**University of São Paulo
“Luiz de Queiroz” College of Agriculture**

**Identification of CNVs in the Nelore genome and its association with
meat tenderness**

Vinicius Henrique da Silva

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

**Piracicaba
2015**

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**Identification of CNVs in the Nelore genome and its association with meat
tenderness**

versão revisada de acordo com a resolução CoPGr 6018 de 2011

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“From the conception the increase, from the increase the swelling, from the swelling the thought, from the thought the remembrance, from the remembrance the consciousness, the desire.”

Maori philosophy

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RESUMO

Identificação de CNVs no genoma de bovinos da raça Nelore e suas associações com maciez da carne

A raça Nelore é predominante no rebanho zebuino brasileiro (*Bos taurus indicus*). A grande adaptabilidade da raça Nelore ao clima tropical brasileiro, no entanto, não está associada à maciez de carne (MT). Sabe-se que MT é influenciada por vários fatores ambientais e pela composição genética. Foi realizada uma análise de todo o genoma para inferir Variação no Número de Cópias de Segmentos Genômicos (*Copy Number Variation* - CNV) a partir de dados oriundos de chip de SNP (Illumina® Bovine High Density), para uma população de 723 machos Nelore, incluindo 30 ancestrais da população. Foram detectadas >2600 regiões de CNV (CNVRs) representando $\approx 6.5\%$ do genoma bovino. O tamanho médio do CNVR foi de 65 kb, variando de 5 kb até 43 Mb. Um total de 1155 CNVRs (43.6%) obtiveram sobreposição com 2750 genes. Estes genes foram enriquecidos para as funções importantes, tais como resposta imunológica, recepção olfativa e processos que envolvem o trifosfato de guanosina (GTP). As vias metabólicas do GTP conhecidamente influenciam a fisiologia e a morfologia do músculo esquelético. Loci de características quantitativas (QTLs) para MT, alguns específicos para Nelore, sobrepueram uma fração substancial das CNVRs encontradas. Dois CNVRs foram encontrados em região proximal à genes do metabolismo da glutathione os quais também são associados com MT. Comparando os resultados com estudos anteriores ≈ 1400 CNVRs (>50%) foram sobrepostos. Nove CNVRs em regiões associadas com MT foram validados nos 30 ancestrais por qPCR. Em conclusão, foram identificadas regiões genômicas de variação estrutural no Nelore, com potenciais implicações sobre o fenótipo MT. No segundo capítulo, um total de 34 animais da população foi submetido à análise do transcriptoma e análise de potencial genético para MT. Foram identificados 170 fragmentos de CNV (CNVFs) mapeados em 20 CNVRs, os quais mostraram frequências significativamente diferentes entre animais com potencial genético para carne mais dura ou mais macia. Uma fração considerável dos CNVFs identificados afetaram a expressão gênica de genes MT (anteriormente descritos como associados à MT ou fisiologia do músculo esquelético), os quais desempenham um papel importante no metabolismo de glicogênio, volume do tecido conjuntivo, transportadores de membrana e vias metabólicas da glutathione. Um número considerável de CNVRs foram associados à expressão de genes sobrepostos e nas proximidades, onde o aumento ou diminuição do número de cópias foi associado com a mudança na expressão gênica. Dois CNVRs associados foram mapeados para os cromossomo 12 e 23, estando próximos a QTLs anteriormente descritos para MT na raça Nelore. Vários CNVFs, entre animais com potencial genético para carne mais macia ou dura, mostraram diferenças significativas na expressão gênica. Essas regiões estão ligadas a importantes funções biológicas com influências altamente relevantes para MT e para a fisiologia do músculo esquelético.

Palavras-chave: SNP-chip; RNA-seq; Integração de dados; Pangenômico

ABSTRACT

Identification of CNVs in the Nelore genome and its association with meat tenderness

The Nelore breed represents the vast majority of Brazilian Zebuine cattle (*Bos taurus indicus*). The great adaptability of the Nelore breed to Brazilian tropical climate, however, is not associated with meat tenderness (MT). It is known that MT is influenced by several environmental factors, but also genetic composition. In the first chapter, we report a genome-wide analysis of copy number variation (CNV) inferred from Illumina® Bovine High Density SNP-chip data for a Nelore population of 723 males including 30 sires. We detected >2600 CNV regions (CNVRs) representing ≈6.5% of the *Bos taurus* genome. The CNVR size was 65 kb on average, ranging from 5 kb to 4.3 Mb. A total of 1155 CNVRs (43.6%) overlapped 2750 genes. They are enriched for important functions such as immune response, olfactory reception and processes involving guanosine triphosphate (GTP). The GTP processes have known influence in skeletal muscle physiology and morphology. Quantitative trait loci for MT, partly specific for Nelore, overlapped a substantial fraction of CNVRs and two CNVRs were found proximal to glutathione metabolism genes that are associated with MT as well. Comparing our results with previous studies revealed an overlap in ≈1400 CNVRs (>50%). We selected 9 CNVRs that overlapped regions associated with MT and we validated them in all 30 sires by qPCR. There was identified many genomic regions of structural variation in Nelore with important implications on the MT phenotype. In the second chapter, a total of 34 animals of the population were subjected to transcriptome analysis and meat tenderness (MT) phenotyping. We identified 170 CNV fragments (CNVFs) residing in 20 CNVRs, which occurred in different frequencies between animals with tougher and softer meat genetic potential. A considerable fraction of the identified CNVFs affected gene expression of the MT genes, which play important roles in glycogen metabolism, connective tissue turnover, membrane transporters and glutathione pathways. We also detected that several CNVRs substantially influenced the expression of overlapped and nearby genes, where the increase or decrease of copy number correlated well with the change in gene expression. Among them are two CNVRs at chromosomes 12 and 23, which are in the vicinity of previously described QTLs for MT in Nelore breed. Several CNVFs, which are more frequent in animals with genetic potential for softer or tougher MT, showed significant differences in gene expression. Those regions are linked to important biological functions with highly relevant influences on MT and skeletal muscle physiology.

Keywords: SNP-chip; RNA-seq; Data integration; Genome-wide

1 INTRODUCTION

Bos taurus is a model organism of great agricultural importance and was the first livestock species for which the complete genome was sequenced (ELSIK et al., 2009; HERATH et al., 2006). Brazil has a large cattle production industry with a herd of estimated 209.541 million head (INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA (IBGE), 2010), being the world's largest beef exporter (INDEX MUNDI, 2014).

The Nelore (*Bos taurus indicus*) is the main cattle breed in Brazil (OLIVEIRA et al., 2014). Nelore animals, like most *Bos indicus* subspecies, are highly adapted to the tropical Brazilian climate but do not have a comparable meat tenderness (MT) as *taurus* breeds (*Bos taurus taurus*) (CONNOR et al., 1997). The MT phenotype was first investigated in the 1920s (WHEELER; SHACKELFORD; KOOHMARAIE, 1997), and is already well-studied in several livestock species (LOMIWES et al., 2014; NONNEMAN et al., 2013; OUALI et al., 2013; PURSLOW; ARCHILE-CONTRERAS; CHA, 2012; WANG et al., 2014; WESTON; ROGERS; ALTHEN, 2002). An established measure of MT is shear force and *Bos taurus indicus* typically requires a higher shear force compared with *Bos taurus taurus* (HOCQUETTE et al., 2006). Larger muscle fibers, cross-bridges between filaments and reduced myofibrillar proteolysis are previously reported features that influence MT of Zebu breeds (CORÓ; YOUSSEF; SHIMOKOMAKI, 1999; WHIPPLE et al., 1990). Stress induced by genetic or environmental sources are also known to negatively affect MT (ZHAO et al., 2012).

Nelore and its crossbreds are widely used in Brazilian beef cattle production and these beef products are exported and consumed in more than 100 countries (PRAKASH; STIGLER, 2012). Since the meat quality is lower mainly due to less tenderness, there is an increasing interest to better understand the molecular biology involved in tenderness traits (MALTIN et al., 2007).

Structural genetic variation associated with traits of interest are promising targets for genetic improvement (BICKHART; LIU, 2014). Frequently observed is copy number variation (CNV) and is, thus, increasingly researched in cattle (BAE et al., 2010; CICCONARDI et al., 2013; HOU et al., 2011, 2012b, 2012c, 2012a; JIANG et al., 2012, 2013; MATUKUMALLI et al., 2009; SEROUSSI et al., 2010). CNVs are

defined as large genomic regions (conventionally >1 kb) with deviation from the normal diploid state due to duplication or deletion events (LIU; BICKHART, 2012).

CNVs are associated with several important phenotypes in humans (KOHLER et al., 2012; PFUNDT; VELTMAN, 2012; VINCENT et al., 2011) and livestock animals (ELFERINK et al., 2008; NORRIS; WHAN, 2008; PAILHOUX et al., 2001). In cattle, anhidrotic ectodermal and renal tubular dysplasia (HIRANO, 2000) as well as osteopetrosis (MEYERS et al., 2010) and birth defects (FLISIKOWSKI et al., 2010) have been previously associated with CNVs. A CNV in the gene encoding CASL-like protein 2 (*MICAL-L2*), which performs a critical role in the development of muscle fibers, is correlated with gene expression in cattle making it an important molecular marker (XU et al., 2013).

