UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE VETERINÁRIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

DIAGNÓSTICO *IN VIVO* E CARACTERIZAÇÃO CELULAR DAS MIOPATIAS *WOODEN BREAST* E *WHITE STRIPING* EM FRANGOS DE CORTE

TAMARA ZINN FERREIRA

PORTO ALEGRE
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Aos meus pais Júlio e Suzete...
DEDICO!

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Agradeço e dedico esta conquista, aos meus pais Júlio e Suzete, os quais sem eles eu nada seria. Aos meus irmãos Júlio César, Carolina, Vanessa e aos meus pequenos amores Felipe e Henrique. Família: Amo vocês demais! Vocês são a base de tudo!

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RESUMO

A fim de elucidar o processo muscular envolvido nas miopatias peitorais de frangos de corte e a obtenção de métodos para detecção destas miopatias no animal *in vivo*, experimentos foram realizados com frangos de corte de rápido crescimento, machos, com diferentes idades de abate. Os estudos que compuseram esta tese objetivaram determinar o processo de crescimento, degeneração e regeneração celular do músculo *Pectoralis major*, bem como determinar o efeito de programas de restrição alimentar quantitativa (70%) e qualitativa (hipoprotéica e hipoenergética) sobre a ocorrência das miopatias *wooden breast* (WB) e *white striping* (WS) e suas consequências na qualidade da carne, além de validar uma metodologia para o diagnóstico *in vivo* da miopatia WB através de imagens ultrassonográficas do peito dos frangos em diferentes idades. A partir dos resultados, pôde-se concluir que dietas restritivas não obtiveram efeitos na redução da ocorrência das miopatias, entretanto, ao se aplicar a restrição entre os 22 e 28 dias de idade foi verificada redução na severidade de WB. A utilização de imagens ultrassonográficas do músculo do peito de aves aos 21 dias de idade pode ser utilizada para predição *in vivo* de WB. O aumento da severidade de WB ocasionou alterações nos atributos de qualidade de carne dos filés como: aumento da perda por cocção, nas coordenadas cromáticas L*, a* e b* e na força de deformação. Em nível celular, o músculo *Pectoralis major* das aves severamente acometidas com WB apresentaram redução na quantidade e aumento do diâmetro das fibras musculares, proliferação de macrófagos, deposição de colágeno Tipo I no endomísio e perimísio e redução de aporte vascular pela diminuição da densidade de pequenos vasos. As atividades das enzimas séricas específicas de lesão muscular apresentaram valores crescentes de acordo com a idade e grau de severidade das miopatias, podendo serem utilizadas como marcadores bioquímicos das miopatias. Filés de peito classificados como de escores mais leves para WB apresentaram maiores valores de células miogênicas (MyoD, Myf-5 e Pax7) quando comparados com os mais severos para esta miopatia. Com base no exposto, o processo regenerativo das células do músculo *Pectoralis major* acometidos com miopatias se estabelece pela desestruturação do tecido muscular e proliferação de tecido conjuntivo, que já se estabelece aos 21 dias de idade e pode ser diagnosticado através de marcadores bioquímicos e imagens ultrassonográficas do peito dos animais *in vivo*.

Palavras chave: miopatias peitorais, células miogênicas, histomorfometria, restrição alimentar, ultrassom.

ABSTRACT

In order to elucidate the muscle development on broilers' breast myopathies and develop methods to detect those myopathies using in vivo experimentation, trials were conducted in fast-growing male broilers with different slaughtering ages. The studies presented in this thesis aimed to determine the growth, degeneration and regeneration cellular processes on pectoralis major muscle, as well as determine the effect of qualitative (hypoproteic and hypoenergetic) and quantitative (70%) feed restriction on the occurrence of wooden breast (WB) and white striping (WS) myopathies, correlating their consequences on meat quality. This thesis also shows a study conducted to validate an in vivo methodology to diagnose the WB myopathy using ultrasound images of broilers' breasts at distinct ages. From the results obtained it is possible to conclude that the feed restrictions applied did not impact on the occurrence of breast myopathies. However, broilers that had feed restriction applied between 22 and 28 d of age presented a decrease on their WB myopathy severity. At 21d of age, ultrasonography on in vivo birds' breast muscles can predict WB. The increase on WB severity lead to changes on the meat quality of breast fillets, such as: higher values for cooking loss, chromaticity coordinates (L, a*, b*) and shear force. At cellular level, pectoralis major muscle of severe WB broilers had a reduction on the quantity and increase of the diameter of muscle fibers, proliferation of macrophages, collagen Type I deposition on endomysium and perimysium, and decrease on vascular supply due to microvessels density. Serum enzymes specific to muscle injury can be used as biochemical markers for the myopathies, as they presented increased activity results directly related to age and WB myopathy severity. Breast fillets classified with lighter scores of WB had more myogenic stem cells (MyoD, Myf-5 and Pax7) when compared to more severe ones for this myopathy. According to the results obtained, the regenerative process of the pectoralis major muscle affected with myopathies is recognized by the deconstruction of the muscle tissue and connective tissue proliferation. These are already established since birds 21 d of age and can be diagnosed using biochemical markers and ultrasonography of in vivo broilers' breasts.*

Keywords*: breast myopathies, feed restriction, histomorphometry, myogenic stem cells, ultrasound.*

LISTA DE ILUSTRAÇÕES

LISTA DE ILUSTRAÇÕES ARTIGO 1

LISTA DE ILUSTRAÇÕES ARTIGO 2

LISTA DE ILUSTRAÇÕES ARTIGO 3

LISTA DE TABELAS ARTIGO 1

LISTA DE TABELAS ARTIGO 2

LISTA DE TABELAS ARTIGO 3

SUMÁRIO

1. INTRODUÇÃO

A elevada taxa de crescimento corporal dos frangos de corte está sendo associada a ocorrência de lesões principalmente no músculo *Pectoralis major*, com destaque especial para as miopatias *wooden breast* e *white striping*, caracterizadas macroscopicamente por filés de peito com consistência dura, coloração pálida e com aumento de volume e por estriações esbranquiçadas, paralelas à fibra muscular e que geralmente ocorrem na superfície ventral do peito, respectivamente.

A velocidade do desenvolvimento do músculo *Pectoralis major* durante o crescimento de frangos de corte pode estar relacionada ao surgimento destas lesões musculares, entretanto atualmente o diagnóstico das miopatias é evidenciado apenas nas carcaças, no final do ciclo de vida dos animais. Dentre os principais métodos de diagnóstico confiáveis por imagem utilizados na medicina veterinária, destaca-se a ultrassonografia, que possibilita a validação de exames *in vivo* como metodologia para diagnóstico de lesões musculares e assim poder selecionar geneticamente animais com características específicas.

A etiologia destas miopatias é desconhecida, porém há hipóteses que suas ocorrências estejam relacionadas com diversos fatores, dentre eles: ambientais, genéticos e nutricionais. Sendo assim, estratégias nutricionais como a restrição alimentar, podem apresentar resultados satisfatórios sobre a velocidade de crescimento dos tecidos, e consequentemente sobre a integridade do tecido muscular.

A restrição alimentar consiste em privar os animais ao livre acesso ao alimento (restrição quantitativa), restringindo a quantidade de ração oferecida ou alterando os níveis de nutrientes (restrição qualitativa), de maneira que o nutriente de interesse esteja disponível da forma restrita na ração. Ambas visam diminuir a taxa de crescimento das aves o que pode reduzir o grau de lesões musculares.

