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DISSERTAÇÃO DE MESTRADO

INVESTIGAÇÃO DE GENES DIFERENCIALMENTE EXPRESSOS ENTRE FRANGOS DE CORTE COM 42 DIAS DE IDADE NORMAIS E AFETADOS COM A MIOPATIA *WHITE STRIPING*

CAROLINE MICHELE MARINHO MARCIANO

CHAPECÓ, 2019

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Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Zootecnia, Área de Concentração Ciência e Produção Animal, da Universidade do Estado de Santa Catarina (UDESC), como requisito parcial para obtenção de grau de **Mestre em Zootecnia**

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Elaborada por
Caroline Michele Marinho Marciano

como requisito parcial para obtenção do grau de
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Chapecó, 28 de fevereiro de 2019.

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RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Zootecnia
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INVESTIGAÇÃO DE GENES DIFERENCIALMENTE EXPRESSOS ENTRE FRANGOS DE CORTE COM 42 DIAS DE IDADE NORMAIS E AFETADOS COM A MIOPATIA *WHITE STRIPING*

AUTOR: Caroline Michele Marinho Marciano
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Chapecó, 28 de fevereiro de 2019

White Striping (WS) é uma miopatia muscular caracterizada pelo aparecimento de estrias brancas paralelas às fibras musculares no peito do frango que afeta a qualidade da carne. É um dos principais problemas da indústria avícola e vem sendo associada à intensa seleção genética realizada para aumento do rendimento de peito e alto desempenho. Devido as perdas econômicas relativas a esta condição, buscou-se identificar genes relacionados com a ocorrência desta miopatia mediante estudos de expressão gênica quantitativa (qPCR) no tecido *pectoralis major* de frangos normais e afetados com WS. Primeiramente, a estabilidade de expressão de 10 genes comumente utilizados como referência foi analisada. Amostras do músculo peitoral de 32 frangos machos com 42 dias de idade foram agrupadas em normais (16) e afetadas (16) com WS. Estas foram coletadas após eutanásia dos frangos, congeladas em nitrogênio líquido e submetidas à extração do RNA total e síntese de cDNA. Os resultados de expressão gênica dos grupos amostrais foram analisados por meio das ferramentas geNorm, NormFinder, BestKeeper e método ΔCt e uma classificação geral foi estabelecida com o uso da ferramenta RankAggreg. Os genes *RPL5* e *RPL30* foram os mais estáveis e, portanto, mais adequados para uso como genes de referência para as condições estudadas. Posteriormente, foram avaliados 15 genes candidatos para a ocorrência de WS, sendo escolhidos com base em genes candidatos funcionais para miopatias. Os iniciadores para cada gene foram desenhados a partir da sequência do genoma aves (*Gallus gallus*) depositada no GenBank e Ensembl. A quantificação relativa dos genes alvos foi realizada por qPCR. Os valores de *fold-change* foram obtidos pelo método de $2^{(-\Delta\Delta Ct)}$ e a expressão diferencial entre os grupos foi obtida pelo teste estatístico não paramétrico Mann-Whitney. Dos 15 genes estudados, 6 foram diferencialmente expressos (DE) por qPCR: *CA2*, *CSRP3* e *PLIN1*, que apresentaram maior expressão e os genes *DNASE1L3*, *MYLK2* e *CALM2* que foram menos expressos em frangos de corte afetados por WS do que em frangos normais. A expressão diferencial destes genes pode estar relacionada ao desencadeamento da miopatia em frangos por afetar a diferenciação muscular, pois os genes DE foram essencialmente relacionados às vias de sinalização de canais de cálcio e metabolismo de carboidratos. Esses resultados contribuem para o melhor entendimento do mecanismo genético envolvido no aparecimento de WS, o que é imprescindível para elaborar alternativas que reduzam a incidência desta miopatia na produção de frangos de corte.

Palavras-chave: avicultura, miopatia peitoral, qPCR.

ABSTRACT
Master's Dissertation
Programa de Pós-Graduação em Zootecnia
Universidade do Estado de Santa Catarina

INVESTIGATION OF DIFFERENTIALY EXPRESSED GENES BETWEEN NORMAL AND WHITE STRIPING-AFFECTED 42 DAYS-OLD BROILERS

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Chapecó, 28 de fevereiro de 2019

White Striping (WS) is a myopathy characterized by the appearance of white streaks parallel to the muscle fibers in the breast muscle of broiler chickens, which affects meat quality. It is one of the main problems in the poultry industry and has been associated with intense genetic selection performed to improve performance and breast yield. Therefore, due to the economic losses related to this condition, the aim of this study was to identify genes related to WS occurrence through quantitative gene expression (qPCR) analysis in pectoralis major tissue of normal and WS-affected broilers. Firstly, pectoral muscle samples from 32 male broilers at 42 days of age, grouped into normal (16) and WS-affected (16) were collected after broilers' euthanasia and frozen in liquid nitrogen. Total RNA extraction and cDNA synthesis were performed. Then, the expression stability of 10 commonly reference candidate genes were analyzed using the geNorm, NormFinder, BestKeeper and ΔCt methods followed by a general classification established using the RankAggreg tool. The *RPL5* and *RPL30* genes were the most stable and therefore more suitable to be used as reference in the studied conditions. Subsequently, 15 candidate target genes for the occurrence of WS, chosen based on their role in myopathies, were evaluated. Primers for each gene were designed based on the *Gallus gallus* genome available in GenBank and Ensembl databases. The relative quantification of those genes was performed by qPCR, using the *RPL5* and *RPL30* as reference genes. The fold-change values were obtained by the $2^{(-\Delta\Delta Ct)}$ method and the differential expression between groups was obtained using the non-parametric Mann-Whitney statistical test. From the 15 studied genes, 6 were differentially expressed (DE) by qPCR: *CA2, CSRP3* and *PLIN1*, which were upregulated and *DNASE1L3, MYLK2* and *CALM2* genes, which were downregulated in WS-affected compared to the normal broilers. The differential expression of these genes might be related to the onset of this myopathy in broilers by affecting muscle differentiation, because the DE genes are essentially related to calcium signaling pathways and carbohydrate metabolism. These results contribute to a better understanding of the genetic mechanisms involved in the appearance of WS, which is essential to elaborate alternatives to reduce the incidence of this myopathy in broiler production.

Keywords: pectoral myopathy, poultry farming, qPCR.

Sumário

1. CAPÍTULO I

REVISÃO DE LITERATURA

1.1 Avicultura

O setor avícola brasileiro ocupa o 1° lugar em exportação (4,32 milhões de toneladas) e 2° lugar em produção de carne de frango (13,056 milhões de toneladas) no ranking mundial (ABPA, 2018). O total exportado em 2017 foi equivalente a US$ 7,236 milhões de dólares, montante 2% superior ao ano anterior, sendo que do montante exportado em 2017, 63% dos produtos foram em forma de cortes (ABPA, 2018). A evolução no setor avícola foi possível devido aos avanços em manejo, nutrição, instalações, sanidade e, sobretudo do melhoramento genético, em função do desenvolvimento genético de linhagens de aves (ALBERS E GROOT, 1998; LEDUR et al., 2003). Dessa maneira, para atender a demanda de carne de aves, empresas focam na seleção para frangos com alta taxa de crescimento e com maior rendimento de peito (PETRACCI et al., 2015).

No entanto, com o desenvolvimento geral de diversas características após o melhoramento genético ocorrido, a seleção genética de características que proporcionam rápido crescimento em curto espaço de tempo ocasionou aumento na incidência de várias miopatias musculares, anormalidades e problemas na qualidade de carne (MUDALAL, et al., 2015; VELLEMAN & CLARK, 2015; MICHELAN FILHO; SOUZA, 2011). Tais anormalidades musculares possuem várias implicações para a qualidade dos produtos frescos e processados, pois a carne do peito pode ser até mesmo descartada devido à aparência inaceitável em frigoríficos (KUTTAPPAN et al., 2012). Devido a isso, a atenção global tem sido voltada para a incidência de miopatias em frangos de corte a fim de se esclarecer as causas dessas anormalidades (ALNAHHAS et al., 2016).

1.2 Miogênese e músculo esquelético

Entender o desenvolvimento e crescimento muscular é um aspecto importante para o estudo das miopias, pois o rendimento de peito (carne) é constituído em maior proporção pelo músculo esquelético. O músculo esquelético é composto por células musculares (fibras musculares ou miofibrilas) e por membranas do tecido conjuntivo que formam vasos e nervos (PEARSON & YOUNG, 2000). Características como diâmetro, densidade e tamanho das fibras musculares, além da deposição de gordura intramuscular, influenciam a qualidade e desempenham papel importante na maciez da carne (SMITH et al., 1963).

A formação do tecido muscular é chamada de miogênese, em que há etapas de hiperplasia e hipertrofia. A hiperplasia ocorre durante a fase embrionária e fixa o número de fibras, e a hipertrofia ocorre durante o período de crescimento do animal em que há aumento do diâmetro das fibras (STAUN, 1968). Em um estudo realizado por Liu et al. (2017) que analisaram linhagens de frango não selecionadas e selecionadas para rápido crescimento, foi observado que há maior contribuição na fase de hiperplasia do que a hipertrofia para o rendimento de peito. Ou seja, a hiperplasia é primordial no desenvolvimento muscular de frangos, em que há diferenciação de células mesodérmicas em mioblastos que se fundem entre si formando os miotubos. Esses miotubos vão se desenvolver em fases distintas que podem ser separadas em primeiro e segundo estágio (DECARY et al., 1997). Os miotubos de primeiro estágio são destinados a originar fibras de contração lenta, enquanto os secundários formam fibras de contração rápida (ROSS et al., 1987).

Os mioblastos que não se diferenciam são chamados de células satélites, que são células mononucleadas e são ativadas durante a fase de hipertrofia, em que se observa aumento no número de núcleos e de miofibrilas nas fibras musculares pré-existentes a partir da proliferação e diferenciação das células satélites (MOSS & LEBLOND, 1971). Inicialmente, as fibras foram classificadas em vermelhas, intermediárias e brancas, e posteriormente, os três tipos principais de fibras musculares foram descritos como fibras do tipo I - de contração lenta e oxidativa, tipo IIA – de contração rápida e oxidativa; e tipo IIB – de contração rápida e glicolítica (BROOKE & KAISER, 1970).

No músculo peitoral de frangos predominam fibras do tipo IIB e IIA. Histologicamente, essas fibras apresentam pouca densidade de capilares sanguíneos e pequeno número de mitocôndrias (MACARI et al., 1994). A integridade estrutural das

fibras musculares é mantida por três camadas de tecido conjuntivo: endomísio, perimísio e epimísio (FIGURA 1, PURSLOW, 2002).

No músculo, cada fibra muscular é envolvida por uma membrana fina formando o endomísio. Do conjunto de endomísio partem septos muitos finos de tecido conjuntivo, que são chamados de perimísio, e por último há uma membrana externa que envolve todos os feixes chamados de epimísio (GONZALES e SARTORI, 2002). Durante a miogênese, fatores genéticos e ambientais definem o número de fibras e consequentemente a massa muscular (REHFELDT et al., 2000).

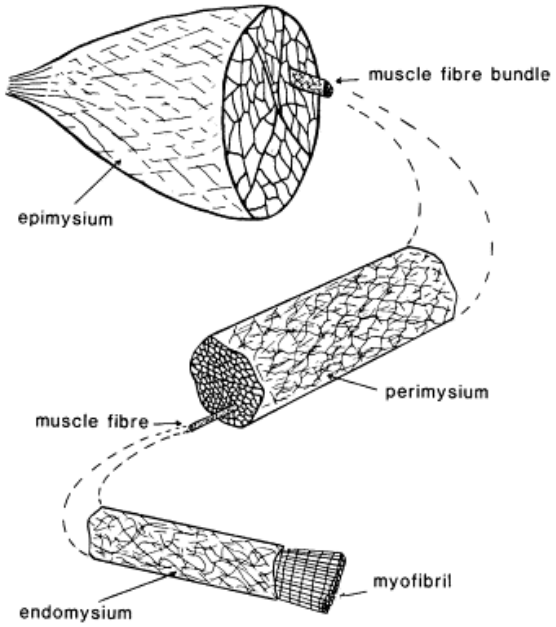

Figura 1: Diagrama esquemático mostrando três estruturas musculares distintas. O músculo todo é cercado pelo epimísio, feixes de fibras são separados pelo perimísio e fibras musculares individuais são separadas pelo endomísio. **Fonte:** Adaptado de Purslow (2002).

Durante o desenvolvimento do músculo do peito de frangos de corte, o aumento na seção transversal da fibra muscular é maior do que no tecido conjuntivo do endomísio e do perimísio (DRANSFIELD E SOSNICKI, 1999). Isso sugere que a seleção para crescimento rápido reduziu a capacidade oxidativa da musculatura dos frangos de corte, resultando em músculos mais anaeróbios (WILSON,1990). Assim, ocorre a formação de grandes espaços intercelulares ocasionando perda de fluídos das fibras musculares comprometendo a integridade muscular de frangos de corte (SWATLAND, 1990).

O comprometimento da integridade muscular dá início ao reparo dos tecidos afetados, desencadeando uma série de mecanismos de regeneração e cicatrização

(ALBINO et al., 2005). Um elemento fundamental na indução do processo de reparo são os macrófagos (DI PIETRO, 1995). Os macrófagos (células inflamatórias) e os fibroblastos são fundamentais na regulação da homeostase do tecido, pois estimulam a deposição do tecido conectivo em caso de lesões musculares, remodelando e substituindo o tecido esquelético (MANN et al., 2011). Em caso de destruição celular de maior proporção, é possível observar proliferação de adipócitos (NATAJARAN *et al.*, 2010 GOMES et al., 2004 e KAARIAINEM et al., 2000). Como consequência, os músculos apresentam alterações histopatológicas (SOIKE e BERGMANN,1998), tais como, presença de tecido conjuntivo e adiposo, ocupando espaços onde ocorreu a degeneração de fibras musculares.

1.3 Miopatias Peitorais

Alvo dos programas de melhoramento genético, a seleção para rendimento de peito de frango e alta taxa de crescimento pode ter respostas correlacionadas indesejáveis como deposição excessiva de gordura, miopatias musculares, problemas locomotores e ascite (ALNAHHAS et al., 2016; VAYEGO et al., 2014). A seleção para frangos de corte resultou em diferenças metabólicas e histológicas nos tecidos musculares (DUCLOS et al., 2007), em que há maior densidade de fibras de contração rápida, caracterizadas por maior diâmetro e menor taxa de degradação de proteínas em comparação com frangos não selecionados (PICARD et al., 2010; SCHEUERMANN et al., 2004). Assim, a seleção em frangos de corte não alterou significativamente o tipo de fibra no músculo, mas afetou o diâmetro e o comprimento das fibras musculares (BERRI et al., 2007). O aumento no tamanho das fibras está associado à menor capilarização, o que pode levar a alterações na homeostase de cátions e suprimento inadequado de oxigênio e nutrientes às células musculares (SANDERCOCK & MITCHELL, 2003). Além disso, pode estar associado à eliminação inadequada de produtos metabólicos intermediários que podem comprometer a funcionalidade da fibra e resultar em desregulação homeostática (MACRAE et al., 2006). Assim, em frangos de crescimento rápido, a seleção para o desenvolvimento da massa muscular reflete na capacidade comprometida dos tecidos vasculares e conectivos de suporte, resultando em desequilíbrios metabólicos (SANDERCOCK et al., 2009).