The experimental detection of CNVs on a large scale can be done using comparative genomic hybridization (CGH, (PINKEL et al., 1998)) or next-generation sequencing (CNV-seq, (KOBOLDT et al., 2012)). Although these are the most accurate methods to detect CNVs, it is still expensive to apply them for many samples (ALKAN; COE; EICHLER, 2011). Thus, several studies in cattle inferred CNVs from SNP-chip data with subsequent validation of selected regions by quantitative polymerase chain reaction (qPCR) (BAE et al., 2010; CICCONARDI et al., 2013; HOU et al., 2011, 2012b, 2012c, 2012a; JIANG et al., 2012, 2013; MATUKUMALLI et al., 2009; SEROUSSI et al., 2010).

Transcriptome studies using next-generation sequencing (RNA-seq) allow a global analysis of transcripts (MORTAZAVI et al., 2008). RNA-seq allows more precise measurements of the level of transcripts and their isoforms as compared to other techniques, such as microarrays (WANG; GERSTEIN; SNYDER, 2009).

Gene transcription can be affected by CNVs especially in overlapped or nearby genes (LEE et al., 2006; MCCARROLL et al., 2006; SOMERVILLE et al., 2005; STRANGER et al., 2007). Particularly for cattle, a CNV in *CASL-like protein 2 gene* (*MICAL-L2*), which performs a critical role in the development of muscle fiber, is correlated with gene expression (XU et al., 2013). CNVs can be thus important molecular markers.

In the first chapter, we inferred CNVs in the genome of Nelore cattle, concatenated into CNV regions (CNVRs) and validated a subset with qPCR. We report identified MT-genes and quantitative trait loci (QTLs) overlapping our and

already known CNVRs to establish important links between structural genomic variation and its association with MT.

In the second chapter, was integrated CNV information and gene expression in genomic regions containing structural variations that are more frequent in animals with genetic potential for softer or tougher meat. Subsequently, we discuss the impact of detected differentially expressed (DE) genes on several pathways associated with MT.

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2 GENOME-WIDE DETECTION OF MEAT-RELEVANT CNVS FROM SNP-CHIP DATA IN NELORE CATTLE BREED

Resumo

A raça Nelore é predominante no rebanho zebuino brasileiro (*Bos taurus indicus*). A grande adaptabilidade da raça Nelore ao clima tropical brasileiro, no entanto, não está associada a maciez de carne (MT). Sabe-se que MT é influenciada por vários fatores ambientais e pela composição genética. Foi realizada uma análise de todo o genoma para inferir *Copy Number Variation* (CNV) a partir de dados oriundos de SNP chip (Illumina® Bovine High Density), para uma população de 723 machos Nelore, incluindo 30 ancestrais da população. Detectamos >2600 regiões de CNV (CNVRs) representando ≈6.5% do genoma bovino. O tamanho médio do CNVR foi de 65 kb, variando de 5 kb até 43 Mb. Um total de 1155 CNVRs (43.6%) obtiveram sobreposição com 2750 genes. Estes genes foram enriquecidos para as funções importantes, tais como resposta imunológica, recepção olfativa e processos que envolvem o trifosfato de guanosina (GTP). As vias metabólicas do GTP conhecidamente influenciam a fisiologia e a morfologia do músculo esquelético. Loci de características quantitativas (QTLs) para MT, alguns específicos para Nelore, sobrepueram uma fração substancial das CNVRs encontradas. Dois CNVRs foram encontrados em região proximal à genes do metabolismo da glutathione os quais também são associados com MT. Comparando os resultados com estudos anteriores ≈1400 CNVRs (>50%) foram sobrepostos. Nove CNVRs em regiões associadas com MT foram validados nos 30 ancestrais por qPCR. Em conclusão, foram identificadas muitas regiões genômicas de variação estrutural no Nelore, com implicações potencialmente importantes sobre o fenótipo MT.

Palavras-chave: Variação estrutural; Validação; Genômica; Estudo comparativo

Abstract

The Nelore breed represents the vast majority of Brazilian Zebuine cattle (*Bos taurus indicus*). The great adaptability of the Nelore breed to Brazilian tropical climate, however, is not associated with meat tenderness (MT). It is known that MT is influenced by several environmental factors, but also genetic composition. In this article, we report a genome-wide analysis of copy number variation (CNV) inferred from Illumina® SNP-chip data for a Nelore population of 723 males including 30 sires. We detected >2600 CNV regions (CNVRs) representing ≈6.5% of the genome. The CNVR size was 65 kb on average, ranging from 5 kb to 4.3 Mb. A total of 1155 CNVRs (43.6%) overlapped 2750 genes enriched for important functions such as immune response, olfactory reception and processes involving *guanosine triphosphate* (GTP). The GTP processes have known influence in skeletal muscle physiology and morphology. Quantitative trait loci for MT, previously identified in Nelore, overlapped a substantial fraction of CNVRs, including two found proximal to glutathione metabolism genes that are associated with MT as well. Comparing our results with previous studies revealed an overlap in ≈1400 CNVRs (>50%). We selected 9 CNVRs that overlapped regions associated with MT and validated them in all 30 sires by qPCR. In conclusion, we identified several genomic regions of structural variation in Nelore, and some of them may influence the MT phenotype.

Keywords: Structural variation; Validation; Genomics; Comparative study

2.1 Introduction

Bos taurus is a model organism of great agricultural importance and was the first livestock species for which the complete genome was sequenced (ELSIK et al., 2009; HERATH et al., 2006). Brazil has a large cattle production industry with a herd of estimated 209.541 million head (INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA (IBGE), 2010), being the world's largest beef exporter (INDEX MUNDI, 2014). The Nelore (*Bos taurus indicus*) is the main cattle breed in Brazil (OLIVEIRA et al., 2014). Nelore animals, like most *Bos indicus* subspecies, are highly adapted to the tropical Brazilian climate but do not have a comparable meat tenderness (MT) as taurine breeds (*Bos taurus taurus*) (CONNOR et al., 1997). The MT phenotype was first investigated in the 1920s (WHEELER; SHACKELFORD; KOOHMARAIE, 1997), and is already well-studied in several livestock species (LOMIWES et al., 2014; NONNEMAN et al., 2013; OUALI et al., 2013; PURSLOW; ARCHILE-CONTRERAS; CHA, 2012; WANG et al., 2014a; WESTON; ROGERS; ALTHEN, 2002). An established measure of MT is shear force and *Bos taurus indicus* typically requires a higher shear force compared with *Bos taurus taurus* (HOCQUETTE et al., 2006). Larger muscle fibers, cross-bridges between filaments and reduced myofibrillar proteolysis are previously reported features that influence MT of Zebu breeds (CORÓ; YOUSSEF; SHIMOKOMAKI, 1999; WHIPPLE et al., 1990). Stress induced by genetic or environmental sources are also known to negatively affect MT (ZHAO et al., 2012a).

Structural genetic variation associated with traits of interest are promising targets for genetic improvement (BICKHART; LIU, 2014). Frequently observed is copy number variation (CNV) which is increasingly researched in cattle (BAE et al., 2010; CICCONARDI et al., 2013; HOU et al., 2011, 2012a, 2012b, 2012c; JIANG et al., 2012, 2013; MATUKUMALLI et al., 2009; SEROUSSI et al., 2010). CNVs are defined as large genomic regions (conventionally >1 kb) with deviation from the normal diploid state due to duplication or deletion events (LIU; BICKHART, 2012).

CNVs are associated with several important phenotypes in humans (KOHLER et al., 2012; PFUNDT; VELTMAN, 2012; VINCENT et al., 2011) and livestock animals (ELFERINK et al., 2008; NORRIS; WHAN, 2008; PAILHOUX et al., 2001). In cattle, anhidrotic ectodermal and renal tubular dysplasia (HIRANO, 2000) as well as osteopetrosis (MEYERS et al., 2010) and birth defects (FLISIKOWSKI et al., 2010)

have been previously associated with CNVs. A CNV in the gene encoding CASL-like protein 2 (MICAL-L2), which performs a critical role in the development of muscle fibers, is correlated with gene expression in cattle making it an important molecular marker (XU et al., 2013).

The experimental detection of CNVs on a large scale can be done using comparative genomic hybridization (CGH, PINKEL et al., 1998) or next-generation sequencing (CNV-seq, (KOBOLDT et al., 2012)). Although these are the most accurate methods to detect CNVs, it is still expensive to apply them for many samples (ALKAN; COE; EICHLER, 2011). Thus, several studies in cattle inferred CNVs from SNP-chip data with subsequent validation of selected regions by quantitative polymerase chain reaction (qPCR) (BAE et al., 2010; CICCONARDI et al., 2013; HOU et al., 2011, 2012a, 2012b, 2012c; JIANG et al., 2012, 2013; MATUKUMALLI et al., 2009; SEROUSSI et al., 2010).

In this article, we infer CNVs in the genome of Nelore cattle, concatenate them into CNV regions (CNVRs) and validate a subset with qPCR. We identified MT associated genes and quantitative trait loci (QTLs) overlapping our and already known CNVRs to establish important links between structural genomic variation and its association with MT.