O músculo esquelético possui uma elevada capacidade de crescimento e regeneração. Este processo ocorre tanto no período de desenvolvimento embrionário como durante o processo de regeneração muscular, através das células satélites presentes no músculo maduro. As células miogênicas envolvidas no processo de regeneração muscular quando na ocorrência de miopatias, no entanto, a capacidade regenerativa das células satélites é finita; e se o músculo perde a sua capacidade regenerativa, ocorre a substituição do tecido muscular por tecido adiposo ou conjuntivo.

Para tanto, o presente trabalho teve como objetivos validar uma metodologia de

diagnóstico *in vivo* da miopatia *wooden breast* através de análises ultrassonográficas do músculo *Pectoralis major*, determinar o processo de crescimento, degeneração e regeneração celular, bem como determinar o efeito de programas de restrição alimentar sobre a ocorrência das miopatias *wooden breast* e *white striping* em frangos de corte abatidos em diferentes idades e as consequências na qualidade da carne.

2. REVISÃO BIBLIOGRÁFICA

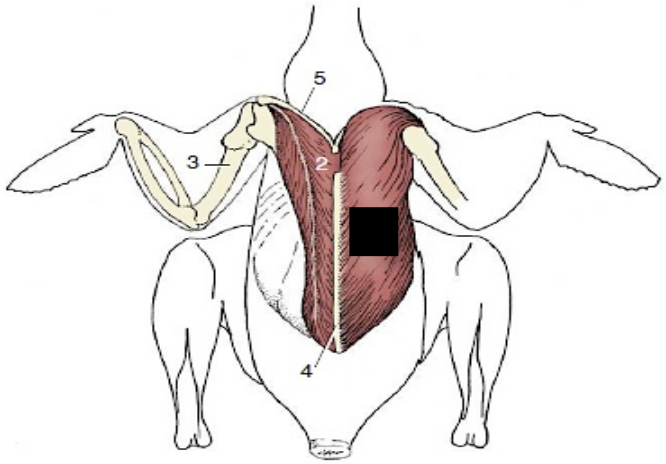
2.1. O músculo *Pectoralis major* e sua evolução genética

O músculo peitoral das aves é composto pelos músculos *Pectoralis major* e *supracoracoideus*. O *Pectoralis major* é o principal contribuinte do aumento de rendimento muscular em frangos de corte, e a seleção para maiores rendimentos de peito possui diferentes respostas no *Pectoralis major* quando comparado ao *supracoracoideus* (ZAPATA *et al.*, 2012).

O *Pectoralis major* é o músculo mais volumoso do corpo da ave formando, em parte, a massa carnosa associada com o peito. Ele tem origem na quilha do esterno e da clavícula, seguindo diretamente para a superfície ventral do tubérculo dorsal do úmero. Localiza-se dorsalmente ao rádio e seu curto tendão passa subcutaneamente sobre a superfície craniodorsal da articulação do carpo e termina na extremidade proximal do osso metacárpico (DYCE; WENSING, 2010).

Anatomicamente, este músculo é dividido em três porções: torácica, de maior massa muscular; propatagial, associada com o tensor propatagial; e abdominal, representada como um feixe muscular cutâneo composto de dois feixes musculares (subcutâneo torácico e abdominal) (Figura 1) (VANDEN BERGE, 1975).

Figura 1 - Localização anatômica do músculo *Pectoralis major*



Fonte: Dyce e Wensing (2010)

1. *Pectoralis major*; 2. *Supracoracoideus*; 3. Úmero; 4. Esterno; 5. Clavícula

Entretanto, as aves utilizadas na avicultura moderna atualmente vêm sofrendo várias alterações na sua composição corpórea e fisiológica. Nas últimas décadas, a indústria avícola sofreu mudanças significativas nas áreas de nutrição, genética e sanidade. De 1957 a 2005, houve aumento de 400% no crescimento de frangos de corte e redução de 50 % na conversão alimentar. Neste mesmo espaço de tempo, houve o incremento de 79 % e de 85 % do músculo *Pectoralis major* de machos e de fêmeas, respectivamente (ZUIDHOF *et al*., 2014).

Autores verificaram que este crescimento muscular difere em frangos de corte e aves poedeiras, sugerindo que, nos animais destinados a produção de carne, a taxa de síntese proteica nos músculos do peito é maior e a taxa de degradação proteica nos músculos do peito e das pernas é menor, quando comparado às poedeiras. Assim, o peso corpóreo do frango de corte tende a ser duas a três vezes maior do que de uma ave poedeira da mesma idade, devido à maior deposição de proteína nas partes comestíveis da carcaça, principalmente no músculo peitoral (GONZALES; SARTORI, 2002).

Segundo diversos autores, a seleção genética resultou em um aumento na tendência de miopatias espontâneas ou geradas pelo estresse (lesão muscular) (SANTE *et al.*, 1991; LE BIHAN-DUVAL; MILLET; REMIGNON, 1999; MITCHELL, 1999 SANDERCOCK *et al.*, 2001; MACRAE *et al.*, 2007). Como consequência disto, por razões comerciais, os músculos da região do peito são os mais susceptíveis a apresentarem alterações histopatológicas (SOIKE; BERGMANN, 1998).

2.2 Desenvolvimento e regeneração muscular

O músculo esquelético possui uma elevada capacidade de crescimento e regeneração. O número de fibras musculares é determinado durante a embriogênese e nesta fase, estruturas denominadas somitos sofrem compartimentalizações e darão origem aos mioblastos, que são células precursoras da musculatura. Essas células irão proliferar e migrar para locais específicos, onde se diferenciarão em miotubos e finalmente se agruparão para formar as fibras musculares (BUCKINGHAM *et al.*, 2003). Este processo ocorre tanto no período de desenvolvimento embrionário como durante o processo de regeneração muscular, através das células satélites presentes no músculo maduro (OLIVEIRA, 2009).

Células satélites são as células precursoras da musculatura esquelética após o nascimento. Estas células estão localizadas entre a membrana plasmática e a lâmina basal

da fibra muscular (SEALE *et al.*, 2000). Após o nascimento, as células satélites são as únicas células musculares que ainda proliferam, diferenciam-se e fundem-se com as fibras musculares adjacentes ou com outras células satélites (MAURO, 1961). Após a mitose, as células satélites incorporam DNA à fibra muscular e, apesar de representarem um pequeno percentual da densidade total de núcleos no tecido muscular, essa deposição de DNA à fibra muscular pode representar mais da metade do DNA acumulado após a eclosão (GRANT; GERRARD, 1998).

No músculo maduro, células satélites são quiescentes, mas podem ser ativadas em reposta a estímulos como crescimento e injúrias, para reparar as miofibras. Este processo consiste em curta fase de proliferação, seguida pela saída do ciclo celular, a diferenciação em mioblastos e finalmente a fusão com os miotubos (KOLEVA *et al*., 2005). Células satélites quiescentes expressam a proteína Pax7 (ZAMMIT; BEAUCHAMP, 2001), durante a proliferação expressam MyoD, Myf5 e Pax7 (ZAMMIT *et al*., 2006; KOLEVA *et al*., 2005), enquanto que MRF4 e Miogenina são necessários para a formação dos miotubos (NINOV, 2010).

A seleção genética para um maior crescimento provocou um aumento na taxa de proliferação dos mioblastos e das células satélites, isto foi observado pelo maior número de mionúcleos, maior síntese de DNA e maior conteúdo total de DNA (REHFELDT *et al*., 2000). De acordo com Halevy *et al*. (1998), as células satélites parecem ser a única fonte de células miogênicas durante o crescimento muscular esquelético de frangos de corte pós-eclosão, sendo que a maioria dos núcleos em células musculares maduras provém de tais células. Durante o crescimento inicial, o número de células satélites é relativamente alto para fornecer um maior conteúdo de DNA para o crescimento da fibra. A medida que o crescimento desacelera, estes valores decrescem até alcançarem uma menor população estável no músculo maduro, variando de 4 a 15 % dos núcleos totais na fibra (ALLEN; RANKIN, 1990 *apud* POPHAL, 2012).

