet, Suzanna Lewis and Paul D. Thomas 2010. PANTHER version 7: improved phylogenetic trees, orthologs and collaboration with the Gene Ontology Consortium. *Nucl. Acids Res.*. 38: D204-D210.

14. Kemp, C.M., Sensky, P.L., Bardsley, R.G., Buttery, P.J., and Parr, T. 2010. Tenderness—An enzymatic view. *Meat Sci.* 84:248–256.

15. Koohmaraie, M., (1994). Muscle proteinases and meat aging. *Meat Sci.* 36, 93–104.

16. Koohmaraie, M. (1996). Biochemical factors regulating the toughening and tenderization processes of meat. *Meat Sci.* 43, 193–201.

17. Koohmaraie, M., Kent, M.P., Shackelford, S.D., Veiseth, E., Wheeler, T.L. (2002). Meat tenderness and muscle growth: is there any relationship*? Meat Science* 62, 345-352.

18. Koohmaraie, M.; Geesink, G.H. (2006). Contribution of postmortem muscle biochemistry to the delivery of consistent meat quality with particular focus on the calpain system. *Meat Sci.* 74, 34-43.

19. Ouali, A.,Herrera-Mendez, C.H., Coulis, G., Becila, S. Boudjellal, A., Aubry, L., Sentandreu, M.A. (2006). Revisiting the conversion of muscle into meat and the underlying mechanisms. *Meat Sci.*74(1):44–58.

20. Purcell, S., Neale, B., Todd-Brown, K., Thomas, L., Ferreira, M. A. R., Bender, D., … Sham, P. C. (2007). PLINK: A Tool Set for Whole-Genome Association and Population-Based Linkage Analyses. *The American Journal of Human Genetics*, *81*(3), 559–575. https://doi.org/10.1086/519795

21. McCarthy, M.I. and Hirschhorn, J.N. (2008) Genome-wide association studies: potential next steps on a genetic journey. Hum Mol Genet, 17, R156-165.

22. Nay, S.L., Lee, D.H., Bates, S.E., O'Connor, T.R. (2012) Alkbh2 protects against lethality and mutation in primary mouse embryonic fibroblasts. *DNA Repair* (Amst). 11(5), 502–510.

23. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning A Laboratory Manual. 2nd Edition, Cold Spring Harbor Laboratory Press, New York.

24. Silva, R. M. de O., Stafuzza, N. B., Fragomeni B, de O., Camargo G., Ceacero T. M. F. de¸alves Cyrillo J, et al. (2017) Genome-Wide Association Study for Carcass Traits in an Experimental Nelore Cattle Population. *PLoS ONE* 12(1): e0169860. doi:10.1371/journal. pone.0169860

25. Tizioto PC, Taylor JF, Decker JE, Gromboni CF, Mudadu MA, Schnabel RD, Coutinho LL, Mourão GB, Oliveira PS, Souza MM. Detection of quantitative trait loci for mineral content of Nelore longissimus dorsi muscle. Genet Sel Evol. 2015;47(1):1.

26. Tizioto, P.C., Gromboni, C.F., de Araujo Nogueira, A.R., de Souza, M.M., de Alvarenga Mudadu, M., Tholon, P., Rosa, A.N., Tullio, R.R., Medeiros, S.R., and Nassu, R.T. 2014. Calcium and potassium content in beef: Influences on tenderness and associations with molecular markers in Nellore cattle. *Meat Sci.* 96:436–440.

27. Tizioto, P. C. ; Decker, J. E. ; Taylor, J. F. ; Schnabel, R. D. ; Mudadu, M. A. ; Silva, F. L. ; Mourao, G. B. ; Coutinho, L. L. ; Tholon, P. ; Sonstegard, T. ; Rosa, A.N. ; Alencar, M.M. ; Tullio, R.R. ; Medeiros, S.R. ; Nassu, R. T. ; Feijó, G.L.D. ; Silva, L.O.C. ; Torres, R.A.A. ; Siqueira, F. ; Higa, R. H. ; Regitano, L.C.A. 2013. A genome scan for meat quality traits in Nelore beef cattle. Physiological Genomics (Print), p. 1012-1020.

28. Shabalin AA. Matrix eQTL: ultra fast eQTL analysis via large matrix operations. Bioinformatics. 2012;28(10):1353–1358. doi: 10.1093/bioinformatics/bts163.

29. Veturi, Y., & Ritchie, M. D. How powerful are summary-based methods for identifying expression-trait associations under different genetic architectures? In:Pacific Symposium on Biocomputing, 23. Hawaii, USA (2018), pp. 228-239

30. Zhou, X., & Stephens, M. (2012). Genome-wide efficient mixed model analysis for association studies. *Nature Genetics*, 44(7): 821–824. https://doi.org/10.1038/ng.2310.Genome-wide

31. Zhou, X. (2014). GEMMA User Manual, 1–27.

32. Zhu Z, Zhang F, Hu H, Bakshi A, Robinson MR, Powell JE, Montgomery GW, Goddard ME, Wray NR, Visscher PM & Yang J (2016) Integration of summary data from GWAS and eQTL studies predicts complex trait gene targets. *Nature Genetics*, 48(1): 481-487.

33. Zinn, R.A., and Shen, Y. 1996. Interaction of dietary calcium and supplemental fat on digestive function and growth performance in feedlot steers. *J. Anim. Sci.* 74:2303–2309.

34. Wazir, U., Sanders, A.J., Wazir, A.M., Ye, L., Jiang, W.G., Ster, I.C. ... Mokbel, K. (2015). Effects of the knockdown of death-associated protein 3 expression on cell adhesion, growth and migration in breast cancer cells. Oncology Reports, 33, 2575-2582. https://doi.org/10.3892/or.2015.382

FINAL CONSIDERATIONS

The identification of QTLs simultaneously affecting pairs of traits studied may explain part of the genetic correlations existing among complex phenotypes. Thus, the approach used proved to be satisfactory when estimating the association of relevant traits to bovine breeding, as well as indicating new candidate genes and metabolic pathways that may explain part of the additive variance of these phenotypes.

Integrative approaches of TWAS contributed to corroborate the influence of genes reported in GWAS and can be a potential tool to understand complex QTL regions. This new approach identifies loci that modulate of phenotype from gene expression, targeting new strategies research on animal genomic selection.