2.2 Materials and methods

2.2.1 Ethical statement

Animals were handled and managed according to the guidelines of the Institutional Animal Care and Use Committee of the Brazilian Agricultural Research Corporation (EMBRAPA) as approved by its director Dr. Rui Machado.

2.2.2 Population and genotyping

The population under study is comprised of 777 Nelore males including 30 population sires. For SNP-array analysis, genomic DNA samples were extracted from semen straws (sires) and blood (offspring). All animals were genotyped using Illumina Bovine HD Beadchip arrays (>770,000 SNP markers).

The call rate (percentage of successfully genotyped SNPs for a given animal) threshold was >95%.

2.2.3 CNV calling

Individual CNVs were called using PennCNV (WANG et al., 2007). PennCNV incorporates Log R Ratio (LRR) and B Allele Frequency (BAF), which respectively denote the log₂ ratio of R (normalized total intensity of 2 SNP alleles) and the frequency of allele B defined as the normalized intensity ratio for each SNP allele.

Regarding SNP-array genomic wave known bias (DISKIN et al., 2008), all individual LRR values were genomic wave corrected by "*genomic_wave.pl -adjust*", considering a genomic window of 1 kb (500 bp up and downstream of each investigated SNP). Sexual chromosomes were excluded from analysis.

Samples with >150 CNV calls ("*-qcnumcnv 150*") and CNV calls <5kb ("*-length 5k*") were excluded to avoid biased samples and CNV calls, respectively. Default settings were used for B allele frequency drift (BAF_drift) and quality control waviness factor (QC_WC). LRR standard deviation (LRR_SD) was set <0.3 ("*-qclrrsd 0.3*") as previously suggested (WAIN et al., 2009).

2.2.4 CNVR compilation

Filtered individual calls from PennCNV were concatenated into CNV regions (CNVRs) using CNVRuler (KIM et al., 2012). Genomic areas with density <10% were excluded (option "recurrence 0.1"). The recurrence trims the CNVR based on its frequency to avoid false positive predictions. Additionally, the option "*Gain/Loss separated regions*" was used to compile CNVRs based on their genotype (gain or loss). Overlapping "gain" and "loss" CNVRs were merged into single regions to account for genomic regions in which both events can occur ("both" CNVRs in the following).

2.2.5 Validation by qPCR

Quantitative PCR was carried out for nine CNVRs in all sires of our population. The CNVRs were selected since we found them to contain SNPs that are associated with MT or are located near to MT-related QTLs and genes.

Primers were designed using Primer3plus (UNTERGASSER et al., 2007) and quality testing was performed with Net Primer (<http://www.premierbiosoft.com/netprimer>). In the genomic region represented by the primer, the presence of SNPs from SNPdb (SHERRY, 2001) was checked against Ensembl-Biomart (<http://www.ensembl.org/biomart/martview>) (KINSELLA et al.,

2011). To ensure qPCR accuracy, only primers that did not contain SNPs from SNPdb were synthesized.

All primers were tested in serial dilutions of pooled genomic DNA to achieve optimal qPCR conditions. A qPCR solution of 10 μ l was used consisting of 5.0 μ l SYBR Green 2x (Roche®), 0.5 μ l forward primer (5 mM), 0.5 μ l reverse primer (5 mM) and 4.0 μ l of genomic DNA (2.5 ng/ μ l). The qPCR steps were as follows: 1) 95 °C for 5 min, 2) 40 repetitions of 95 °C and 51-60 °C (primer-dependent), 3) 72 °C for 10-15 seconds (primer dependent). Each animal was tested in technical triplicates carried out in Light cycle 480 (Roche®).

Cycle thresholds ($\log_2$ Ct) were corrected by primer mean efficiency as calculated with LinReg (RAMAKERS et al., 2003). The Δ Ct denotes Ct of targeted region minus Ct of control region (primer pair targeting the *BTF3* gene, which has been used before as a control gene for CNV studies in cattle (BAE et al., 2010; JIANG et al., 2013). The $\Delta\Delta$ Ct was calculated as Δ Ct from the animal to be tested minus Δ Ct of a diploid (2n) control (D'HAENE; VANDESOMPELE; HELLEMANS, 2010). This Δ Ct control value was estimated based on Δ Ct average value from all 2n animals (estimated with PennCNV).

The copy number estimation was obtained by the normalized ratio (NR): $2^{x2(\Delta\Delta Ct)}$ (JIANG et al., 2013). Copy number states were categorized as 1n (partial deletion), 2n (normal state), 3n (one copy gain), 4n (two copy gains) and 5n (more than two copy gains) based on the geometric average of two states (D'HAENE; VANDESOMPELE; HELLEMANS, 2010). Lack of amplification was considered as 0n (complete deletion).

In PennCNV, each CNV is typically assigned to one of four possible states: 0n (complete deletion), 1n (partial deletion), 2n (normal state), 3n (one copy gain), and 4n (two copy gains). As qPCR can result in >4n copies (see above), a frequently used simplification is to separate all states in just two CNV types: gain or loss (JIANG et al., 2013; LIU et al., 2010).

Accurate CNV detection can be biased due to biological or technical variation (CAHAN et al., 2009; FADISTA et al., 2010; REDON et al., 2006). Moreover, qPCR amplicons usually represent only a fragment of the CNVR. Hence, to consider a sample as concordant, we required it to be detected by PennCNV (≥ 1 CNV call) as well as qPCR (≥ 1 CNVR primer pair) in the same CNV type (loss or gain). CNVRs with ≥ 1 concordant sample were considered as validated.

2.2.6 Annotation (QTLs, genes and enrichment)

CNVRs were screened for cattle QTLs from cattleQTLdb (HU et al., 2013), specific MT-QTLs in this Nelore population (TIZIOTO et al., 2013), genes annotated in Biomart-Ensembl (GUBERMAN et al., 2011), and 25,620 predicted target transcripts of 676 miRNAs listed in the Microcosm Targets database, version 5 (GRIFFITHS-JONES et al., 2006).

The genes overlapping CNVRs were subjected to an enrichment analysis with DAVID (HUANG; SHERMAN; LEMPICKI, 2009) to identify metabolic pathways of Nelore that are predominantly associated with genes prone to CNVs. The enrichment analysis was carried out for the subset of regions in which CNVs occurred frequently (per definition in >1% of the population, region of polymorphic CNV).

2.2.7 Comparison with previous studies

The detected CNVRs (UMD_3.1 genomic coordinates, ZIMIN et al., 2009) were compared to CNVRs from 14 previous cattle studies (BAE et al., 2010; BICKHART et al., 2012; CHOI et al., 2013; CICCONARDI et al., 2013; FADISTA et al., 2010; HOU et al., 2011, 2012a, 2012b, 2012c; JIANG et al., 2013, 2012; KIJAS et al., 2011; LIU et al., 2010; STOTHARD et al., 2011; ZHAN et al., 2011) and structural variation data for bovine from Ensembl (KINSELLA et al., 2011). From those, eleven were Btau_4.0 -> UMD_3.1 liftOver converted [<https://genome.ucsc.edu/cgi-bin/hgLiftOver>]. Two CNVRs from two different studies that overlapped >70% with each other were considered as detected by both studies. CNVRs from external sources are denoted as known CNVRs in this article.

2.3 Results

2.3.1 A genome-wide map of CNV regions in Nelore

We detected 2,649 CNVRs based on SNP genotype data from 723 male Nelore animals (see Material and Methods for genotyping, CNV calling and CNVR concatenation). The CNVRs represent 6.4% of the *Bos taurus* genome (170.6 Mb). The chromosomal proportion covered by CNVRs varies significantly from 2.3% (BTA22) to 19.7% (BTA15).

Regarding CNV type, the number of regions with copy loss and gain are 1,455 and 891, respectively. Occurrence of both types are observed for 304 regions. The CNVR size is 64.4 kb on average, ranging from 5 kb (minimum threshold) to 4.3 Mb. For each CNVR, the relative frequency of animals with a certain CNV overlapping ranges from 0.1% (1 of 723) to 99.8% (722 of 723). CNVRs with 5 kb < size < 50 kb represent the majority of our findings (71.4%) while CNVRs >1 Mb are rarely observed (0.5%, Figure 1).

Figure 1 - Distribution of CNVR length detected for Nelore

We found 521 regions in which CNVs are polymorphic (per definition in >1% of the population; denoted as 'regions of polymorphic CNV' in the following) representing 3.2% of the *Bos taurus* genome (86.4 Mb, Figure 2).

Figure 2 - Chromosomal distribution of 521 regions in which frequently CNVs occurred (>1% of the population). The regions were categorized into loss or gain of copy number and both states

When comparing our CNVRs to previously reported cattle CNVRs (denoted as 'known CNVRs' in the following; Material and Methods for details), we found 1,387 (52.3%) overlapping regions. These CNVRs make up 126.6 Mb of the total CNVR length (74.2%, Figure 3). Additionally, 268 CNVRs (10.1%) covering 11 Mb (6.4%) overlapped mutually $\geq 70\%$ with known CNVRs (Figure 3).