Esta população de células precursoras musculares pode suportar diversos silos de degeneração e regeneração (CHARGÉ; RUDINICKI, 2004). Na degeneração é possível observar a morte das fibras musculares por apoptose ou necrose. Este evento é marcado pelo rompimento do sarcolema das miofibras, refletido por uma elevação no nível sérico de proteínas musculares como a creatina quinase, geralmente restrita ao citoplasma da célula muscular. Assim, em decorrência deste processo, é possível observar a ativação de células mononucleadas, especialmente células inflamatórias e células-satélites, que determinam a indução da regeneração muscular (OLIVEIRA, 2009).

No entanto, a capacidade regenerativa das células-satélite é finita; se o músculo perde a sua capacidade regenerativa, ocorre a substituição do tecido muscular por tecido adiposo ou conjuntivo.

2.3 Miopatias peitorais em frangos de corte: *Wooden Breast* e *White Striping*

Alterações musculares caracterizadas por uma miodegeneração no filé do peito de frangos de corte tem causado preocupações à indústria avícola, as quais incluem estriações esbranquiçadas, paralelas à fibra muscular e que geralmente ocorrem na superfície ventral do filé do peito (*white striping* (WS)), Kuttappan *et al*., 2009), filés de consistência dura, pálidos e com aumento de volume (*wooden breast* (WB)), Sihvo *et al*., 2014), e filés com uma tendência de separação das bandas de fibras musculares (*spaghetti meat* (SM)), SIRRI *et al*., 2016).

Estas miopatias possuem etiologia desconhecida, porém são associadas a frangos de corte machos, de rápido crescimento, ganho de peso e possuem aumento da severidade conforme o peso do filé (KUTTAPPAN *et al*., 2012; 2013, PETRACCI *et al*., 2013; FERREIRA *et al*., 2014; TROCINO *et al*., 2015; RADAELLI *et al*., 2017).

A menor densidade capilar resultante da seleção genética realizada ao longo dos anos pode estar relacionada com uma redução no fornecimento de nutrientes, oxigênio e também na remoção mais lenta de ácido lático nos músculos, conduzindo a danos musculares e com o aparecimento destas miopatias (BILGILI, 2013).

Nestas miopatias, alterações macroscópicas são restritas ao músculo peitoral maior, que mostra-se pálido com áreas extensas de dureza substancial acompanhados de estrias brancas; outros músculos esqueléticos parecem não serem afetados. Nenhum sintoma *ante-mortem* particular tem sido relacionado com esta condição. Essa condição gera rejeição de consumo, o que traz perdas econômicas significativas para indústria e aumenta o interesse para resolver a etiologia e, posteriormente, encontrar meios para evitá-la. Miopatias descritas anteriormente que possam afetar o músculo peitoral major de frangos de corte incluem distrofia muscular hereditária, tensão térmica, trauma, esforço, nutricional e toxicológico. Contudo a consistência endurecida do músculo não é, tipicamente, um achado dentro dessas etiologias. Atualmente, a etiologia e os fatores que causam o rápido aumento desta desordem no músculo peitoral ainda é desconhecida.

Petracci *et al.* (2013) relataram que os híbridos para alto rendimento de peito apresentaram uma maior incidência de *white striping* em comparação com aves de

rendimento padrão. De fato, a questão genética vem sendo reportada como uma das principais responsáveis pelo aumento do aparecimento das lesões musculares principalmente no peito das aves, mas falta definição da causa que podem ser multifatoriais, associados além de fatores genéticos, por fatores ambientais, nutricionais, de manejo ou fisiológicos (ZAMBONELLI, 2017).

Com o intuito de reduzir a ocorrência destas miopatias uma vez que possuem uma estreita relação com o rápido ganho de peso dos animais, estudos com estratégias nutricionais foram elaborados, demonstrando que a suplementação com vitamina E (KUTTAPPAN *et al*., 2012), selênio (FERREIRA *et al*., 2016; SIHVO *et al*., 2017), não possuem efeito na prevenção destas miopatias, entretanto, dietas com diminuição do conteúdo proteico (KUTTAPPAN *et al.*, 2012) e dos níveis de lisina (CRUZ *et al*., 2017) sugerem uma efeito positivo na ocorrência das lesões de peito, porém estas alterações também foram associadas com uma diminuição na taxa de crescimento e/ou peso da ave ao abate.

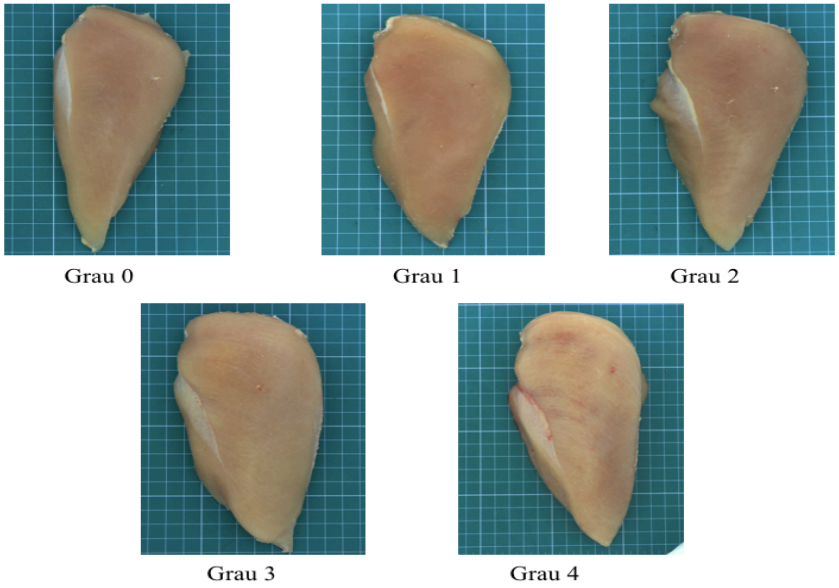
Programas de restrição alimentar também foram avaliados, com uma frequência menor de lesões degenerativas da fibra muscular no período de restrição (80% entre os 13 e 21 dias de idade), porém não se obteve um efeito residual após a re-alimentação, sem diferenças na ocorrência de lesões a partir dos 35 dias de idade entre os animais dos grupos ad libitum e os restritivos (TROCINO *et al*., 2015; RADAELLI *et al*., 2017).

De acordo com Zambonelli *et al*. (2017), há uma alteração nas vias de expressão dos genes nos filés afetados com WS/WB, sendo estas relacionadas ao desenvolvimento muscular, processos no metabolismo de polissacarídeos, síntese de proteoglicanos, inflamação e vias de sinalização de cálcio, tendo os autores classificados estas miopatias como de etiologia multifatorial e complexa.

2.3.1 *Wooden Breast*

Nos últimos anos, foi descrito uma nova miopatia associada à qualidade da carne de peito de frango, chamada *wooden breast*. Macroscopicamente, estas alterações estão restritas ao músculo *Pectoralis major* e são caracterizadas por áreas pálidas e com diversos níveis de rigidez aumentada (SIHVO *et al*., 2014, VIEIRA *et al*., 2017). A lesão é detectada manualmente, por meio da palpação, e acomete as aves a partir de 3 semanas de idade, podendo afetar mais de 50% de um lote (MUTRYN *et al*., 2015).