As alterações que ocorrem no músculo *Pectoralis major* das aves são denominadas de miopatias peitorais, que são modificações degenerativas no músculo e perda das funções das fibras (KUTTAPPAN et al., 2012). A ocorrência das miopatias musculares não só prejudica a aparência visual, mas afeta significativamente a qualidade e propriedades funcionais da carne, em que a capacidade de retenção de água e deposição de proteína é afetada (MUDALAL et al., 2015 & SOGLIA et al., 2015). Além da resistência do consumidor em comprar produtos *in natura*, muitas vezes o destino final das carnes afetadas é a fabricação de produtos processados com menor valor agregado (KUTTAPPAN et al., 2012; MUDALAL et al., 2015).

As miopatias prejudicam a capacidade de retenção de água durante o processamento e armazenamento da carne (BARBUT et al. 2008, PETRACCI et al., 2009; ZHU et al., 2012). Além disso, outras alterações como fraca coesão e tendência para a separação de feixes de fibras musculares ocorrem devido à imaturidade do tecido conjuntivo intramuscular (VELLEMAN et al., 2003; PETRACCI et al., 2012). Ainda que as observações histológicas demonstrem a presença de fibras degenerativas e infiltração de células com lipidose e fibrose (SOGLIA et al., 2015; VELLEMAN & CLARK, 2015), nenhum risco para a saúde humana está associado ao consumo de carne com essas miopatias.

As diferentes miopatias em frangos modernos afetam quesitos fundamentais da qualidade da carne, como aparência, textura e aspectos nutricionais (AGUIAR, 2006). Das miopatias relatadas, há o peito amadeirado (*Woody Breast*) caracterizado pelo endurecimento do músculo do peito, com uma incidência que chega a 50% em níveis moderados e em níveis severos em torno de 15% (OWEN, 2014). A miopatia Peitoral Profunda envolve a necrose isquêmica do músculo do peito e apresenta incidência de 1% nos lotes de frangos (BIANCHI et al., 2006). A mais recente miopatia registrada é chamada de *Spaghetti breast,* em que os músculos peitorais apresentam textura flácida e com as fibras muito desfiadas (SIHVO et al., 2014). Já a miopatia *White Striping* (WS), caracterizada pelo aparecimento de estrias brancas paralelas às fibras musculares na superfície do músculo peitoral maior (KUTTAPPAN, et al., 2012), foi pela primeira vez estudada por Bauermeister et al. (2009) e Kuttappan et al. (2009). A incidência dessa miopatia pode ser de até 12% em condições comerciais (PETRACCI et al., 2013). Em condições experimentais a incidência de WS pode ser superior a 50% (OWENS, 2012, PETRACCI et al., 2013). Pelo fato de ser uma das principais miopatias que afetam a

produção de frangos, a presente Dissertação se concentrou em entender os mecanismos genéticos que atuam para a ocorrência de WS.

1.4 *White Striping*

White Striping (WS) é uma miopatia muito frequente na produção de frangos de corte que afeta negativamente a qualidade da carne. Peitos com WS contém maior teor de gordura e colágeno e menor teor de proteína (MUDALAL et al., 2014), sendo o resultado de alterações estruturais, morfológicas e bioquímicas do tecido muscular, com consequente alteração nas fibras musculares (FERREIRA, 2014). Lorenzi (2014) destaca que peitos estriados são utilizados para retalhos ou subprodutos ou ainda comercializados no mercado interno, o que causa perdas econômicas para as agroindústrias.

As estrias brancas geralmente começam na parte cranial do filé de peito, próximo ao ponto de fixação da asa e à medida que as estrias aumentam de tamanho, estas se tornam mais visíveis (OWENS & VIERA, 2012). A intensidade das estrias no filé pode ser classificada como peito normal, moderada ou severa (FIGURA 1).

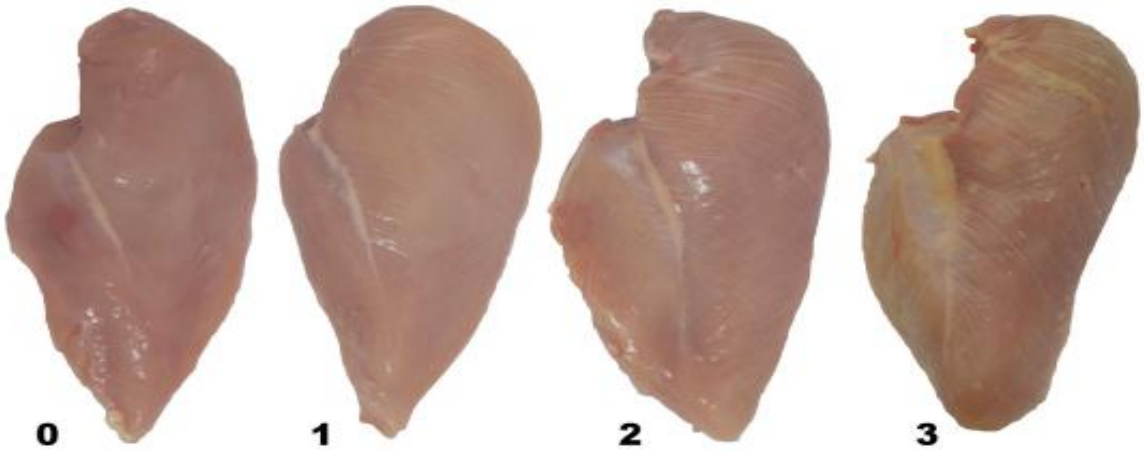

Figura 1: Escala de intensidade de *White Striping* no músculo peitoral de frangos de corte. **Fonte:** Kuttappan et al. (2016). 0 – peito normal, 1 e 2 – peito afetado moderamente e 3 – peito afetado severamente.

Como consequência da formação de espaços intercelulares e do aumento no diâmetro das fibras musculares, os capilares que cercam as fibras são deslocados,

limitando o fornecimento de oxigênio na fibra muscular (JOINER, 2014). Os baixos teores de oxigênio em tecidos levam a hipóxia e estudos recentes com análise de RNA-Seq sugerem que, além de hipóxia, altos níveis de cálcio intracelular podem estar associados à ocorrência dessas miopatias (MARCHESI et al., 2018, MUTRYN et al., 2015).

A hipóxia é conhecida por causar mudanças na arquitetura mitocondrial e, sabe-se que frangos de crescimento rápido têm menor quantidade total de mitocôndrias, tornando-os mais suscetíveis a danos devido ao metabolismo anaeróbico prolongado (POLAK, et al., 2009; PAPAH et al., 2017). Os músculos peitorais são quase inteiramente compostos de fibras do tipo IIB que possuem pequenas quantidades de mitocôndrias (GONZALES e SARTORI, 2002). Isso explica por que as alterações patológicas ocorrem principalmente no músculo *Pectoralis major* (GONZALES e SARTORI, 2002; HUDSON et al., 2017).

Concomitantemente à hipóxia, a demanda por ATP aumenta e o fluxo sanguíneo não é suficiente para fornecer oxigênio, resultando na formação de radicais livres que causam dano tecidual, ou seja, a produção de compostos tóxicos ou danosos aos tecidos, isto é, estresse oxidativo (LUNDBERG e WEITZBERG, 2008; LUNDBERG e WEITZBERG, 2009). Além das alterações metabólicas e bioquímicas envolvidas no WS, há alterações nas fibras associadas à regeneração, variação no tamanho das fibras musculares, infiltração de células mononucleares, aumento de adipócitos e tecido conjuntivo aonde ocorre degeneração das fibras musculares (KUTTAPPAN et al., 2013).

À medida que aumenta a gravidade de WS, o percentual de gordura nas estrias eleva-se e em graus severos de WS há rompimento da fibra resultando no aumento na circulação de marcadores de dano muscular, como: creatina quinase (CK), alanina aminotransferase (ALT), aspartato aminotransferase (AST) e lactato desidrogênase (LDH). Entretanto, não são encontradas diferenças no perfil hematológico entre os graus de WS, sugerindo que esta miopatia não é causada por uma infecção (KUTTAPPAN et al., 2013).

A incidência de estrias musculares nos frangos de corte tem sido atribuída ao desenvolvimento genético das aves, entretanto alguns trabalhos relataram influências nutricionais (MELOCHE et al., 2005, KUTTAPPAN et al., 2013) e de fatores ambientais (BAILEY et al., 2015). Ao avaliar o efeito de dietas com alto teor energético aos 42 e 49 dias, Kuttappan et al. (2012a) observaram maior ocorrência de peitos com

estrias brancas em graus severos. Alguns trabalhos buscam minimizar os graus de WS através da nutrição. Kuttappan et al. (2012b) avaliou a inclusão de maiores níveis de vitamina E em dietas de frangos e observou que a adição não apresentava efeito na incidência de WS. Esses autores salientam a necessidade de mais estudos sobre inclusão de suplementos nas dietas de frangos de corte a fim de reduzir o WS (KUTTAPPAN et al., 2012b).

Avaliações patológicas observadas por Kuttappan et al. (2013) nos músculos afetados por WS foram similares as encontradas por Mitchell et al. (1999) em aves sob condições de estresse por calor. Fatores ambientais e de manejo são destacados no trabalho realizado por Bailey et al. (2015) em que estimou que a condição ambiental influencia 65% da variância das estrias brancas no músculo peitoral. De modo geral, sugere-se que a etiologia de WS é multifatorial e complexa (ZAMBONELLI et al., 2017).

1.5 Efeitos genéticos na manifestação de *White Striping*

Além de fatores nutricionais e de manejo, os componentes genéticos possuem grande influência nessa anomalia. As herdabilidades para WS foram estimadas de moderadas (h^2= 0.33; BAILEY et al., 2015) a altas (h^2 = 0,65; ALNAHHAS et al., 2016). Essas diferenças podem ser explicadas pelo uso de distintas populações base onde diferentes critérios de seleção são utilizados. Das miopatias peitorais, WS possui maior influência genética (BAILEY et al., 2015) se comparada às outras miopatias, como Miopatia Peitoral Profunda e *Woody Breast*, que apresentam herdabilidades inferiores a 0,1, consideradas baixas (BAILEY et al., 2015). Um dos desafios do setor avícola é identificar a etiologia e prever a ocorrência de WS em aves vivas e, assim, utilizar ferramentas que reduzam a ocorrência desse defeito em frangos de corte (KUTTAPPAN et al., 2016).

Dada a base genética na manifestação de WS, utilizar o conhecimento sobre ação dos genes ligados a características de interesse econômico pode complementar os métodos quantitativos de melhoramento (LEDUR, 2001; CASSAR-MALEK et al., 2008). Ao entender os processos fisiológicos resultantes da expressão de vários genes combinados, torna possível identificar biomarcadores específicos que poderiam estar

associados a esta condição (KUTTAPPAN et al.,2016). Por isso, pesquisas que esclareçam a etiologia complexa envolvida nessa miopatia são necessárias.

1.6 Genes Candidatos

Na abordagem de genes candidatos pode-se utilizar estudos prévios para inferir o papel biológico de um gene na variação observada em uma característica (ELZO et al., 2012). Dessa forma, um gene candidato pode ser escolhido com base na função que ele exerce em outra espécie. Neste caso, este é definido como gene candidato com base na biologia. Os genes classificados como genes candidatos posicionais são aqueles localizados em região cromossômica associada com alguma característica de interesse, como as regiões detectadas em estudos de associação genômica - GWAS (WANG et al., 2010). Os genes classificados como genes candidatos funcionais são aqueles envolvidos em vias metabólicas de interesse, codificando uma proteína relacionada com o fenótipo do estudo (WANG et al., 2010) e normalmente evidenciados em estudos de expressão gênica, tanto pontuais como globais (microarranjos e RNA-Seq). Assim, identificar genes responsáveis por determinada condição é importante para associar uma variante genética com algum distúrbio ou característica produtiva. Os estudos de genes candidatos possibilitam evidenciar genes que podem estar envolvidos com características de desenvolvimento, do metabolismo e da biologia do animal (COUTINHO et al., 2010).

Vários genes candidatos funcionais associados à miopatias peitorais foram identificados recentemente por meio da tecnologia de RNA-Seq (MUTRYN et al., 2015; MARCHESI et al., 2018 e ZAMBONELLI et al., 2017), sugerindo que essas anomalias são de herança poligênica. Dessa forma, a hipótese é que há uma rede de modificações biológicas que atuam simultaneamente e são responsáveis por evidências fenotípicas dessas miopatias. Por isso, pesquisas que esclareçam a etiologia complexa envolvida nessa miopatia são necessárias. Assim, para validar esses genes prospectados em estudos globais, a técnica de qPCR é a mais indicada para quantificar a expressão de um gene por ser mais acurada (GINZINGER, 2002; VANGUILDER et al., 2008; TANIGUCHI et al., 2009).

No presente estudo, os genes candidatos avaliados foram selecionados a partir do conjunto de genes que se apresentaram diferencialmente expressos (DE) entre os

transcriptomas de frangos normais e afetados com a miopatia WS, de acordo com dados da literatura (MUTRYN et al., 2015; MARCHESI et al., 2018). Dessa forma, o perfil de expressão de 15 genes candidatos funcionais foi avaliado pela técnica de PCR quantitativa em tempo real (qPCR), sendo eles: *Actin, gamma 1 (ACTG1), Carbonic Anhydrase II (CA2), Calmodulin 2 (CALM2), Carbohydrate Sulfotransferase 1 (CHST1), Cysteine and Glycine Rich Protein 3 (CSRP3), Deoxyribonuclease 1 like (DNASE1L3), Histone Deacetylase 1 (HDAC1), Hypoxia Inducible Factor 1 Alpha Subunit (HIF1A), Mitogen-Activated Protein Kinase 13 (MAPK13), Myosin light chain kinase 2 (MYLK2), Phosphorylase kinase regulatory subunit beta (PHKB), Perilipin 1 (PLIN1) Ryanodine Receptor 2 (RYR2), SMAD Family Member 3 (SMAD3), e Troponin C2, Fast Skeletal Type (TNNC2).* A seguir, a função destes genes será brevemente descrita. Contudo, poucos são os estudos funcionais envolvendo esses genes na galinha.

1.6.1 *Actin, gamma 1 (ACTG1)*

O gene *Actin gama 1* se encontra no cromossomo 10 da espécie *Gallus gallus* (NCBI, 2019). A proteína gerada por esse gene interage com a miosina para gerar força contrátil, sendo responsável pela motilidade celular e manutenção do citoesqueleto (BERGSMA et al., 1985). Um estudo envolvendo o *knockout* desse gene em camundongos demonstrou a influência desse gene sobre padrões progressivos de necrose e regeneração das células musculares, afetando predominantemente as fibras do tipo II (SONNEMANN et al., 2006). O gene *ACTG1* foi descrito como DE em aves afetadas com WS e está envolvido na regulação do citoesqueleto de actina (MARCHESI et al., 2018). As actinas são uma família de proteínas altamente conservadas do citoesqueleto que desempenham papéis fundamentais em quase todos os aspectos da biologia celular e possuem natureza essencial na fisiologia muscular normal.