Figure 3 - Overlap of detected Nelore CNVRs with known cattle CNVRs. All CNVRs: total size of the covered genomic region. Overlapping CNVRs: total size of genomic region covered by known and our Nelore CNVRs that overlap. Effective overlap: size of region that is jointly covered by overlapping CNVRs. >70% overlapping CNVRs: total size of genomic region covered by known and our Nelore CNVRs which overlap >70% mutually. Effective overlap (>70%): size of region that is jointly covered by CNVRs mutually overlapping >70%.

2.3.2 Association of CNVs with biological functions and processes

We found 2,531 CNVRs (95.5%) to overlap with QTLs from Cattle Animal QTLdb (HU et al., 2013). A total of 2,310 QTLs overlapped with 482 regions of polymorphic CNV. This yielded 282 traits, which we found enriched for several milk-related phenotypes (Figure 4). Regarding meat quality, marbling score was found frequently among those overlapped QTL traits.

Figure 4 - Enriched QTL-traits that are overlapped by regions of polymorphic CNV in the Nelore population. Depicted are top 20 frequently overlapped traits

The genomic positions of $\approx 44\%$ of our CNVRs overlapped with 2,750 different genes either entirely, partially $\geq 50\%$, or partially $< 50\%$ (69.2%, 8.6%, and 22.4% of the genes, respectively) and 64 genes overlapped ≥ 1 CNVR. As depicted in Table 1, we found genes overlapping regions of polymorphic CNV to be enriched for immunological functions (including the gene sets: major histocompatibility complex, antigen processing and presentation, and immunoglobulin). A similar observation has been reported for mammals, where immunological genes were found enriched inside CNVRs (AGAM et al., 2010; NICHOLAS et al., 2011; TUZUN et al., 2005; WANG et al., 2014b) and associated with susceptibility to diseases (AITMAN et al., 2006; SCHASCHL; AITMAN; VYSE, 2009).

Table 1 - Functional groups associated with regions of polymorphic CNV (>1% of the population). The statistical significance level was set to 0.01

GO sets		
GO ID	Description	P-value
GO:0004984	olfactory receptor activity	1e-23
GO:0007186	G-protein coupled receptor protein signaling pathway	3.5e-15
GO:0007166	cell surface receptor linked signal transduction	4.7e-12
GO:0016021	integral component of membrane	2.5e-06
GO:0031224	intrinsic component of membrane	2.7e-06
GO:0019882	antigen processing and presentation	4.5e-06
GO:0042611	MHC protein complex	0.000011
GO:0032561	guanyl ribonucleotide binding	0.00018
GO:0019001	guanyl nucleotide binding	0.00022
GO:0042612	MHC class I protein complex	0.00059
GO:0005525	GTP binding	0.0018
GO:0050909	sensory perception of taste	0.0024
GO:0002474	antigen processing and presentation of peptide antigen via MHC class I	0.0025
GO:0005833	hemoglobin complex	0.0076
GO:0007606	sensory perception of chemical stimulus	0.0087
Protein sets		
Protein set ID	Description	P-value
IPR003597	Immunoglobulin C1-set	6.1e-06
IPR007110	Immunoglobulin-like domain	0.000012
IPR003006	Immunoglobulin/major histocompatibility complex, conserved site	0.000026
IPR013783	Immunoglobulin-like fold	0.000086
IPR010579	MHC class I, alpha chain, C-terminal	0.00018
IPR011161	MHC class I-like antigen recognition	0.001
IPR001400	Somatotropin hormone	0.0016
IPR007960	Mammalian taste receptor	0.0016
IPR018116	Somatotropin hormone, conserved site	0.0021
IPR013106	Immunoglobulin V-set domain	0.0036
IPR002338	Haemoglobin, alpha	0.0038
IPR001039	MHC class I, alpha chain, alpha1 and alpha2	0.004
IPR001461	Propeptide, peptidase A1	0.0099

Olfactory receptors (OR) were most significantly enriched (P value = 1e-23). This is again in good agreement with previous studies finding the OR gene family enriched in mammalian CNVRs (GRAUBERT et al., 2007; HASIN et al., 2008; NOZAWA; KAWAHARA; NEI, 2007; YOUNG et al., 2008), and particularly in cattle CNVRs (BICKHART et al., 2012; FADISTA et al., 2010; HOU et al., 2011, 2012b;

JIANG et al., 2012, 2013; LIU et al., 2010; MATUKUMALLI et al., 2009; SEROUSSI et al., 2010).

Biological functions involving GTP were also significantly enriched, which is consistent with previous findings in cattle (CHOI et al., 2013; ZHAO et al., 2012b) and goat (FONTANESI et al., 2010).

2.3.3 Association with meat tenderness (MT)

MT-related QTLs from Cattle Animal QTLdb (“Tenderness score” and “Shear force”) fall into 8.9% of our CNVRs (18.2 Mb). The genes overlapping those CNVRs were particularly enriched for peptidases and proteases related pathways (*Aspartic peptidase, N-terminal*: IPR012848; *Aspartic peptidase, active site*: IPR001969, *aspartic-type peptidase and endopeptidase activity*: GO:0070001 and GO:0004190).

Additionally, from 132 Nelore-specific QTLs associated with shear force measured at 0, 7 and 14 days after slaughter in a previous study conducted with the same population (TIZIOTO et al., 2013), we found 75 (56.8%) to overlap with 109 of the CNVRs. Among them, 24 regions overlap with polymorphic CNV.

2.3.4 CNV validation by qPCR

We validated 9 MT-related CNVRs using qPCR (see Material and methods for details). The concordance of copy number state (loss, normal and gain) between the qPCR and SNP-array results ranged from 46% to 86% of tested samples. The inferred copy number state (from 0n to 4n) is calculated as a normalized ratio of PCR cycle threshold values (see Material and methods for details, Figure 5).

Figure 5 - Average normalized ratios of qPCR cycle thresholds with indicated standard deviation, shown is the average for each copy number (CN) state for 16 tested primers. The lack of signal (NR = 0) was settled as 0n.

2.4 Discussion

Investigation of genetic variants associated with phenotypes of interest are sometimes restricted to single nucleotide polymorphism, however extension to larger regions of variation such as CNVs is feasible and beneficial (SU et al., 2010; YAU; HOLMES, 2008).

To investigate structural variation in the Nelore breed we applied a computational approach and inferred a genome-wide CNV map from SNP-chip genotyping data. Our results revealed that variable regions are scattered across the genome and represent a large portion of the genome, as reported for other breeds of cattle (BAE et al., 2010; SEROUSSI et al., 2010).

Comparison of detected CNVs, even within the same species, is not trivial as their mapping is typically based on different reference genomes and the exact genomic start and end coordinates (CNV boundaries) are additionally subject to biological and technical variation (CAHAN et al., 2009; FADISTA et al., 2010; REDON et al., 2006).

Our comparison was conducted with *Bos taurus* from 14 existing cattle CNV studies, which are predominantly based on the UMD_3.1 and Btau_4.0 reference assembly. Conversion of genomic coordinates resulted in a considerable data loss, as we could not convert 36.3% of the CNVRs from Btau_4.0 to UMD_3.1 using

liftOver (KUHN; HAUSSLER; KENT, 2013). We nevertheless found, for the remaining fraction, a substantial overlap between known CNV regions and the ones we report here.

This includes several regions of polymorphic CNV (found in >1% of the population) to overlap with MT-QTLs from cattle QTLdb (HU et al., 2013). The enriched peptidase and protease gene families at these regions have a remarkable role in post-mortem tenderization as they are involved in connective tissue turnover (SENTANDREU; COULIS; OUALI, 2002). Investigation of genes at MT-QTLs in this Nelore population (TIZIOTO et al., 2013) overlapping our CNVRs showed an enrichment for immunological functions. A notable exception is the diaphanous-related formin 3 gene (*DIAPH3*), which overlaps two regions of polymorphic CNV (CNVR_1432 and 1434). Actin-based processes are regulated by diaphanous-related formins (GARDBERG et al., 2010) and influence MT-associated proteolysis (LI; XU; ZHOU, 2012). Moreover, diaphanous-related formins affect specific GTPases (OLSON, 2003), which showed a different prevalence between animals with tough and soft meat (ZHAO et al., 2012b).

We also found enriched processes involving GTP (Table 1, *G-protein coupled receptor protein signaling pathway* and *GTP binding*). Previously, MT and skeletal muscle reports described the influence of GTP-related genes on myotube morphology, skeletal muscle myogenesis, regeneration and calcium physiology (BANNISTER; COLECRAFT; BEAM, 2008; HAWKE et al., 2006; ROMBERG et al., 2014; SUZUKI; KAZIRO; KOIDE, 2000). The impact on calcium content is especially notable as it affects MT through postmortem protein degradation in skeletal muscle (GEESINK; KOOHMARAIE, 1999; TIZIOTO et al., 2014). A representative example is the proteolysis-associated ATP/GTP binding protein-like 3 gene (*AGBL3*), present in less copies in Korean beef cattle (Hanwoo) when compared to Holstein dairy breed (CHOI et al., 2013). In Nelore, we found a region of polymorphic CNV (CNVR_507) ≈96 kb downstream of *AGBL3*.