Figura 2 – Classificação do músculo *Pectoralis major* de frangos de corte para a miopatia *wooden breast*. Grau 0 - peito normal; 1- leve endurecimento na região cranial do filé; 2 - moderado endurecimento na região cranial do filé; 3 – severo endurecimento do filé; 4 – severo endurecimento com presença de lesões hemorrágicas, aumento de volume e presença de fluído amarelado.



Fonte: Vieira *et al*. (2017)

Visualmente, pode-se observar extensa área pálida, rígida e com ondulações, além de material viscoso com petéquias ou pequenas hemorragias nos casos aonde o grau de severidade é maior, concomitante com lesões de *white striping* (SIHVO *et al*., 2014). Microscopicamente, observa-se degeneração multifocal e necrose caracterizadas pela perda das estriações e por ser infiltrado de células inflamatórias, principalmente macrófagos e heterófilos. As áreas afetadas apresentam espessamento difuso do interstício com quantidade variada de tecido conjuntivo, tecido de granulação ou fibrose separando as fibras musculares (SIHVO *et al*., 2014).

A prevalência e etiologia desta miopatia ainda é desconhecida. Há indícios de maior expressão gênica à hipóxia e ao estresse oxidativo em aves acometidas, porém não está claro se é primária ou secundária a doença (MUTRYN *et al*., 2015).

Os mesmos autores, objetivando caracterizar a miopatia *wooden breast* através de expressão gênica, verificaram que muitos genes envolvidos na reparação celular foram expressos nos peitos de frangos acometidos e que provavelmente estejam ligados com a *wooden breast* em um esforço para compensar os danos musculares. Além disto, os

autores notaram que genes envolvidos no sistema de coagulação não estavam expressos significantemente no músculo, dificultando a associação com a doença (MUTRYN *et al*., 2015).

Neste contexto, estudos demonstram a mudança no metabolismo glicolítico de aves selecionadas geneticamente, através da diminuição da capilaridade em relação ao número de fibras (SOSNICKI; WILSON, 1991). Segundo Mudalal *et al*. (2015), a seleção genética para ganho de peso e para rendimento de peito é a hipótese com maior suporte e os fatores que apresentam maior influência no aparecimento dessa anormalidade.

Além da aparência e da coloração da carne, a *wooden breast* afeta a qualidade da carne refrigerada ou marinada. A carne de peito se apresenta endurecida, com diminuição na absorção da salmoura e maior perda por cocção que peitos afetados por *white striping* ou normais (sem miopatia). O fator principal na redução da qualidade decorrente dos peitos amadeirados é a diminuição da capacidade de retenção de água o que pode estar associada a dureza e aspecto de "madeira" (MUDALAL *et al*., 2015).

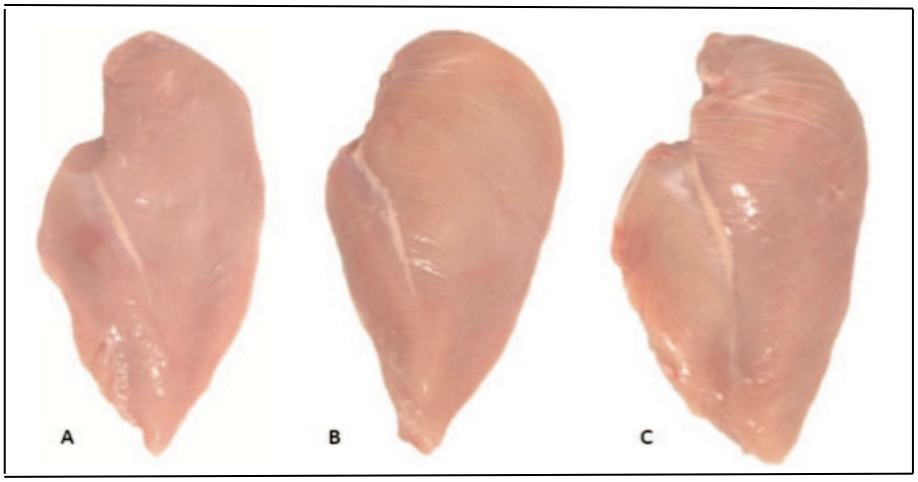
Com relação as características tecnológicas dos filés afetados, observa-se um aumento nos valores de pH, perda por gotejamento, perda por cocção e valores de b* (tom de amarelo) com uma diminuição na capacidade de retenção de água e maciez (PETRACCI *et al*., 2013; MUDALAL *et al*., 2015; SOGLIA *et al*., 2015; TIJARE *et al*., 2016; KUTTAPPAN *et al*., 2017). As composições proximais destes filés demonstraram aumentos no conteúdo de lipídios, cálcio, sódio e umidade, com reduzidos valores de proteína (PETRACCI *et al*., 2014; SOGLIA *et al*., 2015; MAZZONI *et al*., 2015; BALDI *et al*., 2018),

2.3.2 *White Striping*

A miopatia *white striping* caracteriza-se pelo aparecimento de vários graus de estrias esbranquiçadas na superfície do músculo *Pectoralis major* de frangos de corte as quais seguem a direção da fibra muscular (BAUERMEISTER *et al.*, 2009; KUTTAPPAN *et al.*, 2009).

Macroscopicamente, categoriza-se esta condição de acordo com o grau de estriações presentes no músculo peitoral em: normal (NORM), sem estriações aparentes; moderado (MOD), estriações visíveis no músculo e inferiores a um (1) mm de espessura, e severo (SEV), com estriações superiores a um (1) mm e facilmente observadas na superfície do músculo (KUTTAPPAN *et al.*, 2009) (Figura 3).

Figura 3 – Classificação de *white striping* no músculo *Pectoralis major* de frangos de corte. A) normal (NORM); B) moderado (MOD), e C) severo (SEV).



Fonte: Kuttappan *et al.* (2012a)

Estudos anteriores mencionaram a presença de estrias esbranquiçadas no músculo peitoral de frangos de corte e, ocasionalmente, no músculo da perna, semelhante às descritas na ocorrência de *white striping*, entretanto, esta alteração foi associada à condição de distrofia muscular induzida pela deficiência nutricional de vitamina E na dieta das aves (DAM; PHANG; SONDERGAARD, 1952; SCOTT *et al.*, 1955; MACHLIN; SHALKOP, 1956; MACHLIN; PEARSON, 1956; NESHEIM; LEONARD; SCOTT, 1959; FERGUSON *et al.*, 1964; BUNYAN *et al.*, 1967; NETKE *et al.*, 1969; KLASING, 2008).

Porém, em pesquisa realizada por Kuttappan *et al.* (2012c), estudou-se a similaridade da aparência entre animais acometidos com *white striping* e distrofia muscular nutricional ocasionada pela deficiência de vitamina E, concluindo que o nível de vitamina E na dieta não está associado com a ocorrência de *white striping* no músculo peitoral de frangos de corte.

Petracci *et al.* (2013a), estudando a incidência desta miopatia em criações comerciais de frangos de corte na Itália, analisaram 28.000 aves oriundas de 56 lotes e encontraram uma incidência total de 12% de frangos acometidos com *white striping*, sendo 8,9% classificados com grau moderado e 3,1% de grau severo.

Com o objetivo de estudar a relação entre a severidade de *white striping* e o crescimento de frangos de corte, Bauermeister *et al.* (2009) verificaram aumento da incidência e da severidade desta miopatia com o aumento do peso vivo e do peso dos filés

de peito, tendo também aumentado a severidade nos animais abatidos com oito semanas de idade quando comparados aos abatidos com seis semanas.