1.6.2 *Carbonic Anhydrase II (CA2)*

O gene *Carbonic Anhydrase II* está localizado no cromossomo 2 da galinha (ENSEMBL, 2019). As anidrases carbônicas (CA) formam uma família de genes que codificam metaloenzimas de zinco de grande importância fisiológica. Como catalisadores da hidratação reversível do dióxido de carbono, essas enzimas participam

de uma variedade de processos biológicos, incluindo respiração, calcificação, equilíbrio ácido-base e reabsorção óssea. Devido a importante função dos genes da família Anidrase Carbônica e dos relatos de associação com miopatias, é interessante avaliar se há diferença na expressão do gene *CA2* em grupos de frangos afetados com a miopatia WS. Genes da família Anidrase Carbônica já foram relatados como diferencialmente expressos em estudos com miopatias musculares, dentre eles o gene *CA2*, participante no metabolismo de nitrogênio, que foi DE entre frangos normais e afetados com *White Striping* em estudo realizado por Marchesi et al. (2018). O gene *CA3* também foi DE em grupos de frangos afetados com a miopatia WB (MUTRYN et al., 2015). Sugere-se que o *CA3* tem a capacidade de atuar como um agente antioxidante contra moléculas prejudiciais, pois possui funções reguladoras e reparadoras e é um fator crítico no sistema de defesa antioxidante no músculo esquelético (ZIMMERMAN et al 2004).

1.6.3 *Calmodulin 2 (CALM2)*

Em galinhas o gene *Calmodulin 2* localiza-se no cromossomo 5 (NCBI, 2019), e codifica uma proteína membro da família da calmodulina. A calmodulina é a reguladora do sinal de Ca^{2+} e atua no controle de um grande número de enzimas e outras proteínas pelo cálcio. Dessa forma, essa proteína é conhecida por regular inúmeros processos celulares, incluindo motilidade celular, proliferação celular e o metabolismo intermediário (TOUTENHOOFD et al., 1998). Quaisquer alterações na concentração intracelular de *CALM2* e Ca^{2+} iniciam o processo de proliferação celular (COHEN & KLEE, 1988). Em bovinos, foi encontrado um SNP no gene *CALM2* que foi associado à quantidade de calpastaína presente no músculo (DUNNER et al., 2013). Isso demonstra que este gene pode estar associado à textura da carne. Em frangos esse gene não foi associado ainda a nenhuma característica envolvendo tecido muscular.

1.6.4 *Carbohydrate Sulfotransferase 1 (CHST1)*

O gene *Carbohydrate Sulfotransferase 1* está localizado no cromossomo 5 da espécie *Gallus gallus* (NCBI, 2019) e codifica uma proteína da família de sulfotransferase da queratina. O gene *CHST1* é amplamente estudado em diversas espécies por ser predominantemente expresso no músculo esquelético. Em humanos

esse gene foi associado ao desenvolvimento de adipócitos (URS et al., 2004) e associado ao metabolismo de ligantes de L-Selectina, moléculas de adesão expressas na superfície de linfócitos (LYNCH et al., 2012). O gene *CHST1* já foi descrito como DE entre aves normais e afetadas com as miopatias *White Striping* e *Wooden Breast* (ZAMBONELLI et al., 2017), em que é mais expresso no grupo afetado em relação ao grupo controle e atua em diferentes processos metabólicos como os de monossacarídeos e polissacarídeos.

1.6.5 *Cysteine and Glycine rich Protein 3 (CSRP3)*

Posicionado no cromossomo 5 da galinha (ENSEMBL, 2019), o gene *cysteine and glycine rich protein 3*, codifica a proteína LIM envolvida em processos regulatórios importantes para desenvolvimento e diferenciação celular (GENECARD, 2019). Este gene atua como regulador positivo da miogênese e auxilia na manutenção da integridade das células musculares através de um mecanismo baseado em actina (ARBER et al., 1994). A expressão de *CSRP3* está presente principalmente no músculo estriado, incluindo o músculo esquelético, e desempenha um grande papel na diferenciação miogênica. Em aves afetadas com miopatias musculares, o *CSRP3* foi um dos genes com maior expressão em frangos afetados (MUTRYN et al., 2015). A expressão deste gene também se correlaciona com o crescimento e formação de miotubos (ARBER et al., 1994), que é uma característica fundamental do reparo muscular.

1.6.6 *Deoxyribonuclease 1 like (DNASE1L3)*

Presente no cromossomo 12 da espécie *Gallus gallus* (NCBI, 2018), a proteína codificada pelo gene *Deoxyribonuclease 1 like* (*DNASE1L3*) age hidrolisando o DNA internucleossomal durante a apoptose e necrose na célula do fígado e baço, e é expressa predominantemente em células mielóides, como os macrófagos (SISIRAK et al., 2016). O gene *DNASE1L3* atua similarmente nas ligações de íons de cálcio (GENECARD, 2019) e já foi descrito como menos expresso em frangos afetados com WS (MARCHESI et al., 2018).

1.6.7 *Histone Deacetylase 1 (HDAC1)*

Localizado no cromossomo 23 da espécie *Gallus gallus* (ENSEMBL, 2019) o gene *HDAC1* codifica a enzima HDAC1 de classe I, tipicamente localizada no núcleo da célula. É pertencente à família de enzimas HDAC que desacetila as caudas de histonas e as proteínas não-histonas, alterando a estrutura da cromatina (MONTGOMERY et al., 2007). O gene *HDAC1* tem papel na organização do DNA em eritrócitos de frangos (SUN et al., 1999). Em um estudo de associação genômica (GWAS), o gene *histona desacetilase 2* (*HDAC2)* foi um dos genes candidatos posicionais para o peso do músculo do peito em galinhas (SHAHJAHAN et al., 2016). Ao avaliar a possível função do *HDAC2*, sugere-se que é um gene candidato funcional para o desenvolvimento de pré-eclosão e pós-eclosão do músculo esquelético de galinha (SHAHJAHAN et al., 2016).

1.6.8 *Hypoxia Inducible Factor 1 Alpha Subunit (HIF1A)*

O gene *HIF1A* está localizado no cromossomo 5 da espécie *Gallus gallus* (ENSEMBL, 2019). Durante períodos de oferta reduzida de oxigênio, as mudanças mais profundas na expressão gênica são mediadas por fatores de transcrição conhecidos como fatores induzidos por hipóxia (GRAHAN & PRESNELL, 2017). O *HIF1A* atua como fator transcricional em que ativa muitos genes em resposta à hipóxia e também está envolvido no metabolismo energético, na angiogênese e na apoptose (MARCHESI et al., 2018).

1.6.9 *Mitogen-Activated Protein Kinase 13 (MAPK13)*

O gene *MAPK13* está localizado no cromossomo 26 na galinha (ENSEMBL, 2019) e codifica uma proteína que é ativada por citocinas pró-inflamatórias e estresse celular (GENECARD, 2019). Além disso, a proteína age como ponto de integração para múltiplos sinais bioquímicos e envolvidos em variados processos celulares, tais como proliferação, diferenciação, regulação de transcrição e desenvolvimento (NCB1, 2019).

O *MAPK13* foi descrito como gene candidato posicional associado ao desenvolvimento muscular em frangos de corte (BOSCHIERO et al., 2018).

1.6.10 *Myosin light chain kinase 2 (MYLK2)*

O gene *MYLK2* codifica a proteína miosina quinase de cadeia leve que é importante para a contração muscular, uma enzima dependente do cálcio e calmodulina, que é expressa no músculo esquelético adulto (GENECARD, 2019). O gene *myosin light chain kinase 2*, localizado no cromossomo 20 em *Gallus gallus*, atua também na regulação do citoesqueleto da actina e na via de sinalização de cálcio (NCBI, 2019). A miosina é essencial para a contração muscular e compreende a maioria das proteínas miofibrilares dentro das células musculares (SMITH et al., 1999).

1.6.11 *Phosphorylase kinase regulatory subunit beta (PHKB)*

O gene *phosphorylase kinase regulatory subunit beta* (*PHKB*) está presente no cromossomo 11 da espécie *Gallus gallus* (NCBI, 2019). Codificando a subunidade beta da fosforilase quinase, esta subunidade é idêntica nas isoformas musculares e hepáticas, atuando no metabolismo do glicogênio e nas vias de sinalização de cálcio (GENECARD, 2018). Xue et al. (2017) investigaram os potenciais mecanismos regulatórios do crescimento inicial do frango e, dentre eles, o *PHKB* possivelmente está envolvido com crescimento e desenvolvimento da ave.

1.6.12 *Perilipin 1 (PLIN1)*

O gene *Perilipin 1 (PLIN1)* está localizado no cromossomo 10 da galinha (NCBI, 2019), sendo amplamente estudado em humanos devido a sua função no armazenamento no tecido adiposo e na regulação do metabolismo lipídico do fígado, influenciando diretamente no aumento da reserva de gordura (RUIZ et al., 2011). Em animais, o gene *PLIN1* afeta o peso corporal e a deposição de gordura (LONDOS et al., 2005). Em patos, vários genótipos do *PLIN1* foram associados ao peso de carcaça e porcentagem de gordura abdominal (ZANG et al., 2013). Em frangos de corte foram detectados polimorfismos do gene *PLIN1* associados com parâmetros de lipídeos

(ZHANG et al., 2015). Do mesmo modo, esse gene foi relacionado às vias metabólicas de lipídeos em frangos afetados com a miopatia WB (PAPAH et al., 2018).

1.6.13 *Ryanodine Receptor 2 (RYR2)*

Posicionado no cromossomo 3 da espécie *Gallus gallus* (NCBI, 2019), o gene *Ryanodine Receptor 2 (RYR2)* codifica um receptor de rianodina encontrado no retículo sarcoplasmático do músculo cardíaco (CORREIA et al., 2012). A proteína codificada é um dos componentes do canal de cálcio, sendo que a contração muscular é controlada pela liberação de cálcio (Ca^2+) do retículo endoplasmática através de vários canais, um dos quais é a proteína do receptor de rianodina (FUJII et al.,1991). A mutação no gene *RYR1* em suínos é conhecida por desencadear a carne PSE, uma vez que esta mutação resulta na seqüência alterada de aminoácidos e provoca maior liberação de cálcio nos tecidos musculares. As características de PSE também foram observadas em frangos de corte e alterações na expressão do gene *RYR1* (CHIANG et al., 2004; DROVAL et al., 2012).

1.6.14 *SMAD Family Member 3 (SMAD3)*

O gene *SMAD3* está presente no cromossomo 10 da espécie *Gallus gallus* (NCBI, 2019). As proteínas codificadas por *SMAD* são conhecidas como as principais moléculas de transdução que participam da rede de sinalização, sendo o principal mediador da inibição da miogênese pela miostatina (ZHU et al., 2004). As proteínas *SMAD* transportam o fator de crescimento transformador β1 (TGF-β1), o qual é um potente inibidor da proliferação e diferenciação de células musculares, influenciando na expressão dos principais fatores reguladores miogênicos, incluindo MyoD e miogenina durante a miogênese (LI et al., 2008).

1.6.15 *Troponin C2 Fast Skeletal Type (TNNC2)*

Posicionado no cromossomo 20 da galinha (NCBI, 2018), o gene *TNNC2* codifica a proteína troponina C, sendo expresso durante a diferenciação de mioblastos e o desenvolvimento do músculo esquelético (BUCHER et al., 1988). Dessa forma, este

gene desempenha um papel crítico na contração do músculo esquelético e pode estar envolvido em características de qualidade da carne em animais de produção. A troponina é um complexo protéico chave na regulação da contração muscular estriada. Assim, os genes envolvidos no desenvolvimento do músculo esquelético, na diferenciação dos mioblastos ou na contração do músculo esquelético são considerados como potenciais genes candidatos para a qualidade da carne (TE PAS e SOUMILLION, 2001). Em suínos, é possível que o gene *TNNC2* esteja ligado a marcadores relacionados à qualidade da carne, como maciez, uma vez que esse gene tem papel crítico na contração muscular (XU et al., 2008).

1.7 Genes referência e qPCR

A PCR quantitativa em tempo real (qPCR) é um dos métodos de quantificação mais precisos para a análise da expressão gênica, em razão da sua alta sensibilidade e reprodutibilidade (KOZERA e RAPACZ, 2003). Além disso, a qPCR é rápida, fácil de usar e fornece medição simultânea da expressão gênica em muitas amostras diferentes (GACHON et al. 2004). Por isso, é uma técnica indicada para validar dados obtidos por outros métodos de análise de expressão gênica (KOZERA e RAPACZ, 2003).

Frequentemente, o nível de expressão de alguns genes é tão pequeno que a qPCR se torna a única técnica capaz de detectar número tão baixo de cópias de mRNA (MALLONA et al. 2010). Entretanto, com o uso crescente da qPCR é necessário introduzir métodos de normalização apropriados e validar os resultados (BUSTIN et al., 2009). Existe uma enorme quantidade de protocolos, metodologias e dados disponíveis sobre qPCR (KOZERA e RAPACZ, 2003). Um dos passos críticos na comparação dos perfis de transcrição é a normalização precisa, que pode ser afetada por quantidade de amostra inicial, qualidade do cDNA, eficiência da reação de transcrição reversa e presença de inibidores em diferentes materiais de amostra (BUSTIN et al., 2009). Assim, a padronização é o passo inicial para trabalhos com qPCR e o método mais popular de normalização de genes é a utilização de genes normalizadores ou genes referência, contra o qual o nível de expressão será determinado (SUZUKI et al., 2000).

Os genes usados como referência são frequentemente chamados de genes de manutenção, assumindo que esses genes são constantemente expressos em certos tecidos e sob certas circunstâncias (THELLIN et al., 1999). O gene de referência adequado deve ser expresso de forma estável em diferentes condições experimentais

(UDVARDI et al., 2008). Em contraste, a seleção inadequada de genes de referência pode levar a discrepâncias na interpretação dos dados (BUSTIN et al., 2009), levando a conclusões erradas em relação à expressão de genes alvo (HUANG et al., 2018).

Além disso, não há genes de referência expressos descritos em nível constante em todas as espécies e sob todas as condições experimentais (ZHU et al., 2012). De acordo com as diretrizes da Informação Mínima para Publicação de Experiências de PCR em Tempo Real Quantitativo (MIQE) (BUSTIN et al., 2009), os genes de referência são selecionados com base em sua especificidade nas interações entre uma espécie ou tecido submetido a diferentes tratamentos experimentais. Assim, é essencial validar sistematicamente a estabilidade de potenciais genes de referência antes da sua utilização em ensaios de qPCR em qualquer organismo experimental (BUSTIN et al., 2009, HUANG et al., 2018).