The GTP-binding protein RAD-like gene is a validated meat quality biomarker (PIERZCHALA et al., 2014) and GTP-related genes are also targets of differently expressed miRNAs between MT phenotypes (ZHAO et al., 2012a). As the number of miRNA-binding sites is significantly higher inside CNVRs (FELEKKIS et al., 2011), this indicates a potential regulatory mechanism that could modulate transcripts synthesized at CNVRs.

We also found two regions of polymorphic CNV (CNVR_2061 and 2443) that overlapped two QTLs for glutathione redox balance (ZHOU et al., 2014), which prompts further investigation as the glutathione dimer has been reported as a MT-predictor (D'ALESSANDRO et al., 2012a). Moreover, an overlapped gene mapped at those QTLs called SMAD specific E3 ubiquitin protein ligase 1 (*SMURF1*) inhibits the transduction pathway of myostatin (ZHU et al., 2004). The chaperonin containing TCP1, subunit 6B (zeta 2) (*CCT6B*) gene was also mapped in glutathione redox balance QTLs (ZHOU et al., 2014). The chaperonins have an important role in protein folding process (SPIESS et al., 2004), which is very important in meat tenderization (LOMIWES et al., 2014).

The enriched *Somatotropin hormone* (Table 1) plays a major role in growth and differentiation of skeletal muscle (FLORINI; EWTON; COOLICAN, 1996). Interestingly, we found the Insulin-like growth factor 2 hormone (*IGF2*) gene is inside a region of polymorphic CNV (CNVR_2647). IGF2 is a stimulator for myoblast differentiation (ALZHANOV; MCINERNEY; ROTWEIN, 2010; FLORINI et al., 1991) and is predicted to be a target for bta-let-7b miRNA (MI0005453), which is inside another region of polymorphic CNV (CNVR_760).

Concerning to genes previously associated with cattle meat tenderness, the μ -calpain (*CAPN1* - ENSBTAG00000010230) and calpastatin (*CAST* - ENSBTAG00000000874) are key ones for cattle in general (CASAS et al., 2005; TAIT et al., 2014) and even particularly for the Nelore breed (CURI et al., 2009; PINTO et al., 2010). The remarkable influence in postmortem protein degradation, from *CAPN1* and *CAST*, can partially explain the association with meat tenderness (TIZIOTO et al., 2014). However, none of our Nelore CNVRs have overlap with *CAPN1/CAST* genes. Likewise none of miRNAs genes, predicted to bind on *CAPN1/CAST* product transcripts, were overlapped by our Nelore CNVRs or even known CNVRs. Conversely, 3 known CNVRs were overlapped with *CAPN1* or *CAST* genes. One known CNVR overlapped with *CAPN1* gene is described for *Bos taurus coreanae* (Hanwoo) (BAE et al., 2010). Two previously described CNVRs encompassing *CAST* gene, one for Holstein-Friesian dairy cattle (*Bos taurus taurus*) (ZHAN et al., 2011) and another in a study with 27 different cattle breeds including Holstein (HOU et al., 2012a).

Heat shock proteins (HSPs) are involved with key processes in meat tenderness (D'ALESSANDRO et al., 2012b). HSPs and other proteins are associated

with Nelore meat tenderness (CARVALHO et al., 2014). However, none of our Nelore CNVRs, or known CNVRs coordinates, have overlap with source genes of these associated proteins (HSP27, HSP70, MLC2, alpha tropomyosin, beta-LG and cytochrome b-c1 subunit 1) (CARVALHO et al., 2014). It can indicate a lack of copy number events in those genomic regions. Similarly, Ensembl *Bos taurus* annotated miRNAs with predicted binding sites inside to these 6 protein genes do not overlap our Nelore CNVRs. The exception was bta-miR-499 (ENSBTAG00000029949) miRNA gene, which is predicted to bind at alpha tropomyosin TPM1-201 transcript (ENSBTAT00000044796) which is located inside a CNVR in a lost state in Hanwoo breed (*Bos taurus coreanae*) and gain state in Angus breed (*Bos taurus taurus*) (CHOI et al., 2013). The tenderness score is higher for Hanwoo when compared with Angus (VAN BA et al., 2013).

2.5 Conclusion

In conclusion, we found that a considerable fraction of the Zebu Nelore genome contains CNVRs with potential functional implications. Regarding the important MT-trait, we identified overlapped genes and QTLs associated with MT and skeletal muscle physiology (Figure 6). We thus proposed research insights that could direct future studies on the mechanisms related with MT and skeletal muscle physiology.

Figure 6 - Regions and pathways previously associated with meat tenderness and skeletal muscle physiology varying in copy number in Nelore breed

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3 FROM CNVS TO GENE EXPRESSION IN NELORE CATTLE BREED: GENETIC IMPACT ON MEAT TENDERNESS

Resumo

A raça Nelore é predominante no rebanho brasileiro (*Bos taurus indicus*). A grande adaptabilidade da raça Nelore ao clima tropical brasileiro, no entanto, não está associada a maciez de carne (MT). Sabe-se que MT é influenciada por vários fatores ambientais e pela composição genética. Um total de 34 animais da população foi submetido à análise do transcriptoma e análise de potencial genético para MT. Foram identificados 170 fragmentos da CNV (CNVFs) mapeados em 20 CNVRs, com diferença significativa na frequência entre animais com potencial genético para carne mais dura ou mais macia. Uma fração considerável dos CNVFs identificados afetaram a expressão gênica de genes importantes para MT, os quais desempenham um papel importante no metabolismo de glicogênio, fração do tecido conjuntivo, transportadores de membrana e vias metabólicas da glutathione. Um número considerável de CNVRs foi associado à expressão de genes sobrepostos e nas proximidades, onde o aumento ou diminuição do número de cópias foi correlacionado com a mudança na expressão gênica. Dois CNVRs correlacionados foram mapeados no cromossomo 12 e 23, estando próximos a QTLs anteriormente descritos para MT na raça Nelore. Vários CNVFs, os quais são mais frequentes em animais com potencial genético para carne mais macia ou dura, mostraram diferenças significativas na expressão gênica. Essas regiões estão ligadas a importantes funções biológicas com influências altamente relevantes para MT e na fisiologia do músculo esquelético.

Palavras-chave: SNP-chip; RNA-seq; Variação estrutural; Integração

Abstract

Brazil has the largest commercial cattle herd in the world and occupies the first position in worldwide meat export. About 80% of Brazilian herd consists of Zebu breeds (*Bos taurus indicus*). The Nelore breed represents 90% of zebuine cattle, and is widespread distributed and well adapted to its environment. The great adaptability of the Nelore breed to Brazilian tropical climate, however, is not associated with meat tenderness. Meat tenderness is influenced by several environmental factors, but also genetic composition. One important genetic source of phenotypic variance investigated in this article is copy number variation (CNV), i.e. deviation from the normal number of copies of certain DNA sections in the genome. We have previously identified 2,649 CNV regions (CNVRs) based on genotyping of a Nelore population of 723 animals. A total of 34 animals of the population were subjected to transcriptome analysis and meat tenderness (MT) phenotyping. We identified 170 CNV fragments (CNVFs) residing in 20 CNVRs, which occurred in different frequencies between animals with tougher and softer meat genetic potential. A considerable fraction of the identified CNVFs affected gene expression of the MT genes, which play important roles in glycogen metabolism, connective tissue turnover, membrane transporters and glutathione pathways. We also detected that several CNVRs substantially influence the expression of overlapped and nearby genes, where the increase or decrease of copy number correlated well with the change in gene expression. Among them are two CNVRs at chromosome 12 and 23, which are in the vicinity of previously described QTLs for MT in Nelore breed. Several CNVFs, which are more frequent in animals with genetic potential for softer or tougher MT, showed significant

differences in gene expression. Those regions are linked to important biological functions with highly relevant influences on MT and skeletal muscle physiology.

Keywords: SNP-chip; RNA-seq; Structural variation; Integration

3.1 Introduction

Nelore cattle (*Bos taurus indicus*) is an adapted breed to Brazilian tropical climate, but has particular disadvantages in meat quality traits, with meat tenderness (MT) being the most important characteristic for beef consumers. Typically, *Bos taurus indicus* breeds have higher values of shear force (SF, method to measure tenderness (WHEELER; SHACKELFORD; KOOHMARAIE, 1997)) as compared to *Bos taurus taurus* breeds (HOCQUETTE et al., 2006). Known reasons for the decreased MT of Nelore cattle are larger muscle fibers, prominent cross-bridges between thick and thin filaments, and reduced proteolysis of myofibrillar proteins (CORÓ; YOUSSEF; SHIMOKOMAKI, 1999; WHIPPLE et al., 1990).

Nelore and its crossbreds are widely used in Brazilian beef cattle production and these beef products are exported and consumed in more than 100 countries (PRAKASH; STIGLER, 2012). Since the meat quality is lower mainly due to less tenderness, there is an increasing interest to better understand the molecular biology involved in tenderness traits (MALTIN et al., 2007).