As causas da formação de *white striping* são desconhecidas, mas avaliações histológicas demonstraram que esta alteração é usualmente associada à degeneração muscular e alterações miopáticas abaixo da área com estriação, como perda das estriações transversais, variabilidade no tamanho da fibra, degeneração flocular/vacuolar e lise das fibras, mineralização moderada, regeneração, infiltração de células mononucleares, lipidose, inflamação intersticial e fibrose (KUTTAPPAN *et al*., 2011 e 2013b; PETRACCI *et al.*, 2013b). De acordo com Kuttappan *et al.* (2011), há um significante aumento de lesões degerativas e necróticas, além de fibrose e lipidose, a medida em que o grau de *white striping* aumenta, tendo os autores classificado as alterações apresentadas como um caso de distrofia muscular idiopática.

Conforme Kuttappan *et al.* (2012b), o aumento das taxas de crescimento resulta em maior incidência de frangos de corte acometidos com *white striping*, e os vários graus desta miopatia estão associados com alterações na composição química destes filés de peito. Corroborando com esta afirmativa, em outro trabalho, Kuttappan *et al.* (2013b) observaram acréscimo no teor de gordura muscular e decréscimo de proteína, conforme o aumento do grau de *white striping,* concluindo que estas alterações histopatológicas indicam uma miopatia degenerativa que pode estar associada com a taxa de crescimento das aves.

De acordo com Kuttappan *et al.* (2012a), este fenômeno de etiologia desconhecida está afetando a aceitação do consumidor com relação à severidade da mesma no filé do peito. O estudo relata que 50% dos consumidores consultados não comprariam a carne que apresentasse filetes de estrias de graus moderado ou severo. Dentre os problemas relacionados à carne de aves, acredita-se que, similarmente a suínos, a incidência de problemas de coloração na carne ocorre em 5-40% das carcaças e compromete a aparência geral do produto final, atributo de grande valia para os europeus e para os consumidores mais exigentes.

Sendo assim, em razão do pouco conhecimento a respeito destas miopatias e por afetar o aspecto visual de um dos cortes de maior valor comercial da carne de frangos, torna-se de suma importância um maior aprofundamento destas.

2.4 Métodos de diagnóstico de lesão muscular e suas consequências para qualidade da carne

Apesar da taxa de crescimento, da conversão alimentar e da quantidade de musculatura terem aumentado em razão dos programas de seleção para frangos de corte, a qualidade da carne foi alterada (DRANSFIELD; SOSNICKI, 1999), o que resultou na criação de condições que favoreçam a susceptibilidade às alterações metabólicas e a mudanças na estrutura celular do músculo esquelético, tanto morfológicas como bioquímicas.

Existem dois tipos de métodos para analisar os danos musculares, os diretos e indiretos. Os métodos diretos são realizados através de análises por imagens ou por amostras musculares. Já os métodos indiretos são obtidos principalmente através de respostas das concentrações de enzimas plasmáticas, proteínas musculares e parâmetros físico-químicos. Através destes métodos torna-se, possível avaliar os efeitos das lesões musculares nas características de qualidade da carne.

2.4.1 Diagnóstico por imagem - Ultrassonografia

Exames por imagem podem ser úteis para auxiliar no diagnóstico de lesões com maior precisão (FERNANDES *et al*., 2011). A ultrassonografia é uma técnica empregada para a obtenção de imagens, que se baseia no fenômeno de interação de som e tecidos, ou seja, a partir da transmissão de onda sonora pelo meio, observando-se as propriedades mecânicas dos tecidos (MOLNÁR; CERRI, 2004).

As ondas ultrassônicas são produzidas e direcionadas para o interior do corpo até atingires uma barreira tissular que irá refleti-las para o transdutor. As ondas produzidas atingem uma velocidade de cerca 1500m/s e, quando refletidas, são denominadas de ecos.

O transdutor é um cristal vibratório acionado eletricamente que tem a função de converter energia elétrica em energia ultrassônica, ele tanto envia quanto recebe as ondas de eco, agindo tanto como transmissor quanto receptor, convertendo as ondas em tensões elétricas novamente. Tal frequência não é transmissível em ar ou gás, apenas em sólidos e líquidos. As imagens são produzidas pela oscilação longitudinal contra a superfície do tecido. A intensidade das ondas são parcialmente refletidas pelo plano tecidual profundo, sendo parte delas recebidas pelo transdutor. Quanto maior a frequência nominal do

transdutor, menor a profundidade de penetração e melhor é a resolução espacial da imagem (BANDEIRA *et al*., 2013)

O exame pode ser utilizado no diagnóstico de lesões musculares, detectando hematomas, abscessos e fibroses. Um sangramento agudo ou difuso pode ser difícil de ser localizado por ultrassom porque o sangue recém-coagulado é inicialmente ecogênico (KOFLER; BUCHNER, 1995). Segundo Heppelmann *et al.* (2009), o fluido é o meio de transporte ideal para as ondas de ultrassom e é responsável pelas diferenças de impedância acústica, que é um pré-requisito para a diferenciação de tecidos. Processos inflamatórios sépticos são visualizados na ultrassonografia devido ao acúmulo de líquido nos tecidos durante a fase exsudativa. Lesões traumáticas edemaciadas de tecidos moles e avaliação da cicatrização de feridas são outras indicações para ultrassonografia.

O aumento da quantidade de gordura intramuscular e fibrose em um tecido muscular resulta no aumento da eco-reflexão e brilho da imagem do ultrassom (HECKMATT; PIER; DUBOVITZ *et al*., 1988; PILLEN *et al*., 2009). Embora ambas as avaliações de ultrassom qualitativos e quantitativos do músculo esquelético são eficazes na identificação de patologias musculares, a ultrassonografia quantitativa oferece superior confiabilidade e maior sensibilidade para detectar tais patologias (HECKMATT *et al*., 1989; ZUBERI *et al*., 1999; MAURITS *et al*., 2003; PILLEN *et al*., 2006).

A quantificação da quantidade de patologia músculo usando ultrassom fornece uma medida sensível, não invasiva e pode ser utilizada na classificação da gravidade das doenças como distrofias musculares (ZAIDMAN; HOLLAND; HUGHES, 2010; JANSEN *et al.*, 2011). Pillen (2009) encontrou uma significativa correlação entre a quantidade de tecido conjuntivo e a intensidade de ecogenicidade muscular em cães afetados com distrofia muscular.

Diferentes autores já propuseram a utilização do método para correlacionar imagens obtidas pelo ultrassom com o peso de peito e suas proporções em relação a carcaça em frangos de corte. Além disso, as imagens ultrassonográficas podem identificar a presença de diversas alterações teciduais, como edema, inflamação, acúmulo de gordura, fibrose e calcificação (MITTAL *et al.*, 2003; CASE *et al*., 2012). Este método vem contribuir com pesquisas da área através do reconhecimento da miopatia no animal vivo, o que pode vir a ser um novo método de diagnóstico de miopatias, possibilitando o desenvolvimento de estratégias na seleção dos animais antes do abate.

2.4.2. Marcadores enzimáticos

A seleção genética para características de produção, como a taxa de crescimento, o peso de abate e a eficiência da conversão alimentar tem sido extremamente bem-sucedida na produção avícola. No entanto, em frangos de corte e perus, estas melhorias no desempenho produtivo podem estar associadas com alguns efeitos prejudiciais sobre o músculo esquelético (MITCHELL, 1999). Partindo deste princípio, pesquisas tem sugerido que mudanças em enzimas plasmáticas sejam um indicativo de dano no músculo esquelético de frangos de corte, pois estas alterações enzimáticas seriam consequência de uma modificação na permeabilidade da membrana de células musculares (MITCHELL; SANDERCOCK, 1995; YAN *et al.*, 2009).