Vários estudos relataram avaliações de genes de referência em animais, como bovinos (LISOWSKI et al., 2008), suínos (LORENZETTI et al., 2018), ovelhas (ZANG et al., 2011) e peixes (YANG et al., 2013). Em frangos de corte, Staines et al. (2016) criou um painel com genes de referência adequados para frangos em vários contextos experimentais. Dada a importância do músculo do peito no setor avícola, alguns estudos buscaram avaliar os genes mais estáveis no músculo peitoral de frangos (CEDRAZ DE OLIVEIRA et al., 2017 e NASCIMENTO et al., 2015). Entretanto, trabalhos avaliando genes mais estáveis em frangos de corte normais e afetados com a miopatia *White Striping* não foram encontrados. Por isso, no presente estudo, o perfil de expressão de 10 genes candidatos a normalizadores nesse tecido foi avaliado, sendo eles: *Hydroxymethylbilane synthase (HMBS), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Mitochondrial ribosomal 27 (MRPS27), Ribosomal protein lateral stalk subunit P1 (MRPS30), Ribosomal Protein, Large P1 (RPLP1), Ribosomal protein L4 (RPL4), Beta-2-microglobulin (B2M), Ribosomal protein 30 (RPL30), Ribosomal protein L5 (RPL5), Hypoxanthine phosphoribosyltransferase 1 (HPRT1).* Após a escolha dos normalizadores mais acurados, estes foram utilizados para a quantificação relativa do conjunto de genes alvo neste estudo.

1.8 Objetivos

1.8.1 Objetivo Geral

Analisar o perfil de expressão de genes candidatos à ocorrência da miopatia *White Striping* no músculo peitoral de frangos normais e afetados com essa miopatia.

1.6.2 Objetivos Específicos

- Quantificar o nível de expressão de 10 genes referência e 15 genes alvo no músculo peitoral de frangos de corte aos 42 dias de idade.
- Identificar os genes referência mais estáveis no músculo peitoral de aves normais e afetadas com *White Striping*.
- Identificar genes diferencialmente expressos entre frangos de corte normais e afetados com *White Striping* e assim evidenciar mecanismos genéticos associados a esta miopatia.

2 - CAPÍTULO II
MANUSCRITOS

Os resultados desta dissertação são apresentados na forma de dois manuscritos, com sua formatação de acordo com as orientações das revistas as quais foram submetidos:

2.1 – MANUSCRITO I

Stable reference genes for expression studies in breast muscle of normal and white striping-affected chickens

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Abstract

The normalization with proper reference genes is a crucial step to obtain accurate mRNA expression levels in quantitative PCR (qPCR) studies. Therefore, in this study, 10 reference candidate genes were evaluated to determine their stability in normal breast muscle of broilers and those affected with White Striping (WS) myopathy at 42 days of age. Four different tools were used for ranking the most stable genes: GeNorm, NormFinder, BestKeeper and Comparative Ct (ΔCt), and a general ranking was performed using the RankAggreg tool to select the best reference genes among all tools. From the 10 genes evaluated in the breast muscle of broilers, 8 were amplified. Most algorithm/tool has indicated the same two genes, as the most stable in broilers breast muscle. In addition, there was agreement among the tools for the least stable genes: *MRPS27, GAPDH* and *RPLP1* in broilers breast muscle. Therefore, it is interesting to note that even with different tools for evaluating gene expression, there was consensus on the most and least stable genes. Our findings showed that Ribosomal protein 30 (*RPL30*) and Ribosomal protein L5 (*RPL5*) were the most stable genes expressed in the *pectoralis major* muscle of normal and WS-affected broilers at 42 days of age. These results indicate that these genes can be recommended for accurate normalization in qPCR studies with chicken *pectoralis major* muscle affected with White Striping myopathy.

Keywords: broilers, endogenous genes, myopathies, normalization, stability.

Introduction

White striping (WS) is a prevalent myopathy in modern broiler chickens causing losses in production due to the reduced sensorial, technological and nutritional quality of the breast meat (Kuttappan et al 2012). WS is characterized by white striations parallel to fibers in the breast fillet, being a consequence of structural, morphological and biochemical alterations of muscle tissue (Kuttappan et al 2013).This condition is an issue for the broiler industry and needs to be controlled to reduce the negative effects on processing and on the consumer acceptability (Kuttappan et al 2012).

In the last years, studies on WS myopathy have been increasing trying to determine the genetic mechanisms involved in this condition (Zambonelli et al., 2016), which could help developing strategies to reduce the incidence and severity of WS in commercial broilers (Alnahhas et al., 2016). Gene expression analysis is one of the options to study the biological processes involved with WS, and the relative quantification using the real-time PCR (qPCR) methodology may provide the analysis of multiple genes to be evaluated (McCulloch et al., 2012).

The qPCR is an efficient tool to evaluated mRNA expression levels because it is highly specific, reproducible and sensitive (Bustin et al., 2009, Rebouceas et al., 2013). Nevertheless, the production of reliable results requires several steps to ensure a quality assay, including the selection of stable reference genes (Taylor et al., 2010). It is known that each experiment requires a specific search for genes with non-variable expression patterns to be used as normalizer to avoid the quantification errors caused by experimental variations (Bustin et al., 2009).

As a requirement, the reference gene should be constitutively expressed in all the experimental conditions and tissues (Dheda et al., 2004). Thus, for gene expression analysis, the reference genes are evaluated through mathematical algorithms for further standardization and normalization for target gene assessment (Pfaffl et al., 2004). The

evaluation of potential reference gene stability depends on the method used for analysis, and the most common normalization algorithms are NormFinder (Andersen et al., 2004), geNorm (Vandesompele et al., 2002), BestKeeper (Pfaffl et al., 2004) and Comparative Ct (ΔCt) (Silver et al., 2006). These algorithms use different approaches that allow evaluation of the appropriateness of a gene or set of genes as a normalization factor (Piehler et al., 2010).

Traditionally, the reference genes most commonly used in many species, including chickens, are beta-2-microglobulin (*B2M*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), hydroxymethylbilane synthase (*HMBS*) and hypoxanthine phosphoribosyltransferase 1 (*HPRT1*). Several studies searching endogenous genes for different breeds, tissues and conditions in broilers have been reported (Zambonelli et al., 2016, Nascimento et al., 2015, Cedraz de Oliveira et al., 2017). However, there is no study on the identification of reference genes in muscle tissues affected with myopathies in broilers. Therefore, knowing the importance of selecting stable genes under this condition to obtain accurate profiling of gene expression, this study evaluated the stability of 10 endogenous candidate genes in breast muscle of normal broilers and those affected with WS myopathy.

Material and Methods

Experimental animals and tissue collection

The animals for this study were raised at the Embrapa Swine and Poultry National Research Center farm, located in Concórdia, Santa Catarina, Brazil. A total of 168 male

broilers from a commercial line (Cobb500) was used. Chicks were vaccinated against fowl pox and Marek disease at the hatchery. The management conditions were followed as recommended for this commercial line, receiving food and water ad libitum. At 42 days of age, animals were weighed and euthanized by cervical dislocation, according to the practices recommended by the Ethics Committee on Animal Use (CEUA protocol 012/2012). Immediately after slaughter, the broilers *pectoralis major* muscles were evaluated by the presence or absence of white striations following Kuttappan et al. (2013) and were grouped in normal (n=16, classified by absence of WS) an affected (n=16, classified with the moderate presence of WS). The broilers average weight for the normal group was 2.850 g and for the affected broilers was 2.880 g. A total of 32 tissue samples was collected, immediately frozen in liquid nitrogen and stored in an -80°C ultrafreezer for further RNA extraction.

Total RNA Extraction and cDNA synthesis

Approximately 100 mg of breast muscle were ground with a mortar and pestle in liquid nitrogen. Subsequently, the total RNA was isolated using Trizol (Invitrogen, USA) according to manufacturer's protocol and quantified in BioDrop spectrophotometer (Biodrop, UK). The RNA was considered pure if OD260:OD280 ratio was higher than 1.8. Integrity of the samples were confirmed in 1% agarose gel, and electrophoresed for 90 minutes. The first strand cDNA was synthesized using 4ug of total RNA using the SuperScript III First-Strand Kit SuperMix Synthesis (ThermoFisher Scientific, EUA), following the manufacturer's recommendations.

Relative quantification using qPCR

The expression pattern of the following putative reference genes were evaluated based on the literature in broilers (Zambonelli et al., 2016, Nascimento et al., 2015, Cedraz de Oliveira et al., 2017): Hydroxymethylbilane synthase (*HMBS*), Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), Mitochondrial ribosomal 27 (*MRPS27*), Mitochondrial ribosomal protein 30 (*MRPS30*), Large Ribosomal Subunit Protein P1 (*RPLP1*), Ribosomal protein L4 (*RPL4*), Beta-2-microglobulin (*B2M*), Ribosomal protein 30 (*RPL30*), Ribosomal protein L5 (*RPL5*) and hypoxanthine phosphoribosyltransferase 1 (*HPRT1*).

The sequence of these 10 genes were obtained from the *Gallus gallus* in the Genbank (http://www.ncbi.nlm.nih.gov/gene/) and Ensembl (http://www.ensembl.org/index.html) databases. Primers were designed in exon-exon junctions to avoid DNA amplification using the Primer-Blast (Ye et al., 2012). The reactions were run in the QuantStudio 6 (Applied Biosystems, Foster City, CA), with cycling of 95ºC for 10 minutes, 40 cycles of 95ºC for 11 seconds and 60ºC for 1 minute. The qPCR reactions were carried out with 15 uL volume containing 1x Maxima SYBR Green (2x) (Fermentas, USA), 0.13 uM of primers for each gene (Table 1) and 2 uL of diluted cDNA. In addition, the melting curve analysis was included with the cycling stage of 70°C to 95°C at 0.1°C/s for all genes to verify the primers specificity. Reactions were performed in duplicate, with negative controls included to detect possible contamination. The average of cycle threshold (Ct) values were collected for further analysis.

Table 1 Primers for the 10 reference candidate genes for the qPCR analysis in the breast muscle of broilers.

Gene	Function	Primer sequence (5' - 3')	Ensembl ID

HMBS[1] Hydroxymethylbilane synthase	The third enzyme of the biosynthetic pathway of the Heme group	F: ACTAGTTCACTTCGGCGAGC R: CTCAGGAGCTGACCTATGCG	ENSGALG00000042939
GAPDH Glyceraldehyde-3-phosphate dehydrogenase	Participates in nuclear transcription, RNA transport, DNA replication and apoptosis	F:TGGGAAGCTTACTGGAATGG R:ATCAGCAGCAGCCTTCACTAC	ENSGALG00000014442
MRPS27[2] Mitochondrial ribosomal 27	S27 Ribosomal protein subunit component	F: GCTCCCAGCTCTATGGTTATG R:ATCACCTGCAAGGCTCTATTT	ENSGALG00000015002
MRPS30[2] Ribosomal protein lateral stalk subunit P1	S30 Ribosomal protein subunit component	F: CCTGAATCCCGAGGTTAACTATT R: GAGGTGCGGCTTATCATCTATC	ENSGALG00000014874
RPLP1 Ribosomal protein lateral stalk subunit P1	Ribosomal protein 60S subunit component, L12P family	F: CCCTCATTCTCCACGACGAC R:CCAGAGCCTTAGCAAAGAGAC	ENSGALG00000030878
RPL4 [3] Ribosomal protein L4	Ribosomal protein 60S subunit component, L4E family	F: TGTTTGCCCCAACCAAGACT R:CTCCTCAATGCGGTGACCTT	ENSGALG00000007711
B2M Beta-2-microglobulin	Codes protein present on all nucleated cells	F: CTTCCACCCACCCAGGATCA R: ACTCGGGATCCCACTTGAAGAC	ENSGALG00000002160
RPL30[3] Ribosomal protein 30	Ribosomal protein 60S subunit component, L30E family	F: ATGATTCGGCAAGGCAAAGC R:GTCAGAGTCACCTGGGTCAA	ENSGALG00000029897
RPL5[2] Ribosomal protein L5	Ribosomal protein 60S subunit component, L18P family	F: AATATAACGCCTGATGGGATGG R: CTTGACTTCTCTCTTGGGTTTCT	ENSGALG00000005922
HPRT1 hypoxanthine phosphoribosyltransferase 1	Enzymatic plays a central role in the generation of purine nucleotides through the purine salvage pathway	F: TGGGGATGACCTCTCAACCT R:TCCAACAAAGTCTGGCCGAT	ENSGALG00000006098

[1]Paludo et al. (2016), [2]Nascimento et al. (2015), [3]Petry et al. (2018)

Determination of expression stability of reference genes

The BestKeeper, geNorm, NormFinder and Comparative ΔCt algorithms were used to select the most stable genes in the present study. The NormFinder program is an application of a mathematical model to describe the expression values measured by qPCR. The program predicts both the intragroup and intergroup expression variation of the genes tested, combining estimates to provide a direct measure of variation in the expression of each gene, according to the stability (S) and similarity of their expression

187 profiles. Values closest to 0 indicate the best genes or the most stable genes to be used
188 as normalizers (Andersen et al., 2004).

189 The geNorm software calculates an internal control stability measurement (M-
190 value) for each reference gene, which is the mean variation of a gene compared to all
191 other genes tested. Genes with the highest M values have the greatest variation in
192 expression, while the most stable genes are determined based on the lowest M values,
193 and values lower than 1.5 indicate stable genes (Vandesompele et al., 2002). The
194 geNorm values were obtained using the SLqPCR package on R
195 (http://bioconductor.org/packages/release/bioc/html/SLqPCR.html).

196 The BestKeeper program was developed using an Excel-based tool (Pfaffl et al.,
197 2004) which analyzes the variability of reference genes expression through the values of
198 Ct, fold-change, standard deviation (SD) and coefficient of variation (CV). The CV and
199 SD of Cts are the main parameters used to evaluate the expression variation of candidate
200 reference genes. Genes with SD of Cts higher than 1.0 are not indicated to be used as
201 endogenous genes (Pfaffl et al., 2004). This tool also combines those values (Ct, fold-
202 change, SD and CV) in an index called power of the gene and genes with lower values
203 are considered the most stable ones.

204 The Comparative Ct method (ΔCt) ranks candidate reference genes by
205 comparing the relative expression of 'pairs of genes' within each sample. Genes with
206 the lowest SD means and with constant ΔCt values are considered the most stable
207 (Silver et al., 2006).

208 RankAggreg package (Pihur et al., 2007) assign the most to the least stable genes,
209 considering the stability values and the frequency that each gene is displayed according
210 to the algorithms of stability analysis tools. This is an R software that uses a Monte
211 Carlo algorithm to calculate the Spearman distance to obtain the overall ranking.

Results

Primers efficiency and specificity

From the ten genes evaluated in the broilers breast muscle, 8 genes amplified, while the *B2M* and *HPRT1* were excluded from further analysis since there was no amplification in the tissue used. The primers' specificity was evaluated for all genes tested and a single peak in all melting curves was obtained, indicating that the amplicons were specific (Fig 1). The efficiency of the primers ranged from 1.9 to 2.1.