Animal breeding is an efficient way to reach improvements in desired phenotypes such as meat tenderness. A better understanding of genetic markers associated with traits of interest in livestock is promising to enhance response to selection (DEKKERS, 2004). Several approaches emerged recently to access genetic information. The “omics” studies such as genomics and transcriptomics play a key role in this scenario. The first one includes the study of the genome-wide DNA composition, involving sequence and structural variation (CLOP; VIDAL; AMILLS, 2012; SELLNER et al., 2007). One type of structural variation is Copy Number Variation (CNV). A CNV is defined as a large region of the genome (typically >1kb), which differs in the number of copies, mainly due to duplication or deletion events between different individuals (LOCKE et al., 2006; SCHERER et al., 2007). On the other hand, transcriptomics represents the holistic view of mRNA molecules in a cell or tissue (HORGAN; KENNY, 2011).

CNV information can be inferred from SNP-arrays and several algorithms are available to perform this task (XU et al., 2013a). The concordance of CNV call

between different algorithms can show substantial variation and a combination of different algorithms is a suitable strategy to reach less false positives (WINCHESTER; YAU; RAGOISSIS, 2009). However, selecting just calls found by multiple algorithms increases the number of false negatives (XU et al., 2013a). A frequently used algorithm to estimate CNVs from SNP-chip data is PennCNV (WANG et al., 2007). PennCNV shows good consistency to the Comparative Genomic Hybridization (CGH) gold standard, which suggests that PennCNV can be applied without using other algorithms (ZHANG et al., 2014).

Transcriptome studies using next-generation sequencing (RNA-seq) allow a global analysis of transcripts (MORTAZAVI et al., 2008). RNA-seq allows more precise measurements of the level of transcripts and their isoforms as compared to other techniques, such as microarrays (WANG; GERSTEIN; SNYDER, 2009).

Gene transcription can be affected by CNVs especially in overlapped or nearby genes (LEE et al., 2006; MCCARROLL et al., 2006; SOMERVILLE et al., 2005; STRANGER et al., 2007). Particularly for cattle, a CNV in *CASL-like protein 2 gene* (*MICAL-L2*), which performs a critical role in the development of muscle fiber, is correlated with gene expression (XU et al., 2013b). CNVs can be thus important molecular markers.

In this article, we integrate CNV information and gene expression in genomic regions containing structural variations that are more frequent in animals with genetic potential for softer or tougher meat. Subsequently, we discuss the impact of detected differentially expressed (DE) genes on several pathways associated with MT.

3.2 Material and methods

3.2.1 Ethical Statement

Animals were handled and managed according to Institutional Animal Care and Use Committee Guidelines from Brazilian Agricultural Research Corporation - EMBRAPA approved by the president, Dr. Rui Machado.

3.2.2 Population

Nelore bred steers (746) in the Brazilian Agricultural Research Corporation (EMBRAPA/Brazil) experimental breeding herd between 2009 and 2011 were used in this study. Steers were sired by 34 unrelated sires, and were selected to represent the main genealogies used in Brazil according to the National Summary of Nelore

produced by the Brazilian Association of Zebu Breeders (ABCZ) and National Research Center for Beef Cattle (CESAR et al., 2014).

3.2.3 Phenotype and MT-EBV estimation

The tenderness evaluation was performed by measuring the shear force (SF) (WHEELER; SHACKELFORD; KOOHMARAIE, 1997) on day 7 and day 14 after slaughtering. The SF values were used to estimate genetic breeding values for meat tenderness (MT-EBV, detailed procedure in (CARVALHO et al., 2014)). A total of 250 animals with MT-EBV values were used herein.

3.2.4 SNP genotyping and CNV estimation

Seven hundred and twenty three Nelore steers were genotyped using SNP-chip Illumina Bovine High-Density Beadchip® technology. After processing and filtering, a total of 2,649 CNV regions (CNVRs), from 723 animals, were detected in this population (unpublished observations carried by our group). These CNVRs cover > 6% of the *Bos taurus* reference genome UMD_3.1. To infer CNVRs, we used the CNV calls obtained from PennCNV (WANG et al., 2007). The total number of inferred CNV calls which were used in this project was 49,997. CNVRuler (KIM et al., 2012) was used to concatenate the CNV calls into CNVRs. The genomic regions commonly shared by two or more animals were considered as Copy Number Variation Fragments (CNVFs). The CNVFs were obtained from individual CNV calls. Like CNV calls, CNVFs were categorized in four states: complete deletion (0n), partial deletion or one copy (1n), three copies (3n) and four copies (4n).

3.2.5 Gene expression data

RNA-seq analysis was carried out for 38 animals (data not published) in skeletal muscle. Sequencing adaptors and low-complexity reads were removed from the raw sequencing data. FASTQC (version 0.10.1) [<http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/>] was used for quality control and statistics of the reads sequenced. TopHat (version 1.2.0) was used to map the reads back to the genome. Cufflinks (version 2.0.2) was applied for transcript counting and the new reference guided assembly with a minimum alignment count of 10 per locus (LANGMEAD et al., 2009).

The number of Fragments Per Kilobase Of Exon Per Million Fragments Mapped (FPKM) was estimated for each annotated gene (Ensembl, (KINSELLA et al., 2011)) using the UMD_3.1 reference genome.

3.2.6 Animals groups, analysis pipeline and applied statistics

A different number of animals were subjected to MT, CNV and RNA-seq analysis. A total of 746 animals have information coming from at least one of the three used methods (Figure 1).

Figure 1 - Venn diagram indicating the number of animals with CNV, Meat tenderness EBV or gene expression information and general pipeline used to integrate MT, CNVs and gene expression information

Using the available data from each of the three analysis types, we applied the pipeline shown in Figure 1 intending two main objectives: 1) detect genomic regions where a certain number of copies (ranging from 0n to 4n) are statistically more frequent in animals with tough or soft meat, 2) infer if the presence of such structural events are correlated with gene expression.

Based on MT-EBV values, tough (MT-EBV > 0.3 kg) and soft (MT-EBV < -0.3 kg) groups were formed. Related to the first objective, it is important to emphasize that CNV calls were used for the estimation of CNVFs (as previous described). Using all estimated CNVFs, logistic regression was applied to infer which of those CNVFs were statistically more frequent in animals grouped as tough or soft meat.

Each of the significant CNVFs was mapped to the CNVR it locates in. Each CNVR with ≥ 1 significant CNVF was tested with Fisher's product test (FISHER, 1925). Fisher's product test was used to combine all P-values from CNVFs overlapping a given CNVR. The combined P-value intends to verify if, beyond each individually significant CNVF, all CNVFs in the same CNVR were in general, more frequent in tough or in soft animals.

For the gene expression analysis step, we used all individually significant CNVFs for soft or tough animals. For each significant CNVF we formed two groups: CNVF⁺ group (animals with CNVF) and the CNVF⁻ group (animals with normal state in the same genomic region). As the CNVFs were divided by state (0n, 1n, 3n and 4n), and each state was separately analysed, the CNVF⁻ group was checked to assure that just 2n animals are used to form it (excluding animals who eventually hold non-diploid state in analysed genomic window). A minimum of 3 animals in each group was used. The FPKM values were used to estimate the fold changes (FC) for each gene we calculated t-test (PAN, 2002) derived P-values to identify differentially expressed genes (DE) between CNVF⁺ and CNVF⁻ groups. Genes with FC = 0 were not included in analysis.

Several t-tests were carried out with a minimum of 3 samples in one group (typically the CNVF⁺ group) while other t-tests with more than 10 animals in each group. To exclude t-tests which give significant results just by chance, we simulated 1,000 times a random grouping for each t-test (using exactly the original number of animals in each CNVF group). The t-tests > 10% of random discovery rate (RDR) were excluded from the analysis. The RDR was used to adjust the difference in the number of animals tested and recognize t-tests with spurious results.

Each of the CNVFs evaluated for gene expression were mapped to its respective CNVR (same genomic location), defining consequently the evaluated genes for each CNVF. All genes overlapping the CNVR or until 450kb away from its boundaries (extension of CNV effect on expression proposed by HENRICHSEN et al., 2009) were analysed (neighbourhood genes).

Additionally, all genes on the same chromosome of each tested CNVF were analysed in a different way. These DE genes outside the defined neighbourhood limits were subjected to gene set analysis (GSA). The GSA was performed to identify functional related genes, outside the defined borders, affected by a CNVF, or CNVFs belonging to the same CNVR.

The combined P-value in the gene expression step was used in two contexts: 1) all CNVFs against a specific gene; 2) all genes against all CNVFs. The first strategy was used to evaluate the influence of all significantly frequent CNVFs of a specific CNVR in the tough or the soft group, on a given gene. The second aimed to contextualize the influence of these CNVFs on gene expression inside the delimited genomic region.

3.3 Results

3.3.1 Meat tenderness in the Nelore Population

Genetic potential for meat tenderness (MT) was predicted from estimated breeding values (EBV, based on SF values). The EBV is a prediction of genetic merit commonly used for important phenotypes in animal husbandry. The MT-EBV was estimated in kg, which basically means that animals with high MT-EBV tend to have offspring with tougher meat as compared to those with low MT-EBV (see Material and Methods for MT-EBV details). The mean MT-EBV in our population was 0.256 kg and 40.4% of the animals have $0.3 \geq \text{MT-EBV} \geq -0.3$ (thresholds for soft and tough group). The minimum MT-EBV was -1.15 kg and the maximum 0.97 kg.