A atividade enzimática é utilizada na rotina como um marcador de detecção em patologias em animais, porém em frangos de corte ainda há pouca informação sobre os níveis enzimáticos. Porém, Gee *et al.* (1981) recomenda o uso das enzimas aspartato aminotransaminase (AST), creatina quinase (CK) e lactato desidrogenase (LDH) como indicadores de lesão em tecido muscular para patologias em aves. Elevações acentuadas na atividade de CK no plasma foram reportadas em várias condições patológicas musculares em frangos, incluindo miopatias (MITCHELL *et al.*, 1992; MITCHELL; SANDERCOCK, 1994, 1995) e distrofia muscular congênita no músculo esquelético (STEWART *et al*., 2004).

De acordo com Mitchell *et al.* (1999), o aumento do crescimento muscular compromete a integridade da membrana do músculo esquelético, o que provoca alterações a nível enzimático sérico, tais como mudanças quantitativas na atividade de LDH, de CK e de AST. Em razão de estas moléculas serem citoplasmáticas e não terem a capacidade de atravessar a barreira da membrana sarcoplasmática, o aumento da concentração sérica dessas moléculas livre na circulação sanguínea é utilizado como indicativo de dano na membrana muscular e em outras estruturas teciduais (BROWN; CHILD; DONNELLY, 1997).

Segundo Szabó *et al*. (2005), a atividade de CK no soro aumenta em paralelo com o peso corporal total ou massa muscular, colocando como hipótese de causa o aparecimento de uma hipertrofia peitoral ou até mesmo danos na membrana muscular, e apresentando como base desta interpretação o aumento de mais de uma de magnitude no soro sanguíneo. Partindo deste princípio, Mahon *et al.* (1995) e Mills *et al.* (1998) relataram que extensivos estudos histológicos, principalmente em perus comerciais, têm

obtido resultados afirmativos ao comparar a elevada atividade plasmática de marcadores enzimáticos intracelulares, como CK, com o aumento da incidência de anormalidades da estrutura muscular conforme a o acréscimo da idade.

Há inúmeros mecanismos, através dos quais o cálcio pode induzir uma disfunção metabólica, incluindo alterações na integridade da membrana muscular (MITCHELL, 1999). Altas elevações descontroladas e sustentada de cálcio intracelular causam dano muscular, sendo característica de muitas doenças e patologias musculares. Está bem claro que estes aumentos prolongados podem ativar vários processos degenerativos intracelulares, o que resulta na quebra estrutural e funcional de células de músculo esquelético (JONES *et al*., 1984; DUNCAN; JACKSON, 1987; JACKSON, 1993). Acredita-se que o extravasamento de CK a partir de células musculares esqueléticas nestas situações seja uma consequência de alterações na integridade da membrana da célula muscular (sarcolema) induzida por elevações de cálcio intramuscular (SANDERCOCK; MITCHELL, 1998; SANDERCOCK *et al.*, 2001).

Com relação ao perfil hematológico e sorológico dos frangos de corte acometidos com *white striping*, Kuttappan *et al.* (2013c) não encontraram diferenças em vários parâmetros hematológicos, porém foram observados aumentos nos níveis séricos de creatina quinase, alanina transaminase, aspartato aminotransferase e lactato desidrogenase nos animais cujos músculos foram classificados como de grau severo. Estes resultados, segundo os autores, sugerem que não há infecção sistêmica ou inflamatória associada ao grau severo de *white striping*. O aumento do nível sérico das enzimas confirma uma lesão muscular associada com miopatia degenerativa nos animais de grau severo.

Guetchom *et al.* (2012), avaliando o efeito da suplementação de vitamina E sobre a integridade muscular do peito de frangos de corte e sobre os marcadores enzimáticos, verificaram que a adição de vitamina E na dieta convencional aumentou os níveis plasmáticos de vitamina E e ligeiramente diminuiu o número de fibras danificadas no músculo peitoral de frangos de corte jovens (28 dias). A atividade da enzima CK não foi um indicador bioquímico confiável de degeneração muscular leve em frangos de corte.

2.4.3 Morfometria do peito e qualidade de carne

A seleção para altas taxas de crescimento nos frangos de corte promove alterações estruturais e metabólicas nos músculos e altera a qualidade da carne. A produção de filés de peito com especificações rígidas de peso, comprimento e espessura para produção de produtos pós-processados ou para consumo em restaurantes de comidas rápidas, possui implicações econômicas importantes para rentabilidade das integrações (MADEIRA, 2008).

O comprimento, espessura e peso do filé de peito de frangos de corte são afetados pela linhagem, sexo e idade das aves. Filés mais longos, espessos e pesados são produzidos pelas aves mais velhas e por machos (ROBINSON *et al*., 1996b). O aumento na massa peitoral se deve, principalmente, ao aumento na espessura do músculo *Pectoralis major* (LUBRITZ, 1997). A espessura da parte mais larga do *Pectoralis major*, próximo à inserção das asas, varia de acordo com a linhagem; o mesmo não ocorre com a ponta mais fina do músculo (ROBINSON *et al.*, 1996b; MADEIRA, 2008).

Esta alta taxa de crescimento morfológico do peito promove maior diâmetro das fibras musculares, maior proporção de fibras glicolíticas e menor proteólise muscular. Estas alterações proporcionam o *rigor mortis* mais rápido, aumentando a palidez da carne e diminuindo a capacidade de retenção de líquidos, piorando a qualidade dos produtos processados. A diminuição da proteólise muscular aumenta a dureza das carnes de aves (DRANSFIELD; SOSNICKI, 1999). O efeito da seleção para ganho de peso apresenta aumento da área transversal das fibras (ABERLE et al., 1979; IWAMOTO *et al*., 1993; BURKE; HENRY, 1997; SCHEUERMANN, 2004) e indica a possibilidade de inclusão desta variável nos programas de seleção. (MADEIRA, 2008).

Dentre os principais atributos avaliados na carne para determinar sua qualidade destacam-se cor, capacidade de retenção de água e textura. A cor é um dos fatores mais importantes na percepção do consumidor quanto à qualidade da carne, pois é uma característica que influencia tanto a escolha inicial do produto pelo consumidor como a aceitação no momento do consumo (FLETCHER, 1999).

De acordo com OLIVO *et al.* (2001), a cor observada na superfície das carnes é o resultado da absorção seletiva da luz pela mioglobina e por outros importantes componentes, como as fibras musculares e suas proteínas, sendo também influenciada pela quantidade de líquido livre presente na carne. Os parâmetros utilizados na avaliação da cor da carne utilizados baseiam-se no sistema colorimétrico denominado CIELab e

suas coordenadas cromáticas (luminosidade, representada por L*, teor de vermelho, representado por a* e teor de amarelo, representado por b*).

A capacidade de retenção de água é um termo originalmente usado para descrever a capacidade do músculo e dos produtos cárneos em manter a água ligada a si (FENNEMA, 1990). A água no músculo é retida em sua maior parte intracelularmente e também entre as miofibrilas (OFFER; KNIGHT, 1988). A capacidade de retenção de água está entre as propriedades funcionais mais importantes da carne (ANADÓN, 2002), pois influencia seu aspecto, sua palatabilidade e está diretamente relacionada às perdas de água antes e durante o cozimento (BRESSAN, 1998).