Insert Fig. 1 here

Descriptive statistics of reference candidate genes

The cycle threshold (Ct) values showed variability among different reference candidate genes, with mean Ct values ranging from 12.5 to 27.3, approximately (Fig. 2), indicating high variation of the mRNA levels in the *pectoral major* muscle among the genes. The *RPL30* (SD=0.66) and *RPL4* (SD=0.40) had the lowest variance Ct, while *GAPDH* (SD=0.79) and *RPLP1* (SD=0.73) had the highest variance.

Insert Fig. 2 here

Determination of expression stability of reference candidate genes

Regarding the different approaches used, the BestKeeper (Table 2, Supplementary file 1), geNorm (Fig. 3) and the ΔCt method agreed with the two most stable reference genes (Fig. 2), classifying the *RPL30/RPL5* with the lowest power of the gene, M values (0.330) the ΔCt, respectively (Table 2). This result demonstrates the stability of these genes in the breast muscle tissue.

Table 2 Gene classification based on the stability values and ranking (in parenthesis) of the four algorithms analyzed and the general rank obtained by RankAggreg.

Gene	BestKeeper (Power of the gene)	geNorm (M-value)	NormFinder (S-value)	ΔCt	RankAggreg
RPL30	**1.60 (1)**	**0.32 (1)**	**0.07 (1)**	**0.63 (1)**	**1**
RPL5	**1.75 (2)**	**0.32 (2)**	**0.13 (3)**	**0.67 (2)**	**2**
MRPS30	1.84 (4)	0.40 (3)	0.17 (5)	0.71 (4)	3
RPL4	1.95 (5)	0.49 (4)	0.13 (2)	0.70 (3)	4
HMBS	2.43 (8)	0.59 (6)	0.19 (6)	0.77 (5)	5
RPLP1	2.16 (6)	0.75 (8)	0.23 (8)	0.96 (6)	6
MRPS27	1.76 (3)	0.67 (7)	0.16 (4)	1.01 (7)	7
GAPDH	2.21 (7)	0.56 (5)	0.22 (7)	1.09 (8)	8

Insert Fig. 3 here

The NormFinder indicated that the *RPL4* and *RPL5* genes were the best combinations of the two genes with S-value = 0.13, while the *RPL30* (S-value = 0.07) was indicated if using just one endogenous gene (Table 2). However, the *RPL4* was classified in the 5th position in BestKeeper and in the 4th position in geNorm and ΔCt. This variation can happen since the algorithms and data transformation of those tools are different. Moreover, the least stable genes had a small variation among the algorithms: the *GAPDH* gene was ranked in the 7th position in BestKeeper and NormFinder, and in the 5th position in the geNorm and ΔCt (Table 2). The BestKeeper ranked the *MRPS27* gene in the 8th position, while geNorm ranked *RPLP1* gene in the 8th position (Table 2).

Once all the results from the 4 tools were obtained, the RankAggreg was used to generate a consensus ranking (Fig. 4). The results obtained in the general ranking were similar to those from the other algorithms, with *RPL30* and *RPL5* genes as the most

stable, while *MRPS27* and *GAPDH* were considered the least stable genes (Fig. 4, Table 2).

Insert Fig. 4 here

Discussion

According to Bustin (2009), in the guidelines for studies of gene expression, the selection of normalizing genes is important for the reliability of the results. Selecting stable reference genes for gene expression analyses has been recently highlighted in several studies (McCulloch et al., 2012, Rebouceas et al., 2013, Nascimento et al., 2015, Cedraz de Oliveira et al., 2017, Lorenzetti et al., 2018). The use of erroneous reference genes to evaluate relative expression data has an impact on the final normalized results, which may lead to future research based on incorrect or biased data. Therefore, the stability of a large panel of potential reference genes (between 10 to 20) should routinely be tested (Chapman et al., 2015). The use of different reference genes to evaluate relative expression data has an impact on the final normalized results. Although several tissues have already been evaluated in different species (Rebouceas et al., 2013, Yang et al., 2013, Mohindra et al., 2014), including broilers´ breast muscle (Nascimento et al., 2015, Cedraz de Oliveira et al., 2017, Mutryn et al., 2015), no information on the expression profile of candidate reference genes in the skeletal muscle affected with WS has been reported to date.

Our study revealed that there was low discordance among the results of the four used tools (geNorm, BestKeeper, NormFinder and ΔCt). The *RPL30* reference gene was pointed as the most stable gene for all tools, which showed the highest uniformity in its expression within the tools and groups: normal and WS-affected (Table 2, Fig. 3). This

gene had stability values within the parameters suggested by each tool: S < 0.5 for NormFinder, M < 1.5 for geNorm and [±Ct] SD < 1.0 for BestKeeper (Supplementary file 1) and ΔCt and, in the general rank obtained with RankAggreg, *RPL30* was also considered the most stable gene (Fig. 4). This gene has already been shown to be stable in chicken embryo fibroblasts (Yang et al., 2013) and in fish with hypoxic conditions (Mohindra et al., 2014), which reinforces the results found in our study. The NormFinder also indicated the *RPL30* gene (S = 0.073) should to be used in combination with *RPL5* (S = 0.138). The *RPL5* was also considered a good reference gene in geNorm, BestKeeper and ΔCt. The two genes (*RPL5* and RPL30) are ribosomal proteins involved in the development and residual cellular homeostasis, encoding small proteins that are component of the 60S subunit and they are responsible for the transport of rRNA. Therefore, they are usually used as normalizing genes in multiple tissues (Cedraz de Oliveira et al., 2017, Andersen et al., 2004, Yang et al., 2013, Mohindra et al., 2014) and their functions can explain why they were ranked as the most stable genes. According to RankAgreeg (Fig. 4), *RPL5* and *MRPS30* were ranked in 2nd and 3rd most stable genes, while there was a divergence between positions of the *MRPS30* gene in the tools used. The position of this gene varied from 3rd, 4th and 5th in geNorm, BestKeeper and ΔCt, respectively (Table 2). The classification of the *MRPS30* and *RPL5* genes (Fig. 2) found in our study corroborate those reported by Cedraz de Oliveira et al. (2017), in which both genes had similar rank in the pectoral muscle tissue of broilers submitted to heat stress. Thus, these two genes can be used in studies involving WS-affected and unaffected muscle tissues as endogenous genes.

The *GAPDH*, *MRPS27* and *HMBS* have been widely used as reference genes in expression studies with breast muscle in broilers (Zambonelli et al., 2016, Nascimento et al., 2015, Cedraz de Oliveira et al., 2017). The *MRPS27* (Cedraz de Oliveira et al.,

2017) and *HMBS* (Nascimento et al., 2015) were the most stable in breast muscle of broilers submitted to heat stress and the *GAPDH* was used as reference gene in a study with White Striping-affected broilers (Zambonelli et al., 2016). However, in our study, these genes were not considered stable, having a high coefficient of variation among samples and groups (Table 2, Fig. 3 and 4). The *GAPDH* gene was differently express (DE) in studies that evaluated normal and affected broilers with myopathies (Mutryn et al., 2015, Marchesi et al., 2018), and it was found to be involved in the glycolysis/gluconeogenesis, one of the main pathways associated to this condition. Therefore, since *GAPDH* could be regulated due to the WS phenotype, this gene is not recommended as normalizer in gene expression studies involving White Striping myopathy. These findings highlight the importance of measuring the stability of genes for each experimental design since tissues and conditions are complex (Chapman et al., 2015).

Another gene with high variation between the analyzed samples was the *RPLP1* (Fig. 1). This gene has already been considered the most stable in different species, such as humans (Nakayama et al., 2018), mouse (Hernández et al., 2015) and fish (Aursnes et al., 2011). The *RPLP1* belongs to the ribosomal protein family, acting on the senescence of fibroblasts and in the elongation of protein synthesis (Artero-Castro et al., 2009). However, in chickens, the *RPLP1* has never been used as reference gene. The *RPLP1* has been identified as differentially expressed in the liver and in the adipose tissue of broiler chickens, apparently playing an essential role in protein translation and energetic metabolism (Wang et al., 2012). This gene was DE in the large intestine, spleen and cecal tonsils in a study evaluating the immune response of broilers (Slawinska et al., 2016) and also in feather development (Ng et al., 2015). Although suitable as a reference gene in other species, in chickens, the *RPLP1* can be co-regulated

with target genes, making it unsuitable for gene expression analysis as a reference gene in broilers breast muscle affected with WS. Moreover, two commonly used endogenous genes, *B2M* and *HPRT1,* did not amplify in several samples evaluated in our study. The non-amplification of these genes could be due to some intrinsic characteristics of the tissue, such as age, WS appearance or even due to a limitation of the qPCR detection. These two genes have already been studied in the muscle of different species, such as mouse (Thomas et al., 2014), swine (Nygard et al., 2007) and bovine (Pérez et al., 2008) in different conditions, being considered the least stable. This could indicate that those genes may not be constitutively expressed in muscle tissue. However, Nascimento et al. (2015) found that *HPRT1* was the 3rd most stable gene among 13 reference genes evaluated in the *pectoralis major* muscle of broilers supplemented with different diets and slaughtered at different ages, and the *B2M* ranked as the least stable gene in the *Pectoralis major* muscle of chickens. This finding reinforces the importance of checking reference genes, even when similar tissues have already been studied.

In our study, there were some variations among the tools used, although most of the 1st and 2nd genes better ranked were the same (Table 2). These different rankings are expected since the tools have different algorithms to define the most stable genes. The NormFinder calculates a stability value (S) based on intra and intergroup variation of genes tested using log2 transformed data (Andersen et al., 2004). The smallest S values indicate the most stable genes to be used as normalizers. The geNorm uses the arithmetic mean to obtain the M value, recommending the two most stable reference genes (Vandesompele et al., 2002). The BestKeeper evaluates the expression of the genes according to the power of the gene and Cts standard deviation (Pfaffl et al., 2004), while the ΔCt tool uses the coefficient of variation and a constant value of Ct (Silver et al., 2006). After the stability values were obtained for all evaluated genes by the distinct

357 analyzed tools (NormFinder, geNorm, BestKeeper and Comparative Ct), the
358 RankAggreg function was used to calculate a Spearman distance based on Monte Carlo
359 algorithm (Pihur et al., 2007) to determine a general ranking of the most stable genes
360 studied. Although the RankAggreg provides a general classification of the genes, the
361 best genes ranked with this tool could not necessarily indicate that the genes are stable
362 (Pihur et al., 2007). Therefore, it is necessary to take into account the standards of
363 stability measures suggested by each tool, i.e., the geNorm indicates that genes with M-
364 values higher than 1.5 are unstable and should not be used as a reference in any case. In
365 our study, besides the general ranking, we also considered those standards to select the
366 best reference genes.

367 The WS myopathy causes modification of the breast muscle tissue being
368 characterized by the presence of white parallel lines in the same orientation of muscle
369 fibers, composed of adipose tissue (Petracci et al., 2012). Moreover, WS-affected
370 breasts have distinct fatty acid profiles, rich in monounsaturated fatty acid, whereas
371 normal breasts are rich in saturated and polyunsaturated fatty acids (Kuttappan et al
372 2012). Furthermore, there is a decrease in protein content, resulting in deposition of
373 adipocyte cells in areas of necrotic muscle fibers (Russo et al., 2015). Therefore, since
374 there is a relevant change of the type of cells between normal and WS-affected muscles,
375 the search for genes that are constitutively expressed in these two conditions become
376 more complex and necessary, especially considering that several biological processes
377 are involved in the WS phenotype. This complexity could be highlighted by the
378 *GAPDH* gene, which is commonly used as a reference gene in several species but was
379 one of the least stable in our study. It is known that this gene is involved in the
380 development of WS myopathy in broilers, as previously reported by Marchesi et al.
381 (2018). Our results have shown that from the 10 reference candidate genes evaluated,

the ribosomal protein genes *RPL30* and *RPL5* were selected as the most stable to be used as endogenous genes in expression studies with broilers affected with White Striping myopathy.

Conclusions

The *RPL30* and RPL5 were considered the most stable genes among the 10 evaluated genes and all analyzed tools and might be recommended for normalization of gene expression data in qPCR studies involving normal and WS-affected breast muscle in broilers.

List of abbreviations

cDNA: complementary DNA
ºC: Degree Celsius
CG: control group
CV: coefficient of variation
CT: cycle threshold
DNA: Deoxyribonucleic Acid
F: Foward
ID: identification
mRNA: messenger Ribonucleic Acid
mg: milligrams
R: Reverse
RT-qPCR: reverse transcription-quantitative PCR
RNA: Ribonucleic Acid
s: second
SD: standard deviation
uL: microliter
uM: microMolar
WS: White Striping

Declarations

Ethics approval: All animal procedures were performed in accordance with the Ethics Committee on Animal Utilization of Embrapa Swine and Poultry National Research Center, under protocol number # 12/2012.

Consent for publication: Not applicable.

Availability of data and material: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: JOP, MCL, and AMGI conceived and designed the experiment. JOP, AMGI and MCL were responsible for the data collection. AMGI, CMMM, KBC, and IRS performed the laboratory experiment. AMGI, CMMM, KBC, and IRS performed the data analysis. AMGI, CMMM, JOP, and MCL interpreted the results and wrote the manuscript. All authors reviewed, edited and approved the final manuscript.

Supplementary material

Supplementary file 1. Results from the BestKeeper tool.

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Fig. 1 Melting curve analyses of the 8 reference candidate genes evaluated in the broiler breast muscle.

Fig. 2 Cycle threshold (Ct) variation in normal and WS-affected chickens. CG: control group; AG: affected group.

Fig. 3 Ranking of reference candidate genes based on the average expression stability using the geNorm software.

Fig. 4 Ranking of reference candidate genes based on the RankAggreg tool.