The MT-EBVs of the 250 animals was analysed. For 245 animals the ancestor is known (for 5 animals with MT-EBV information the ancestor is unknown).

A total of 34 animals have both RNA-seq and MT-EBV data. The MT-EBV average for these 34 animals was 0.0495 kg (range: -1.15 kg to 0.95 kg).

We observe presumably inherited MT-EBV differences between sons from different sires. The number of sons for each sire ranged from 1 to 16 animals. The lowest offspring median (MT-EBV of -0.35) and mean (-0.36) were obtained by the sires NE001385 and NE001707, respectively. The highest median (0.4) and mean (0.29) for MT-EBV, on the other hand, was obtained from the sires NE001387 and NE003322, respectively.

3.3.2 CNVFs in the Nelore Population

We detected 9,565 CNVFs from CNV calls ≥ 5 kb in our population using PennCNV (WANG et al., 2007). Most CNVFs are 1n (4,603, 48.12%) and 3n (4,120, 43.07%). A total of 820 CNVFs belong to 0n (8.57%). Less common is 4n (22 CNVFs, 0.23%). Thus, 5,423 (56.69 %) were events of copy loss.

The detected CNVFs ranged from 1 bp to 580 kb. As we inferred the CNVs from SNP-chip data, it can occur that one CNV ends in the same SNP position in which another CNV starts (corresponds to a CNVF of 1 bp).

3.3.3 Frequency of CNVFs for animals with tougher or softer meat

Both meat groups contain ~50 animals (48 animals belong to the soft and 53 to the tough group). A total of 170 CNVFs were more frequent in one of the two groups (65 tough and 105 soft MT-CNVFs). As depicted in Table 2, we detected ≥ 1 significant CNVF for 20 distinct CNVRs. Eight CNVRs from these had a significant combined P-value for ≥ 1 CNV state (see Material and Methods for details).

Table 2 - CNVRs with ≥ 1 CNVF more frequent in animals with either soft or tough meat (logistic regression, P-value ≤ 0.05)

CNVR	Chr	Start	End	Ratio 0n	Group 0n	Ratio 1n	Group 1n	Ratio 3n	Group 3n
CNVR_3*	1	1893592	2009127	-	-	-	-	11/8	Soft
CNVR_323	3	22796624	22829139	-	-	-	-	6/1	Tough
CNVR_656	5	57951588	58316375	2/0	-	28/6	Tough	32/0	-
CNVR_723	5	100568796	100645237	-	-	-	-	19/1	Soft
CNVR_725*	5	102375978	102695158	7/0	-	31/15	Soft	11/0	-
CNVR_727*	5	103105996	103454169	11/0	-	15/0	-	19/10	Soft
CNVR_760*	5	117220685	118203101	22/0	-	71/3	Soft	47/12	Tough
CNVR_950	7	9626435	10150703	1/0	-	24/1	Tough	15/0	-
CNVR_1180	9	16413696	16924662	-	-	-	-	24/13	Tough
CNVR_1267	10	22184374	25493882	11/0	-	179/4	Soft	251/1	Tough
CNVR_1540*	12	70357278	72147564	35/2	Tough	81/0	-	114/6	Soft
CNVR_1541**	12	72409908	76756892	104/1	Tough	267/18	Tough	410/15	Soft
CNVR_1566*	13	12536047	12822615	-	-	-	-	46/36	Soft
CNVR_1724	15	11980564	13291161	-	-	5/0	-	47/1	Tough
CNVR_1831*	15	80260269	80879074	6/5	Tough	14/0	-	43/0	-
CNVR_1849	16	5447029	6146432	6/1	Tough	20/0	-	26/0	-
CNVR_1858*	16	8737260	9002301	-	-	8/4	Soft	-	-
CNVR_2279	23	25255521	25450746	10/0	-	18/0	-	14/1	Soft
CNVR_2280	23	25626467	26663258	19/1	Soft	22/0	-	35/0	-
CNVR_2637	29	42437944	42578956	3/0	-	14/0	-	30/4	Tough

Ratio: Number of CNVFs in CNVR / Number of MT-EBV frequent CNVFs in CNVR.

Group: More frequent in the tough or soft group.

- No fragments belonging to this state.

*Significant combined P-value for one state.

** Significant combined P-value for two states.

A total of 16 CNVRs obtained significant CNVFs just in one tested state. Interestingly, all 4 CNVRs with significant CNVFs in >1 state showed a different group prevalence between the states. For CNVR 760 and CNVR 1267 (genomic

positions in Table 2), we observed more significant tough CNVFs in 3n and more soft CNVFs in 1n suggesting a negative correlation between copy number and meat tenderness. Conversely, the CNVR 1540 and 1541 showed significantly more copies in softer meat genetic potential group.

3.3.4 Impact of MT-CNVRs on gene expression

From all tested CNVRs, mainly four CNVRs showed possible effect on genes, molecules or/and biological functions previously described as associated with MT (Figure 2).

Figure 2 - The CNVRs and its putative cascade effect ending in impact on Meat tenderness (MT). *The effect on calcium homeostasis is restricted for specific ATP-binding transporters family members

These were: CNVR 656, 760, 1541, and 2280 (genomic positions in Table 2). Further, CNVR 1541 and 2280 are in the vicinity of QTLs for MT in this Nelore population (TIZIOTO et al., 2013).

Two CNVFs mapped at CNVR 656 (chr5:58,024,309-58,039,917) were associated with the matrix metalloproteinase19 gene (*MMP-19*). The animals with 1n in those CNVFs showed significantly decreased expression of *MMP-19* compared to 2n animals.

In CNVR 760, the majority of 3n CNVFs were more frequent in the tough group with a statistically significant combined P-value. After grouping and filtering (see Material and Methods in Section animals groups, analysis pipeline and applied statistics) 8 CNVFs in CNVR 760 showed a significant association to gene expression: 7 genes (5 overlapping, 2 nearby) were DE between the CNVF⁺ and the

CNVF⁻ group for ≥ 1 CNVF. Different DE gene sets were associated with CNVFs at distinct genomic regions. The different gene expression patterns in genes associated with distinct 3n CNVFs, suggest that the influence of one specific state is highly dependent on its genomic context. Moreover, despite of a reported weak association, the increase in copy number is not necessarily followed by increase in gene expression (HAREWOOD; CHAIGNAT; REYMOND, 2012).

CNVR 1541 show significant influence on gene expression in its neighborhood considering the combined P-value of all CNVFs detected in this region. From those, 9 CNVFs (4 for 3n and 5 for 1n) were individually associated with MT-EBV and gene expression (Figure 3). The DE genes include claudin 10 (*CLDN10*) and three uncharacterized genes (Ensembl:ENSBTAG00000026070, ENSBTAG00000039714 and ENSBTAG00000023309). Those genes are ATP-binding cassette, sub-family C orthologs (Ensembl, (KINSELLA et al., 2011)). All genes showed decreased expression in 1n compared to normal 2n.

Figure 3 - Statistically significant increase (green lines) and decrease (orange lines) of gene expression in neighbourhood of CNVR 1541 that corresponds to an increase (green bars) and decrease (orange bars), respectively, in copy number

For each significant CNVF we performed an overrepresentation analysis of GO and InterPro categories of all DE genes on the same chromosome. This revealed GO and protein categories associated with glutathione (GO:0004364, InterPRO:IPR003080 and InterPRO:IPR004045). A 0n CNVF (chr:23 25,848,219-25,854,175) in CNVR 2280 was associated with more expression of glutathione-related genes (Figure 4).

Figure 4 - Statistically significant gene expression changes between normal (2n) and loss (0n) state in the neighbourhood of CNVR 2280.

3.4 Discussion

3.4.1 CNVR concept

Most of cattle CNV studies use copy number variation regions (CNVRs) to arrange individual CNVs in a given population (BAE et al., 2010; CICCONARDI et al., 2013; HOU et al., 2011, 2012b, 2012c, 2012a; JIANG et al., 2012, 2013; SEROUSSI et

al., 2010). The CNVR is a simple concatenation of all overlapping individual CNVs calls. Methods like CNVRuler (KIM et al., 2012) provide optional filters for CNVR concatenation as well as fragments and reciprocal overlap.

The CNVR concept is suitable to detect genomic regions containing structural variations in a given population. However, individual CNVs can vary considerably in length and position inside a CNVR. This has been observed especially for large CNVRs in this studied Nelore population. CNVRs with size above 320 kb rarely holds CNVs with the same size.

This variation can be due to the technical or biological sources (CAHAN et al., 2009; FADISTA et al., 2010; REDON et al., 2006). The use of CNVRs, to group animals with the same structural event, can result in a non-normal structural state (gain or loss) at genomic regions never diagnosed as such by individual CNV calls (Figure 5). In addition, the same sample can contain more than one individual CNV, even gain and loss simultaneously, overlapping the same CNVR. Thus, CNV association studies based on CNVRs alone can produce substantially biased results.