Trocino *et al.* (2015) verificaram que peitos de frangos acometidos com White striping exibiram pequenas diferenças nos valores pH final e cor, e os acometidos com *wooden breast* demonstraram valores similares de pH e cor porém perderam mais água e exibiram maiores forças de cisalhamento quando comparados com peitos normais ou com *white striping*.

A textura é outro fator bastante importante na percepção do consumidor quanto à qualidade da carne (BRESSAN, 1998). A textura da carne, determinada por sua força de cisalhamento, está intimamente relacionada à quantidade de água intramuscular e, portanto, à capacidade de retenção de água da carne, de modo que quanto maior o conteúdo de água fixada no músculo, maior a maciez da carne (ANADÓN, 2002).

Avaliando as características físico-químicas de filés de peito de aves acometidas com *white striping*, Kuttappan *et al.* (2013a) aferiram as dimensões dos filés (comprimento, largura, espessura cranial e caudal), o pH, a cor (L*[luminosidade], a*[tom de vermelho] e b* [tom de amarelo]), a perda de peso por cocção e a força de cisalhamento e concluíram que o grau de *white striping* não influenciou ($P > 0,05$) os valores de pH, L*, a*, perda de peso por cocção e força de cisalhamento. Entretanto, nas amostras consideradas de grau severo de *white striping* foram observados aumentos significativos na espessura cranial e no tom de amarelo (b*), o que pode comprometer a qualidade estética do produto comercializado.

Dalle Zotte *et al.* (2014) avaliaram as características físico-químicas do peito de frangos acometidos com wooden breast e verificaram valores de pH mais elevados, cor mais clara e valores de L*, a* e b* mais elevados quando comparados com peitos considerados normais (sem miopatia). Além disto, os peitos acometidos apresentaram menor capacidade de retenção de água e maiores perdas de rendimento de corte pós-

congelamento e pós-cocção. Em contrapartida, os valores de força de cisalhamento não apresentaram diferença significativa.

Mazzoni *et al.* (2015) constataram que o grau de miodegeneração do músculo peitoral de frangos de corte refletiu em uma ampla modificação da composição química e uma deficiência notável na capacidade de processamento da carne.

2.5 Restrição nutricional na prevenção de miopatias

Em sistemas de produção de carne, o conhecimento dos fatores que determinam o crescimento e desenvolvimento dos tecidos e do organismo como um todo é fundamental para a adequação de programas de melhoramento, de manejo nutricional, ambiência e definição da idade de abate, para melhorar a qualidade e a quantidade de carne produzida. Para que isso ocorra é necessário que o sistema digestório apresente características funcionais estruturais para a digestão e a absorção de nutrientes (FURLAN, 2014).

O método atualmente utilizado para formulação de dietas para frangos de corte baseia-se em tabelas de exigências nutricionais que somente leva em conta as diferentes fases de crescimento das aves. A utilização de uma exigência mínima fixa impossibilita a determinação do efeito sobre o crescimento, consumo ou composição de carcaça (GOUS, 1998; POPHAL, 2004).

A seleção genética para carne magra, os grandes músculos do peito e as rápidas taxas de crescimento fazem com que as aves domésticas particularmente como frangos de corte sejam suscetíveis ao estresse oxidativo (SIHVO *et al*., 2013).

Diversos estudos sobre programas de manejo que restringem o rápido crescimento têm mostrado que a prática da restrição alimentar é uma proposta viável a ser explorada.

A restrição alimentar pode ser realizada em termos qualitativos ou quantitativos, porém no primeiro caso a ração utilizada possui um menor nível proteico ou energético, com isso as aves tendem a ingerir maiores quantidades de ração na tentativa de equilibrar suas necessidades, levando a um consumo desiquilibrado de alguns nutrientes tais como, aminoácidos, minerais e vitaminas. Na restrição quantitativa, a ração fornecida contém os níveis recomendados de nutrientes, variando apenas a quantidade fornecida ou tempo de acesso aos alimentos (RAMOS, 2007).

Uma das preocupações com relação à introdução da técnica de restrição alimentar está associada ao ganho compensatório, pois o peso de abate é um parâmetro importante para a comercialização (FURLAN *et al.*, 2002).

A restrição alimentar diminui o crescimento das aves no período da limitação da ingestão do alimento, que pode ser compensado durante o período de realimentação, denominado período de ganho compensatório (YU *et al*., 1990). No entanto, alguns estudos, como os de Plavinik e Hurwitz (1985), mostraram que o ganho compensatório ocorre somente após curto período de restrição.

De acordo com Gonzales (1992), a restrição alimentar (50%) em frangos de corte deve ser seguida de pelo menos três semanas de realimentação, período necessário para que as aves apresentem ganho compensatório e, consequentemente, peso final semelhante ao dos frangos com consumo à vontade. Entretanto, Moran Jr. (1992), sugere que seriam necessárias seis semanas de realimentação para que as aves consigam recuperar a perda de peso ocasionada pela restrição alimentar precoce. Outros autores, no entanto, não encontraram os mesmos resultados, ficando os animais com perdas no peso corporal após a restrição alimentar e, sugeriram que o ganho compensatório não existe (MOLLINSON *et al*., 1984; CABEL; WALDROUP, 1990; YU *et al*., 1990 *apud* RAMOS, 2007).

Togashi (2004), avaliando a restrição quantitativa em frangos de corte, observaram que os diferentes programas alimentares não influenciaram ($P > 0,05$) os ganhos de peso dos animais aos 21 dias de idade, já aos 45 dias de idade, a mesma autora verificou que houve diferença para o ganho de peso dos animais avaliados, onde os animais submetidos aos programas alimentares alcançaram peso vivo inferior aos do tratamento que recebeu ração à vontade. No entanto, Camacho *et al.* (2004), ao avaliarem o efeito da idade e da restrição alimentar e da suplementação com microelementos, indicaram que a restrição quantitativa, iniciada no 7º dia de idade com a suplementação levou a redução da mortalidade, dos problemas de patas e permitiu o crescimento compensatório aos 49 dias de idade. Da mesma forma, Sugeta (2002) evidenciou que a restrição alimentar mais severa (70% do consumo diário, do 8º até 14º dia de idade) afetou de forma negativa o desempenho das aves.

Furlan *et al*. (2001) e Leone *et al.* (2001), ao estudarem o desempenho produtivo de frangos de corte submetidos a diferentes programas de restrição alimentar, observaram que o ganho de peso, o peso vivo e o consumo de ração não foram influenciados estatisticamente pelos programas aplicados. Por outro lado, FURLAN *et al.* (2002) indicaram que a forma de arraçoamento por 12 horas, tanto diurna quanto noturna, não se

mostrou satisfatória no manejo diário, uma vez que houve prejuízo no desempenho produtivo com menor peso vivo das aves.

Rosa *et al.* (1994) citam redução significativa do peso vivo nos grupos de aves que sofreram a restrição, mas estes programas não influenciaram no rendimento de carcaça e na composição química.

Trabalhando com diferentes programas de restrição alimentar Figueiredo *et al*. (1998) constataram diminuição do rendimento da carcaça dos frangos de corte em relação aos alimentados à vontade. Em contrapartida, Sugeta *et al*. (2002), estudando a restrição alimentar na segunda semana de vida das aves (8º ao 14º dia), relataram que frangos restritos a 30% do consumo diário dos animais alimentados à vontade não diferiram ($P > 0,05$) dos animais do tratamento que recebeu ração *ad libitum*. Já os frangos restritos em 70% do consumo diário, tiveram um peso de carcaça significativamente inferior ($P < 0,01$) ao dos outros dois tratamentos (restrito em 30% e *ad libitum*), evidenciando, assim, que a qualidade da carcaça foi influenciada negativamente, por esse programa ser muito severo (RAMOS, 2007).