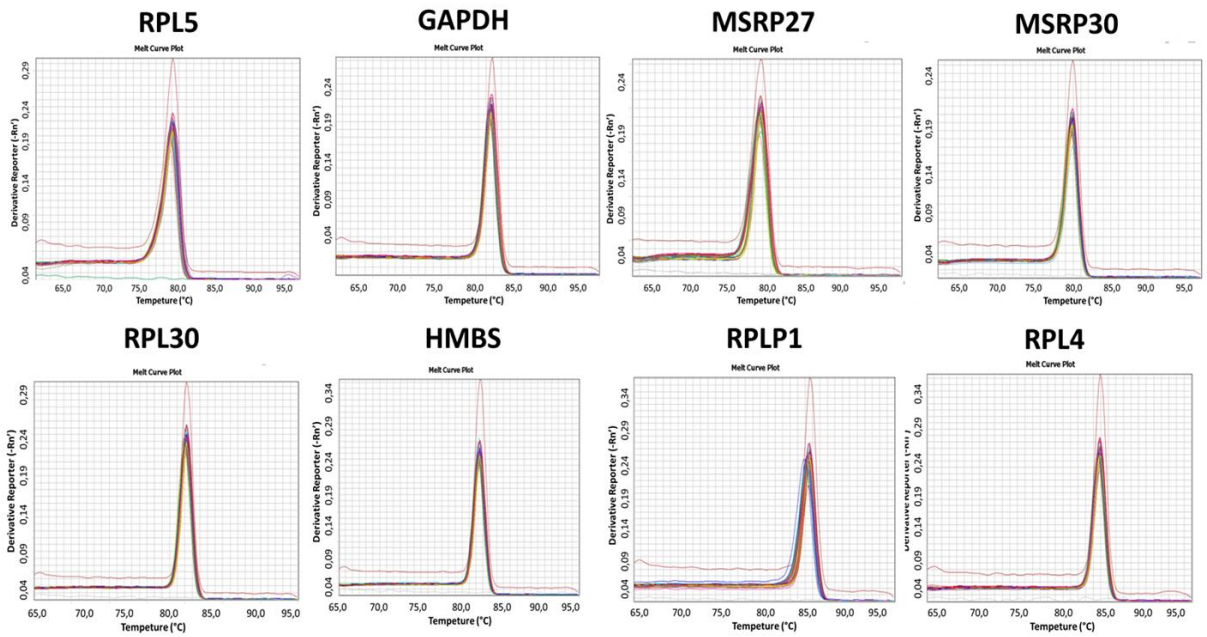
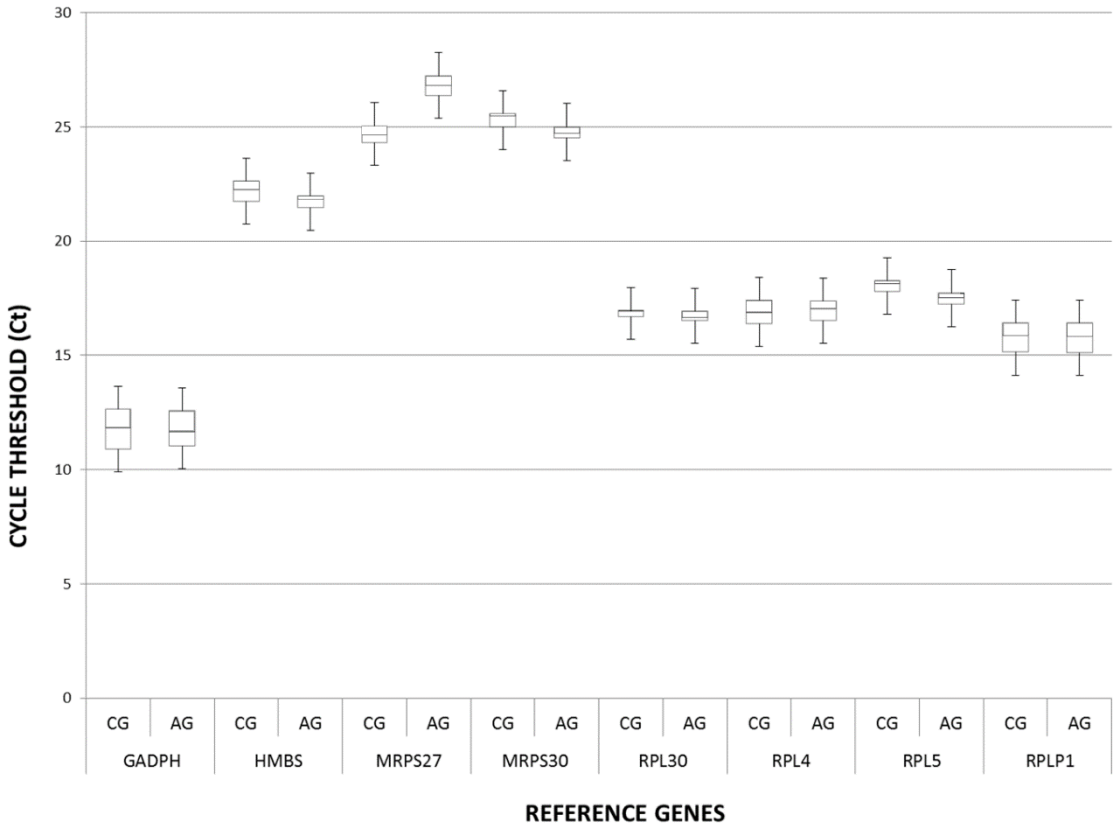


Figure 1. Melting curve analyses of the 8 reference candidate genes evaluated in the broiler breast muscle.



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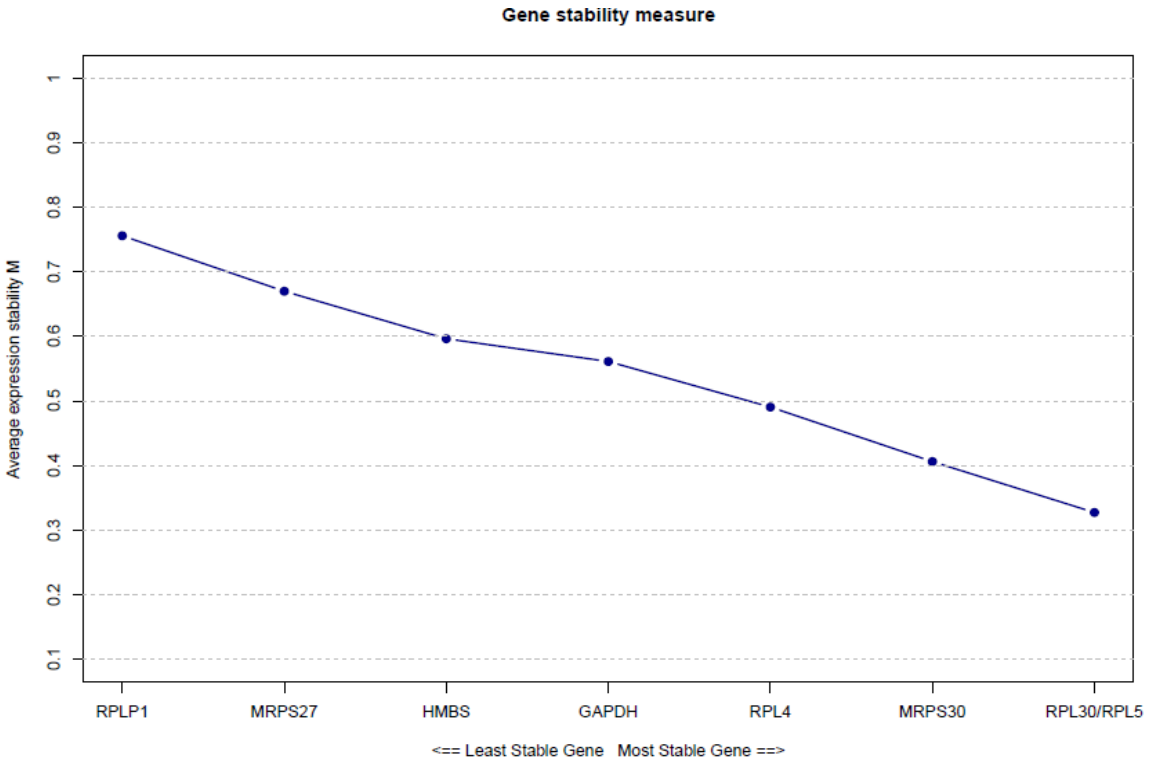

Figure 3. Ranking of reference candidate genes based on the average expression stability using the geNorm software.

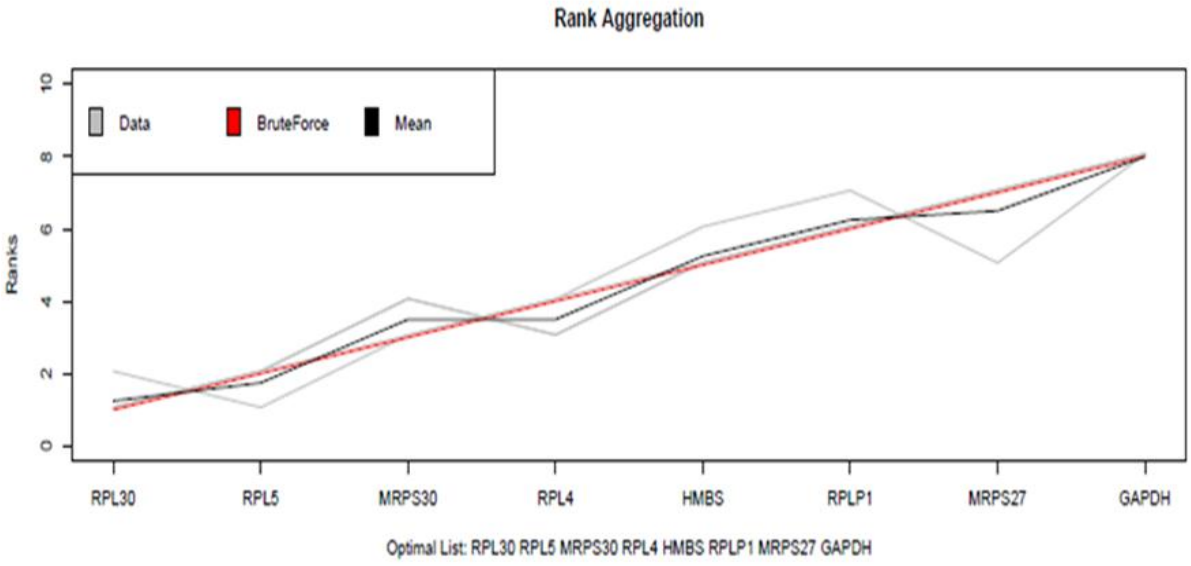

Figure 4. Ranking of reference candidate genes based on the RankAggreg tool.

2.2 MANUSCRITO II

Differential expression of myogenic and calcium signaling-related genes in broilers affected with White Striping

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Abstract

White Striping (WS) has been one of the main issues in poultry production in the last years, which affects meat quality. Studies have been conducted to understand WS and other myopathies in chickens and some biological pathways have been associated to the prevalence of these conditions, such as extracellular calcium level, oxidative stress, localized hypoxia, possible fiber-type switching and cellular repairing. Therefore, to understand the genetic mechanisms involved in WS, 15 functional candidate genes were chosen to be analyzed by qPCR in breast muscle of normal and WS-affected chickens trying to clarify the involvement of these genes on the occurrence of this myopathy. To this, the pectoral major muscle of 16 normal and 16 WS-affected broilers were collected at 42 days of age and submitted to qRT-PCR analysis. Out of the 15 genes studied, 6 were differentially expressed between groups. The *CA2*, *CSRP3* and *PLIN1* were upregulated, while *CALM2*, *DNASE1L3* and *MYLK2* genes were downregulated in the WS-affected when compared to the normal broilers. These findings highlight that the disruption on muscle and calcium signaling pathways can be possibly triggering WS in chickens. Improving our understanding on the genetic basis involved with this myopathy might contribute for reducing WS in poultry production.

Keywords: chickens, gene expression, glycogen metabolism, pectoral myopathy.

Introduction

The White Striping (WS) is one of the most prevalent myopathies occurring in broilers, being characterized by parallel white striations across the muscle fibers in the breast fillets and thighs, affecting the meat quality [1]**.** Variation in the size of the muscle fibers, degeneration and myofibrils necrosis, an inflammatory influx and other immune cells, and irregular adipose tissue throughout the muscle have been observed in WS-affected pectoral major muscle of broiler chickens [2, 3, 4].

Swatland et al (1990) suggest that selection for rapid growth has created muscle that outgrow their life support systems and cause muscle damage. Therefore, the formation of large intercellular spaces causes loss of muscle fiber fluids, compromising the muscular integrity of the chickens. Thus, the selected modern hybrids have differences in muscle tissue histology and metabolism [7], with higher density of fast twitch fiber, which is characterized by a higher diameter and a lower rate of protein degradation, compared to unselected broilers [8].

Different WS levels based on the white striations have been reported among commercial lines as normal, moderate and severe [1], being the etiology of this condition still unclear. Genetic studies have shown heritability estimates of 0.65 for WS [9, 10], indicating an important genetic component involved in the occurrence of WS in broilers. Several studies searching for the causes of the WS development have been conducted to date, and advances were obtained. However, efforts are still needed to reduce this problem, and gene expression studies evaluating normal and affected tissues would provide additional support to find the triggering pathways of this myopathy [11].

Recently, many genes have been associated with the occurrence of myopathies in chickens [13, 10, 14, 15] and the identification of specific biomarkers can help to accurately assess the effect of genetics, environment or management conditions in improving or aggravating WS myopathy in broilers. Some candidates genes involved

with the development of WS in fast-growing broilers suggest that these genes play important role in mechanisms such as muscle differentiation, oxidative stress, signaling calcium, hypoxia and muscle fiber type replacement [13, 15]. Therefore, understanding the genetic mechanisms involved in WS would help counteracting this problem. Thus, the profile of 15 functional candidate genes were analyzed by qPCR in breast muscle of normal and WS-affected chickens to clarify the involvement of these genes on the development of this myopathy.

Macrophages and fibroblasts are fundamental in regulating tissue homeostasis and they are responsible for damage control and tissue remodeling on muscle lesions [5].

Material and Methods

Experimental animals and tissue collection

A total of 168 male broilers from the commercial line Cobb500 were raised at the Embrapa Swine and Poultry National Research Center farm, located in Concórdia, Santa Catarina State, Brazil. Chicks were vaccinated in the hatchery against fowl pox and Marek disease. The management conditions followed the guidelines of the line, with water and feed provided *ad libitum.* At 42 days of age, broilers were weighed and slaughtered by cervical dislocation following the procedures of the Committee for Ethics in Animal Use (CEUA) from the Embrapa Swine and Poultry National Research Center under protocol No. 012/12. Immediately after slaughter, the breasts were classified by moderate presence or absence of WS, according to Kuttappan et al. [3]. Fillets with white striations generally less than 1 mm thick observed on the surface of the breast were considered to have a moderate degree of WS. From the 168 broilers evaluated, samples of from the cranial region were collected by removing approximately 1 g of the breast muscle of 16 normal broilers (with no WS) and 16 WS-

affected chickens, were frozen in liquid nitrogen and stored at -80 °C for further molecular analysis.

RNA extraction and cDNA synthesis

Frozen samples were ground in liquid nitrogen and 100 mg of PMM muscle was submitted to RNA extraction using Trizol (Invitrogen, USA) following the manufacturer's protocol. Briefly, 1 mL of Trizol was added to the PMM, homogenized at room temperature (RT) for 5 minutes and then 200 µL of chloroform was added and the samples were shaken vigorously. The tubes were centrifuged at 10,000 xg at 4°C for 15 min and the aqueous phase was transferred to a microtube containing 500 uL of 100% isopropyl alcohol. Another RT incubation for 10 min was performed, followed by a 12,000 xg centrifugation for 10 min. The RNA pellet was washed with 75% ethanol and centrifuged at 7500 xg for 5 minutes at 4°C. Pellet was dried for 15 minutes at RT and resuspended in DEPC-treated water. The total RNA was quantified in BioDrop spectrophotometer (Biodrop, UK), Samples with 260nm:280nm ratio higher than 1.8 were considered pure. The integrity of the RNAs were confirmed in 1.5% agarose gel after electrophorese for 90 min. The first strand cDNA was synthesized using 4 ug of total RNA and SuperScript III First-Strand Kit SuperMix Synthesis (Thermo Fischer Scientific, EUA), using oligo dT primer, following the manufacturer's recommendations.