Figure 5 - Exemplification of individual CNV calls, CNVF and CNVR concepts for hypothetical animals. Overlapping 0n CNV calls from Animal 1 and 2 yield the 0n CNVF below (dark blue bars). The 1n CNV calls (green bars) overlap only in one SNP probe (first track), thus resulting in a CNVF of 1 bp. The 3n CNV call of Animal 1 locates inside the one of Animal 2 resulting in a corresponding CNVF (orange bars). The CNVR derived from all CNV calls is shown in the last track (soft blue bar). The same colors were used to tag the state for each CNV call

3.4.2 Fragment concept

To avoid the CNVR bias described in the previous section, CNVs calls were joined to CNV fragments (CNVF) as previously proposed (KIM et al., 2012). A CNVF intends to cover only shared CNV regions (by at least 2 animals in the population). Thereby, it is possible to infer common states at one certain genomic region. It can occur that different samples yield different sizes of overlapping CNVs (with different genomic coordinates), which is ignored by the CNVF approach. However, the same individual CNV can generate several CNVFs, which allows a better resolution to evaluate different structural events inside a given CNV or CNVR (Figure 6).

Figure 6 - CNVs in CNVR 1541, at chromosome 12, in a representative subset of 50 animals (one animal per track). The first 3 tracks are the frequencies (% for all 723 animals with CNV information, note differences in scale between states) for 0n (blue), 1n (green) and 3n (orange) states

The CNVFs were categorized based on the CNV state (which ranges from 0n, i.e. complete deletion to 4n, i.e. four copies). Consequently, CNVFs can overlap with other CNVFs of a different state.

3.4.3 Biological mechanisms for CNVRs associated with MT and expression

3.4.3.1 CNVR 656

The muscle connective tissue has influence on meat tenderness (WESTON; ROGERS; ALTHEN, 2002). The diameter and collagen content of perimysial and endomysial connective tissue is correlated with meat toughness in cattle (LIGHT et al.,

1985). The matrix metalloproteinases (MMP) are the main enzymes of connective tissue turnover (PURSLOW; ARCHILE-CONTRERAS; CHA, 2012).

The expression level of *MMP-9* increases skeletal muscle fiber cross-section area, reduces the deposition of collagen I and IV and increases the proportion of fast-type muscle fiber (DAHIYA et al., 2011). All these traits are associated with shear force values (CHRIKI et al., 2012).

The associated CNVF in CNVR 656 is 266kb upstream of the DE *MMP-19* gene and distant regulatory elements associated with certain transcripts can be located until several Mbs from the associated transcriptional site (NOBREGA et al., 2003; SAGAI et al., 2005).

3.4.3.2 CNVR 760

The increase of number of copies in different regions of CNVR 760 seems to decrease the *GTSE1* expression. The G-2 and S-phase expressed 1 (*GTSE1*, Ensembl:ENSBTAG00000007102) gene was DE for 3n and 1n inside CNVR 760. The fold change (FC) was - 0.41 for 2n-1n comparison and 0.35 for 2n-3n comparison. *GTSE1* is involved in cell migration (SCOLZ et al., 2012) and apoptosis (MONTE et al., 2003). During the conversion from muscle to meat, apoptosis is a first step of intrinsic physiological process (OUALI et al., 2006). Moreover, the known role of *GTSE1* in apoptosis is to interact with transcription factor p53, which in turn interacts with small heat shock protein 60 (*sHSP60*) (GHOSH et al., 2008; MONTE et al., 2003). The sHSPs have a remarkable role in meat tenderization by protecting against muscle ageing and myofibrillar protein degradation and additionally supporting cellular homeostasis (LOMIWES et al., 2014).

The role of the Tetratricopeptide repeat domain 38 (*TTC38*, Ensembl:ENSBTAG00000003017) DE gene is not well understood, but it interacts on the protein level with prefoldin subunit 1 (*PFD1*) and the thymidine kinase 1 (*TK1*) (VINAYAGAM et al., 2011). The *PFD1* gene is a molecular chaperone subunit required for protein folding (ABE et al., 2013). Moreover, *PDF1* cooperates with sHSPs like *HSP70* and *HSP40* (GEISSLER; SIEGERS; SCHIEBEL, 1998; HARTL; HAYER-HARTL,

2002; SIEGERS et al., 1999; VAINBERG et al., 1998). The *HSP70* and other several sHSPs were previously associated with beef tenderness (LOMIWES et al., 2014), including this Nelore population (CARVALHO et al., 2014).

The *PPAR α* overlap with CNVR 760 in the downstream breakpoint position. *PPAR α* was DE (P-value = 0.006) in one 3n CNVF (chr5:117,340,184-117,340,184) belonging to CNVR 760. The combined P-value for all tested 3n CNVFs (belonging to CNVR 760) for *PPAR α* expression was also significant (P-value = 0.003).

Peroxisome proliferator-activated receptors (*PPARs*) are transcription factors involved in the regulation of important genes in fat and glucose homeostasis (GRYGIEL-GÓRNIAK, 2014). Peroxisome proliferator-activated receptor alpha (*PPAR α* , Ensembl:ENSBTAG00000008063) is one *PPAR* group member involved in fat metabolism and distinct molecular mechanisms through interaction with other *PPARs* members (HONDARES et al., 2011; MARRAPODI; CHIANG, 2000). In glucose metabolism the *Glycogen synthase type 2* is a rate limiting enzyme in glycogen storage, which have its gene targeted by *PPAR α* (MANDARD et al., 2007).

ATP and consequently glucose metabolism enzymes remain active post-mortem under anaerobic conditions (HAMM, 1977). The glycogen content is associated with ultimate pH, which influences post-mortem enzymatic dynamics, which in turn is relevant for tenderization process (SILVA; PATARATA; MARTINS, 1999). Additionally, higher glycogen potential (GP), which is a measure of glycogen *in vivo* is associated with meat tenderness in cattle (WULF; EMNETT, 2002).

3.4.3.3 CNVR 1541

In gain state (3n) *CLDN10* was influenced by a CNVF between 73,863,426 and 73,892,475 bp on chromosome 12 (Figure 3). *CLDN10* was less expressed in animals with 3n in this region. However, the 1n state at 72,432,362-72,439,440 bp is also associated with less expression. Based on these findings, distinct genomic windows with distinct states (overlapping the same CNVR) can perform similar effects on expression.

The claudins are key players in the tight junction structure (GÜNZEL; YU, 2013). Moreover, high expression of claudins in fibroblasts can simulate tight junction-like

networks of strands (FURUSE et al., 1998; GÜNZEL; YU, 2013; KUBOTA et al., 1999). Fibroblasts produce collagen, involved in several physiological processes associated with meat tenderization (CHANG et al., 2002; PURSLOW; ARCHILE-CONTRERAS; CHA, 2012).

Based on 3n state CNVFs were obtained a total of 3 DE genes. All DE genes are ATP-binding cassette, sub-family C orthologs (Ensembl:ENSBTAG00000026070, ENSBTAG00000046041 and ENSBTAG00000045751). All of them were DE between animals CNVF⁺ and CNVF⁻ in three different CNVFs at genomic windows between 75,563,831 bp and 75,618,647 bp on chromosome 12 (Figure 3).

The transport of molecules across lipid membranes is mainly performed by ATP-binding cassette transporters (DEAN; HAMON; CHIMINI, 2001). ATP-binding cassette transporters are ATP-powered pumps, which have a high conserved nucleotide-binding domain (NBD) (VASILIOU; VASILIOU; NEBERT, 2009). Several ATP-binding cassette transporters are involved with calcium homeostasis, which influence enzymes associated with MT, like calpains (GEESINK; KOOHMARAIE, 1999; TUTULAN-CUNITA et al., 2005).

3.4.3.4 CNVR 2280

A complete deletion (0n) CNVF (chr:23 25,848,219-25,854,175 bp) that belongs to a CNVR 2280 was associated with more expression of glutathione metabolism genes (Figure 4).

The starting point of this CNVF is 1.02 Mb away from the starting point of the *GSTA2* gene (most distant of the three *GSTA* DE genes). The GST gene family via conjugation with reduced glutathione (GSH) determine its interaction with several substrates (NEBERT; VASILIOU, 2004). The dimer form of glutathione (GSSG) metabolic level is GSH(glutathione)/GSSG ratio interdependent (glutathione redox balance) (ZHOU et al., 2014). Interestingly, the GSSG predicts meat tenderness (D'ALESSANDRO et al., 2012).

Moreover, the CNVR 2280 is located 627.14 kb downstream of a previously described QTL for MT in this Nelore population (TIZIOTO et al., 2013), which overlap the three detected GSTA genes.

3.5 Conclusions

Certain structural events in the genome of Nelore cattle are more frequent in animals with genetic potential for tougher or softer meat. We detected that animals with these frequent events often displayed significant changes in gene expression. The influence of CNV on gene expression can be interpreted as an intermediate layer between genomic structural variation and the MT phenotype. The identification of CNVRs, candidate genes and pathways associated with MT in this study contributes to the knowledge of the genomic structure in Nelore breed and gives important insights to its link to MT. Moreover, the integration genomic and transcriptomic information coming from different platforms seems to be a suitable strategy.

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