Real time RT-PCR

A total of 15 functional candidate genes (Table 1) possibly involved with WS development in chickens were chosen to be evaluated for gene expression analysis. The

sequences were obtained in the Genbank (https://www.ncbi.nlm.nih.gov/genbank/) and Ensembl (https://www.ensembl.org) databases. Primers for each gene (Table 1) were designed in exon-exon junctions to avoid DNA amplification using the Primer-Blast online tool [16] and primer´s quality were evaluated in the Netprimer online tool (http://www.premierbiosoft.com/netprimer).

The qPCR reactions were performed in the QuantStudio 6 (Applied Biosystems, Foster City, CA) equipment, in a final volume of 15 µL containing 1x Maxima SYBR Green (Fermentas, USA), 0,13 µM of each primer and 2 µL of 1:10 diluted cDNA. The reactions for all primers followed the cycling condition: 95ºC for 10 min, 40 cycles of 15 seconds at 95ºC and 60ºC. A melting curve stage of 70°C to 95°C was added in all qPCR reactions to verify their specificity. Primer specificity was also confirmed by 1% agarose gel. The reactions were analyzed in duplicates and negative controls were included to detect contamination. Primers efficiency were obtained by linear regression (efficiency = $10^{(-1/\text{slope})}$), according to [17] and primers with efficiency between 95 to 105% were considered for gene expression analysis.

Differential gene expression analysis

The average of cycle thresholds (Ct) values was collected and the $2^{-\Delta\Delta CT}$ was calculated [18] for each sample to obtain the fold-change. The *RPL30* (Ribosomal protein 30) and *RPL5* (Ribosomal protein L5) reference genes were used for normalization. The reference genes were chosen based on the stability values. The groups comparison of relative gene expression was performed using Mann-Whitney test. Genes with p-values < 0.05 were considered differentially expressed (DE).

Table 1 Primers for the 15 candidate genes used for the qPCR analysis in the breast muscle of normal and WS-affected broilers.

Gene	Ensembl ID	Primer (5' to 3')	Size (bp)
ACTG1 Actin, gamma 1	ENSGALG00000001381	F: 5'-CTCTGTTCCAACCCTCTTTCCT-3' R: 3'-GTGTTGGCGTACAGATCCTTC-5'	112
CA2 Carbonic Anhydrase 2	ENSGALG00000030781	F: 5'-GCTCTGAGCAGATGTGCAAAC-3' R: 3'-CTCATCGCTGAGGTTACTGGAAG-5'	144
CALM2* calmodulin 2	ENSGALG00000010023	F: 5´-CCACCATGGCTGATCAACTG-3´ R: 5´-GCCATTGCCATCAGCGTCTA-3´	191
PLIN1 Perilipin 1	ENSGALG00000023395	F: 5'-GGCTATGGAGACGGTGGATG-3' R: 5'-CTGGCTTGCTCTCCTCTTCC-3'	173
CHST1 Carbohydrate Sulfotransferase 1	ENSGALG00000008440	F: 5'-CGCCCCTCTTTCTCGTCTTC-3' R: 5'-CTCATCGCTGAGGTTACTGGAAG-3'	133
CSRP3 Cysteine And Glycine Rich Protein 3	ENSGALG00000004044	F: 5'-GCTCTGAGCAGATGTGCAAAC-3' R: 3'-GCTTGGAGAGACCCGATTCC-5'	202
CTGF Connective Tissue Growth Factor	ENSGALG00000037402	F: 5'-TCACCAACGATAATGCTTTCTG-3' R: 3'-GAATGCACTTTTTGCCTTTCTT-5'	111
DNASE1L3 Deoxyribonuclease I-like 3	ENSGALG00000005688	F: 5'-GAGTTTGCGTGGCTCATCG-3' R: 3'-CACGATCCTGTCATAGGGGC-5'	78
HDAC1 Histone Deacetylase 1	ENSGALG00000003297	F: 5'-GGGGCGGGTTGCGTT-3' R: 3'-ACATCACCGTCGTAGTAGTAGC-5'	115
HIF1A Hypoxia Inducible Factor 1 Alpha Subunit	ENSGALG00000011870	F: 5'-CGTCACCGACAAGAAGAGGATT-3' R: 5'-GTCAGCCTCATAATGGATGCCT-3'	171
MAPK13 Mitogen-Activated Protein Kinase 13	ENSGALG00000030966	F: 5'-TCTGCTCCGCCATAGACAAG-3' R: 5'-CAAGCAGCCCAATGACATTCTC-3'	150
MYLK2 Myosin Light Chain Kinase 2	ENSGALG00000006273	F: 5'-ACCCTTTTGAGATATTGGACGA-3' R: 5'-TCCTTGGAGCTGAGGTTGTACT-3'	112
RYR2 Ryanodine Receptor 2	ENSGALG00000010812	F: 5'-ATACAAGGGACCTGCTGGGT-3' R: 5'-GGGAGGCAAAACAATCTGGC-3'	92
SMAD3 SMAD Family Member 3	ENSGALG00000035701	F: 5'-CCCCATGTCATCTACTGCCG-3' R: 5'-GGTAACACTGGGGTCTCCAC-3'	158
TNNC2 Troponin C2, Fast Skeletal Type	ENSGALG00000006835	F: 5'-GTCAATGACGGACCAGCAG-3' R: 5'-CGTCCGCATCAAACATGTCA-3'	95

F: Forward (5'-3'); R: Reverse (3'-5').*Paludo et al. (2016)

Gene interaction analysis

The STRING online software (https://string-db.org/) was used to evaluate the interactions among the DE genes. This software predicts the evidence of gene interactions, based on co-expression and co-localization. The gene network was constructed using both *Gallus gallus* and *Homo sapiens* databases, in order to improve the information about the interaction among genes.

Results

The expression of all 15 genes studied was obtained from the breast muscle tissue of normal and WS-affected 42 days-old broilers (Table 2). The *CSRP3* gene had the highest fold-change (11.17) and the others had a magnitude ranging from 1 to 2.11 (Table 2, Fig 1).

Table 2: Relative expression (fold-change) between normal and WS-affected groups, respective p-values, and express levels calculated for each group.

Genes	Relative Expression	p-value	Fold-change by group (Mean± SD)	
			Normal	WS-Affected
ACTG1	-1	0.717	1.12±0.49	1.12±0.56
CA2	1.58*****	0.006	1.07±0.38	1.68±0.75
CALM2	-1,79*	0.007	1.11±0.55	0.62±0.62
CHST1	1.176	0.945	1.57±1.61	1.84±2.92
CSRP3	11.17*****	0.0001	1.97±2.55	21.98±29.61
DNASE1L3	-1.85*****	0.005	1.11±0.50	0.60±0.34
HDAC1	-1.01	0.508	1.11±0.48	1.02±0.44
HIF1A	-1.12	0.151	1.12±0.58	0.99±0.85
MAPK13	-1.03	0.703	1.21±0.83	1.17±0.58
MYLK2	-2.08*****	0.035	1.66±2.19	0.79±1.00
PLIN1	2.11*****	0.009	1.47±1.11	3.10±1.97
RYR2	-1.45	0.773	1.57±1.47	1.08±0.67
SMAD3	1.14	0.816	1.37±0.86	1.51±1.26
TNNC2	-1.17	0.386	1.14±0.71	0.97±0.66

SD: standard deviation; *p<0.05.

Regarding the differentially expressed (p<0.05) genes, the *CALM2, DNASE1L3* and *MYLK2* were downregulated in the WS-affected broilers when compared to the normal group (Fig 1). The expression levels of these 3 genes were, respectively, 1.79, 1.85 and 2.08 lower in WS-affected than in the normal broilers.

The *CA2*, *CSRP3* and *PLIN1* genes were 1.58, 11.17, and 2.11x upregulated in WS-affected broilers when compared to the control group (Table 2, Fig 1). For the 8 remaining evaluated genes, no differential expression was observed between groups (Fig 1).

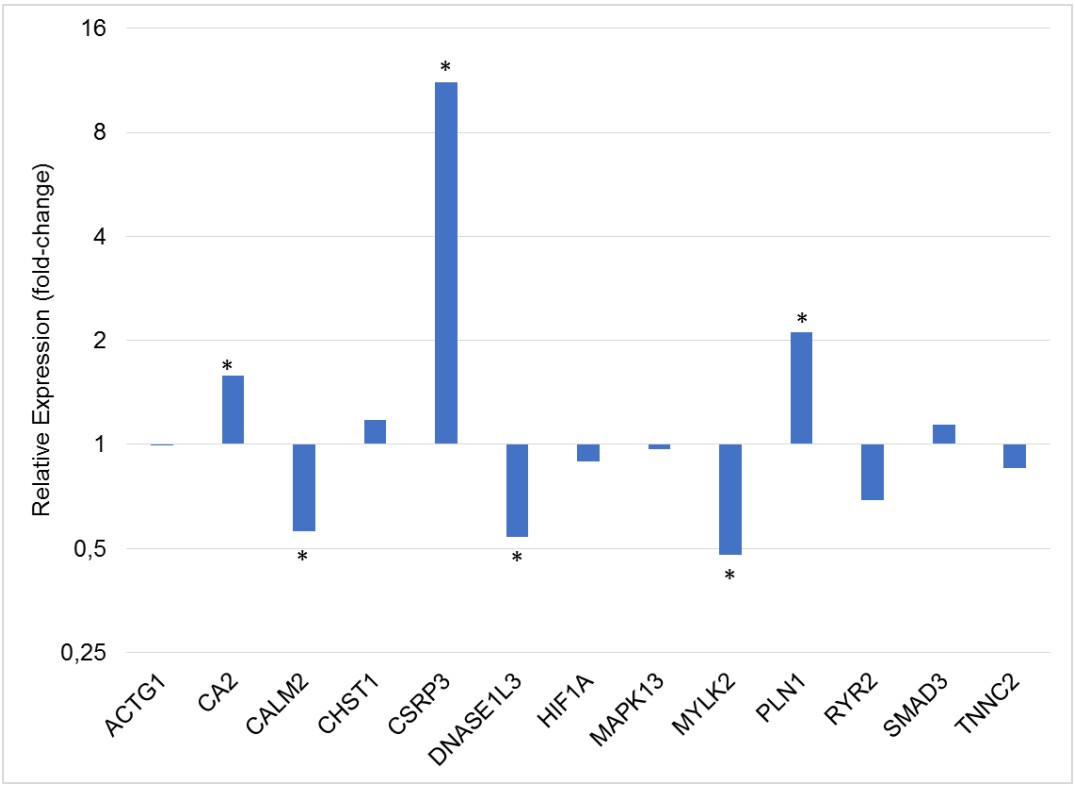

Fig 1. Ratio of gene expression between normal and WS-affected broilers at 42 days of age, normalized for *RPL30* and *RPL5* reference genes. (*p<0.05)

In the gene network analysis performed with the six DE genes, the *CALM2* and *MYLK2* genes, which were downregulated in the WS-affected group (Table 2), were the main interactors of two gene clusters constructed from both chicken (Fig 2A) and human database (Fig 2B). One of the clusters was related to myogenesis and included the *CSRP3*, *MYOZ2*, *MYPBC3*, *MYL2* and *MYL9* genes (Fig 2A) and the other was composed by phosphorylase kinase genic family (*PHKB, PHKA1*, *PHKA2*, *PHKG1 and PYGL*) (Fig 2A), which play an important role in providing cell energy for muscle and liver tissue. The *CA2*, *DNASE1L3 and PLIN1* were not clustered with other genes according to the information available for *Gallus gallus* (Fig 2A). However, with the information from humans, the *PLIN1* gene was clustered with genes related to adipocyte differentiation, fatty acids and glucose homeostasis (Fig 2B).

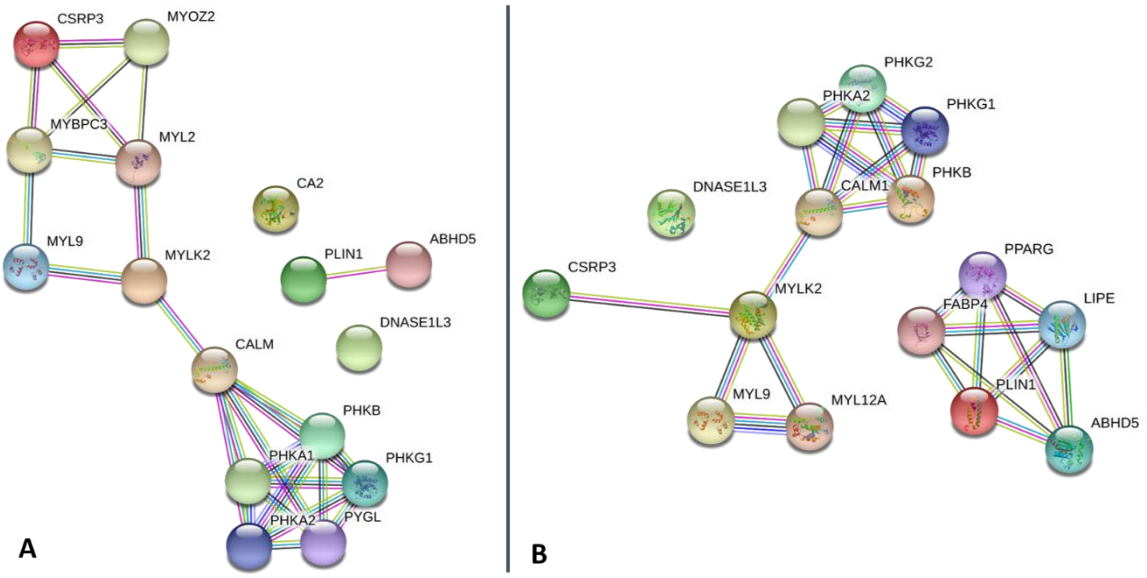

Fig 2 Gene network performed with differentially expressed genes between normal and WS-affected groups, obtained with the String database using *Gallus gallus* (A) and *Homo sapiens* (B) protein information. Circles represent the genes. Lines represent the interaction among the DEG and other related genes based on the prediction methods: known interaction in curated databases (light blue) and experimentally determined (magenta), predicted co-occurrence (blue), gene fusion (red) or neighborhood (green), homology (purple), text mining information (yellow) and co-expression (black).

Discussion

White Striping has been one of the main issues in poultry production in the last years, which affects meat quality. Studies have been conducted to understand this and other myopathies in chickens and some biological pathways have been associated to the prevalence of these conditions, such as extracellular calcium level, oxidative stress, localized hypoxia, possible fiber-type switching and cellular repairing [13, 15]. Here, 15 functional candidate genes important for tissue development were investigated in normal and WS-affected broilers. From the 15 genes evaluated, *CA2*, *CSRP3* and *PLIN1* were upregulated in WS-affected broilers, whereas the *CALM2*, *DNASE1L3* and *MYLK2* were downregulated.

The *CA2* and *CSRP3* genes, which were upregulated in WS-affected broilers in this study, are mainly involved in oxidative stress and cell repair, respectively (Fig. 1). Genes of carbonic anhydrase (CA) family have regulatory and repair functions [19] with ability to act as an oxidant agent in both physiological and physiopathological conditions in skeletal muscle. This gene family is important to efficiently transport and eliminate CO^2 from tissues [20]. Some genes of this family were reported as DE in muscle myopathies in broilers. Among them, *CA3* could be highlighted since it was approximately 25 times more expressed in chickens affected with Wooden Breast myopathy than in normal broilers [13]. Nishita et al (2012) also found that *CA3* was upregulated in the pectoral muscle of broilers with muscular dystrophy compared to normal chickens. The *CA4* gene is involved in the regulation of extracellular pH, reducing the muscular contraction and possibly increasing the intracellular acidosis [22]. The *CA2* encodes zinc metalloenzymes and participates in a variety of biological processes including, calcification, acid-base balance and bone resorption [19, 20]. The specific function of *CA2* associated to muscle development or myopathies, such as WS, has not yet been described, but it is known that CAs are involved with lipid metabolism and obesity, and increasing mitochondrial oxidative stress [23]. Therefore, the upregulation of *CA2* in WS-affected broilers could be related to the adipose tissue deposition between muscle fibers. However, the function of this gene should be further explored, since our results (Fig. 3) differ from the expression pattern obtained by Marchesi et al. [15], in which the *CA2* gene was downregulated.

Regarding the oxidative stress in the skeletal muscle, the increase in reactive oxygen species (ROS) causes changes in the cell signaling pathways, affecting the release of calcium from the sarcoplasmic reticulum, resulting in damage to the contractile capacity of muscle cells [24]. The increase in ROS is cytotoxic and can alter

246 cell integrity, inducing severe stress and leading to the production of carbonic
247 anhydrases signaling to the non-recoverable muscle damage [19]. In our study, the
248 upregulation of the gene *CA2* in WS-affected broilers may have an oxidative action in
249 the cell, which could lead to a hypoxic state in the tissue. This pattern has also been
250 described in WB/WS chicken myopathies [13,15].

251 The *CSRP3* gene encodes the LIM protein, which regulates processes important
252 for the development and differentiation of satellite cells. In the muscle LIM interacts
253 with various proteins, such as titin, participating on intracellular signaling cascades and
254 in the maintenance of sarcomere integrity [25]. In addition, the LIM protein seems to be
255 involved with stress response through compensatory signaling pathway. This protein
256 appears to be essential to the structure and maintenance of the sarcomere [26] and its
257 expression occurs mainly in slow skeletal muscle [27], helping the formation and
258 growth of myotubes, which are key features for muscle repair [28]. Alterations in
259 *CSRP3* expression profile changed the type of fibers from fast to slow, in a study with
260 rats [27]. The upregulation of this gene has already been reported in *pectoral major*
261 muscle of chickens affected with myopathies, such as Wooden Breast [13] and WS
262 [15].

263 According to Arber et al. [29], the *CSRP3* promotes muscle differentiation, acting
264 on regeneration, structural repair and genetic regulation of the skeletal muscle. In WS-
265 affected chickens, there is an increase in extracellular space because the muscle
266 differentiation gradually leaves gaps between neighboring fibers and bundle fibers. In
267 these gaps, the infiltration of mononuclear, adipose and fibrous cells occurs and may be
268 involved as a secondary response to myopathy [30]. Offer & Cousins et al. [31]
269 suggested that the upregulation of the *CSRP3* gene in chickens with myopathy is a
270 repair mechanism of myofibers trying to regenetare the affected muscle. They also

271 suggest that the positive regulation of *CSRP3* affects meat quality, since this gene is
272 involved in the development of myofibers acting on the interchange of muscle fiber
273 type. This gene has already been associated with different meat quality traits in bovine
274 [33] and porcine [31, 32], but no information is available in broilers. Moreover, *CSRP3*
275 regulates the autophagy in muscle cells [34] and has already been associated with
276 myopathies in humans [35] and chickens [10], which make this gene a good molecular
277 marker for myopathies occurrence.

278 In the gene network (Figure 2), the *CSRP3* was grouped with several myogenic
279 genes, including the *MYLK2*, *MYL2* and *MYOZ2*, which were DE in a previous chicken
280 WS study [15]. The *MYLK2*, a myosin light chain kinase gene, was downregulated in
281 our study and it is essential for muscle contraction (Fig 1), composing the main
282 myofibrillar proteins in muscle cells [36]. This gene is a calcium/calmodulin dependent
283 and it is responsible for light chain phosphorylation, facilitating its interaction with
284 actin filaments and then inducing a contractile activity [37]. *MYLK2* is expressed
285 predominantly in fast skeletal muscle fibers, studies with *MYLK2* knockout mice
286 showed a decrease in the phosphorylation of the myosin regulatory light chain in the
287 skeletal muscle [38]. Reduced levels of *MYLK2* in turkeys *pectoralis* muscle with PSE
288 meat favor a low integrity of myofibrillar proteins reducing the skeletal muscle
289 contraction [39]. In broilers, it has been observed that breast muscle with severe WS
290 myopathy have low integrity of myofibrillar proteins, resulting in reduced water
291 retention capacity compared to normal breasts [40] and, consequently, affecting meat
292 quality [9]. The hypothesis is that the structural alteration of myosin facilitates water
293 loss [41, 42]. Therefore, the presence of myopathies impairs the water holding capacity
294 during the meat processing and storage [43, 44, 45]. Moreover, the reduced *MYLK2*
295 mRNA levels in WS-affected chickens could lead to a decrease of the light chain

296 phosphorylation, changing the myosin formation and muscle contraction. A similar
297 pattern of *MYLK2* expression was observed in a study evaluating broilers affected with
298 deep pectoral myopathies [46], showing a similar expression pattern of this gene across
299 different myopathies.

300 It is interesting to note that besides the *MYLK2*, the *CALM2* gene was also
301 downregulated (-1.8) in WS-affected when compared to normal broilers (Table 2).
302 *MYLK2* and *CALM2* were the two genes linking the main branches of the gene network,
303 one composed by muscle development and other by gluconeogenesis related genes,
304 respectively (Figure 2). The *CALM2* is the most important Ca^{2+} signal transducer in the
305 cells, regulating several types of protein, such as kinases, transcription factors and ion
306 channels in most of the eukaryotes [47]. The lack of Ca^{2+} transport to muscle fibers
307 could increase the calcium concentration in the sarcoplasmic reticulum [48,15]
308 affecting several biological processes, such as muscle contraction, oxidative stress,
309 inflammation and also glycogen metabolism [49, 50, 51, 47]. The disruption of calcium
310 signaling pathway has been associated with different myopathies in humans [52] and in
311 chickens [13, 15]. In our study, the downregulation of *CALM2* could be affecting
312 myogenesis, since the intracellular calcium concentration is essential for myosin light
313 chain phosphorylation [46]. The *MYLK2* is a calcium dependent kinase and its
314 downregulation could be a consequence of insufficient intracellular calcium
315 transportation caused by the reduced levels of *CALM2*, which leads to a defective
316 muscle contraction ratio due to the change in the myosin fibers.

317 In this study, the *CALM2* gene was also grouped with several genes from
318 phosphorylase kinase family (PHKs) (Figure 2). This family is responsible for the
319 phosphorylation of certain muscle substrates, such as troponin I [53] and is involved in
320 the glycogen metabolism/catabolism [54]. In humans, the gamma subunit of PHKs

encodes the calmodulins 1, 2 and 3, and in chickens, the *CALM2* is also known as *PHKD*. The carbohydrate metabolism is an essential part of the skeletal muscle physiology, since the glucose. In humans, several metabolic myopathies have been described as associated to the impairment of the carbohydrates and lipid metabolism [55]. Some of them are characterized as disturbances in the glycogenolysis, affecting the glycogen metabolism [56, 54, 53]. Therefore, reducing levels of calmodulin could be involved with deficiency of PHKs, which prevents the catalysis of glycogen in G-6-phosphate. Some *PHKs* genes have already been associated to myopathies in chickens [10, 15], which reinforces that calcium signaling and carbohydrate metabolism genes are possibly involved on triggering myopathies in chickens.

In our study, the expression of the *DNASE1L3* gene was also reduced (Figure 1) in the WS-affected group. This gene is a member of the *DNASE1* family, which acts on the DNA catabolic processes, regulating inflammatory response, cytotoxicity, hypoxia and presenting key functions related to tissue structure and development [57, 58, 59]. The *DNASE1L3* is also known as DNASE gamma, being part of an endonuclease gene family that depends of Ca^{+2} and Mg^{+2} ions to be activated, usually participating on DNA cleavage and apoptosis [60, 61]. It is known that apoptosis is an important process involved in skeletal muscle development in vertebrates, since it is necessary during the myogenic differentiation [62, 63, 60]. The activation of *DNASE1L3* is considered to be responsible for DNA fragmentation needed to myoblasts differentiation [60]. Therefore, the downregulation of this gene in the WS-affected broilers could prevent the occurrence of the correct myogenesis and consequently the cell hyperplasia. Although the DNASE1L3 has not been previously associated with myopathies, another gene of this family, the *DNASE1L1*, was related to Pompe's disease in humans, which is a glycogen storage disease and characterized by muscle weakness [64]**.** In chickens, there

346 are few studies involving *DNASE1L3* gene function, especially considering its
347 involvement with WS. However, this gene should be further explored in terms of its
348 function in WS, since it is involved with important pathways associated to this
349 condition. Furthermore, its calcium ion dependency could indicate some relationship
350 between the downregulation of *CALM2* and *DNASE1L3*, narrowing the hypothesis that
351 the dysregulation of calcium signaling is one of the most important pathways
352 contributing to WS myopathy.

353 The *PLIN1* gene was 2x upregulated in broilers affected with WS (Figure 1) and,
354 in the gene network analysis, this gene was not grouped with the main network in
355 chickens neither in humans (Figure 2). The *PLIN1* is one of the main proteins found in
356 adipocytes, being required for the maintenance of the lipid metabolism, and normally
357 inhibits the lipolysis of the cells [65]. Therefore, the upregulation of *PLIN1* in WS-
358 affected broilers could explain the adipocyte differentiation and fat deposition between
359 muscle fibers. This gene has already been associated to intramuscular fat in swine [66]
360 and lipodystrophy in humans [67].
361 Finally, the other genes evaluated in this study were not differentially expressed
362 between groups, besides their possible biological function involvement with
363 myopathies, such as hypoxia, oxidative stress, immune response and cellular repair.
364 However, previous studies have shown that some of those genes were related to chicken
365 myopathies [10, 15]. The difference in these results could be due to a high variation
366 within the studied groups, the low magnitude of fold-changes or even to differences in
367 the severity of the myopathies.
368

369 **Conclusions**

The genes *CA2, CSRP3, MYLK2, CALM2, PLIN1* and *DNASE1L3* were differentially expressed between normal and WS-affected broilers. These findings highlight that the disruption on muscle and calcium signaling pathways can possibly be triggering WS in chickens. Improving our understanding on the genetic basis involved with this myopathy might help finding alternatives to reduce WS in poultry production.

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Author Contributions

JOP, MCL and AMGI conceived and designed the experiment. JOP and MCL were responsible for the data collection. AMGI, CMMM, KBC and IRS performed the laboratory experiment. AMGI, CMMM, KBC and IRS performed the data analysis. AMGI, CMMM, JOP and MCL interpreted the results and wrote the manuscript. All authors reviewed, edited and approved the final manuscript.

Competing Interests

The authors declare that they have no competing interests.

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Fig 1. Ratio of gene expression between normal and WS-affected broilers at 42 days of age, normalized for *RPL30* and *RPL5* reference genes. *p<0.05

Fig 2. Gene network performed with differentially expressed genes between normal and WS-affected groups, obtained with the String database using *Gallus gallus* (A) and *Homo sapiens* (B) protein information. Circles represent the genes. Lines represent the interaction among the DEG and other related genes based on the prediction methods: known interaction in curated databases (light blue) and experimentally determined (magenta), predicted co-occurrence (blue), gene fusion (red) or neighborhood (green), homology (purple), text mining information (yellow) and co-expression (black).

3 – CONSIDERAÇOES FINAIS

Neste estudo, os níveis de expressão de genes no tecido p*ectoralis major* em frangos de corte normais e afetados com *White Striping* foram analisados. Primeiramente, foram avaliados os padrões de expressão de 10 genes referência candidatos neste tecido peitoral dos dois grupos de aves experimentais. Por meio das ferramentas utilizadas, foi possível definir dois genes codificadores de proteínas ribossomais, *RPL5* e *RPL30,* que foram indicados como os melhores normalizadores nas condições avaliadas. Neste trabalho, foi possível observar que houve uma concordância na classificação dos melhores genes constitutivos elencados.

Posteriormente, os níveis de expressão relativa de 15 genes candidatos funcionais à miopatia *White Striping* foram avaliados e 6 destes genes foram diferencialmente expressos entre frangos normais e afetados com a miopatia. De acordo com os resultados, os genes *CA2, CSRP3, PLIN1, DNASE1L3, MYLK2* e *CALM2* apresentam possível atuação no desenvolvimento da anomalia uma vez que estes genes DE participam de importantes processos biológicos, tais como diferenciação muscular, metabolismo de sinalização de cálcio e carboidratos. Alguns desses genes interagem entre si, indicando a existência de uma rede de modificações biológicas que atua simultaneamente para a manifestação do *White Striping*, reforçando que o mecanismo de herança do fenótipo WS é poligênico e complexo. O conhecimento da atuação destes genes contribui para futuras pesquisas que visam reduzir os impactos dessa miopatia na indústria avícola.

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Embrapa Suínos e Aves	Certificado de Conduta Ética	ETICA 1/1

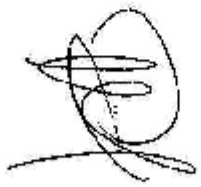
CERTIFICADO

Certificamos que o Protocolo nº**(000/AAAA): 012/2012**, sob título **"Identificação de genes associados à problemas locomotores em frango de corte por meio de RNAseq do fêmur",** sob responsabilidade de **Jane de Oliveira Peixoto** está de acordo com os Princípios Éticos na Experimentação Animal, adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), **TENDO SIDO CONSIDERADO APROVADO PELA** Comissão de Ética no Uso de Animais (CEUA/CNPSA) em reunião realizada em **23/08/2012**.

CERTIFICATE

We certify that the Protocol nº **(000/YYYY): 012/2012**, under the following **title "Identification of genes associated with leg weakness in broilers by RNAseq femur"** is in agreement with the Ethical Principles in Animal Research adopted by Brazilian College of Animal Experimentation (COBEA) and was approved by the Embrapa Swines and Poultry Ethical Committee for Animals utilization in experimentation (CEUA/CNPSA) in **08/23/2012.**

Concórdia, **23/08/2012**.



Presidente CEUA/CNPSA

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