No trabalho de Trocino *et al*. (2015) os animais que receberam restrição alimentar no período de 13 a 21 dias de idade apresentaram ganho compensatório no período de re-alimentação o que ocasionou redução na diferença de peso vivo e conversão alimentar quando comparados aos animais sem restrição. Entretanto, a restrição alimentar aumentou em 10% a ocorrência de *white striping*.

Dietas de baixa energia reduzem tanto a taxa de crescimento como a ocorrência de miopatias, similarmente a restrição alimentar pode reduzir a incidência de miopatias e doenças metabólicas por controle do crescimento inicial (DE JONG *et al.*, 2012; SAHRAEI, 2012; BUTZEN *et al.,* 2013).

3. OBJETIVOS

3.1. Objetivos Gerais

O presente trabalho teve como objetivos validar uma metodologia para diagnóstico *in vivo* da miopatia *wooden breast* através de análises de imagens ultrassonográficas, determinar o processo de crescimento, degeneração e regeneração celular do músculo *Pectoralis major*, bem como determinar o efeito de programas de restrição alimentar sobre a ocorrência das miopatias *wooden breast* e *white striping* em frangos de corte abatidos em diferentes idades e as consequências na qualidade da carne.

3.2. Objetivos Específicos

3.2.1. Experimento 1

Avaliar o efeito das dietas com restrição alimentar (quantitativa e qualitativa) no desempenho zootécnico de frangos de corte

Determinar a ocorrência das miopatias peitorais (*wooden breast* e *white striping*) em frangos de corte submetidos a programas de restrição alimentar.

Correlacionar as medidas ultrassonográficas (ecogenicidade e profundidade) do peito com a severidade da miopatia *wooden breast* em frangos de corte e, assim, propor uma metodologia para diagnóstico *in vivo* desta lesão.

Analisar as características da qualidade da carne (pH, cor, capacidade de retenção de água, força de deformação, perda por cocção e força de cisalhamento) de peitos de frangos de corte acometidos com diferentes graus da miopatia *wooden breast* submetidos a programas de restrição alimentar.

3.2.2. Experimento 2

Avaliar o processo de crescimento, degeneração e regeneração do músculo *Pectoralis major* de frangos de corte de rápido crescimento.

Analisar as medidas histomorfométricas de diâmetro e densidade da fibra muscular, espessura do endomísio e perimísio, densidade de pequenos vasos e volume parcial do

tecido muscular em frangos de corte aos 21 e 49 dias de idade, com diferentes graus de severidade da miopatia wooden breast.

Avaliar os marcadores bioquímicos séricos de lesão muscular ALT, AST, CK e LDH de frangos de corte aos 21 e 49 dias de idade acometidos com a miopatia *wooden breast.*

3.2.3. Experimento 3

Analisar a população das células miogênicas MyoD, Myf-5 e Pax7, ativas e não ativas envolvidas no processo de regeneração muscular em frangos de corte acometidos com a miopatia *wooden breast*.

Identificar e quantificar, através do uso de anticorpos imunomarcadores, a proliferação de colágeno no músculo *Pectoralis major* de frangos de corte com *wooden breast.*

4. MATERIAIS E MÉTODOS

Para elaboração desta tese, foram realizados 2 experimentos.

O primeiro experimento foi conduzido entre os meses de setembro e outubro de 2016, gerando os artigos 1 e 2. O alojamento e abate dos animais foram realizados no Aviário de Ensino e Pesquisa e, as análises laboratoriais, no Centro de Ensino e Pesquisa em Tecnologia de Carnes, ambos localizados na Universidade Federal do Rio Grande do Sul.

O segundo experimento foi realizado durante o período de "Doutorado Sanduíche", entre os meses de abril a setembro de 2017, na Auburn University – Auburn, Alabama, Estados Unidos, no Poultry Science Departament, sob supervisão da Dra. Jéssica Starkey, o qual gerou o terceiro artigo desta tese.

Os referidos artigos foram redigidos de acordo com as normas da revista Poultry Science, cujas normas para publicação encontram-se em anexo.

5. ARTIGO CIENTÍFICO 1

PROCESSING AND PRODUCTS

IN VIVO MEASUREMENTS AND BREAST MYOPATHIES

Use of in-vivo measurements to estimate breast myopathies in broilers subjected to feed restriction and the effects on meat quality

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ABSTRACT

This study aimed determine the occurrence of wooden breast (WB) and white striping (WS) myopathies in broilers breast fillets submitted to feed restriction (quantitative or qualitative) at different ages and their effects on meat quality and, in concomitance to ultrasound images (US), estimate the WB myopathy in vivo. A total of 1,800 Cobb 500 male chicks were fed 9 treatments with 8 replicates of 25 each. Restrictive quantitative (70% of ad libitum – 70% FR) and qualitative (low energy and protein – Low EP) diets was applied in a 3-phase program (1 to 7 d, 8 to 14 d, 15 to 21 d and 22 to 28 d). After restriction periods, birds were fed ad libitum until the end of the trial at 49 d. US images on pectoralis major muscle was weekly conducted from 7 to 49 d to evaluate the echo intensity and breast depth. Weekly, one bird per pen was slaughtered, breast fillets were weighed, given visual scores for WB (0 to 4) and WS (1 to 3) and proceeded to meat quality analysis. A decrease in body weight gain was observed in broilers submitted to both feed restrictions; however, after 28 d, birds demonstrated to compensate their weight and presented similar performance than those fed ad libitum. No fillets where scored as 4 of WB on the periods from 22 to 28 d for both quantitative and qualitative feed restrictions. Fillets presenting WB score 4 had higher values of echo at 28 d, 42 d and 49 d, as well as the depth of breast at 21 d. Cooking loss increased among age and severity. At 49 d, shear force (MORS), shear energy (MORSE) and color increased in fillets classified as WB score 4. The US images can be utilized to predict the WB of broilers in vivo from 21 d. Broilers had a compensatory performance after the restrictions periods, and the increased severity of WB affects the meat quality parameters.

Key words: breast myopathies, compensatory growth, feed restriction, meat quality, ultrasound

INTRODUCTION

A series of myopathies affecting the pectoralis major muscle have arisen, which have been related to the increased growth rate and breast meat yields of broiler chickens, i.e., wooden breast (**WB**) and white striping (**WS**) (Kuttappan et al., 2013a; Lorenzi et al., 2014; Sihvo et al., 2014; Trocino et al., 2015). They are only detected at slaughter and the industry is facing great economic losses due to the undesirable appearance of these fillets and changes on meat quality characteristics (Tijare et al., 2016). Wooden breast meat has more fat and moisture, less protein, leading to higher water losses during cooking (Mudalal et al., 2014; Trocino et al., 2015; Cai et al., 2017; Radaelli et al., 2017). Fillets with white striping present higher final pH and higher cooking losses (Mudalal et al., 2014; Mazzoni et al., 2014; Trocino et al., 2015; Radaelli et al., 2017).

Muscle ultrasound (**US**) is an easy technique to visualize normal and pathological muscle tissues, as it is non-invasive and real-time. Once the WB myopathy is characterized as an accumulation of interstitial and connective tissue (Sihvo et al., 2014), and a significant correlation has already been found between the amount of fibrous tissue and muscle echo intensity in dogs affected with muscular dystrophy (Pillen, 2009), this technique could be used to estimate this myopathy in vivo. The application of US had already been successfully used for genetic selection in broilers to estimate in vivo carcass and breast muscle weight and yields on broilers and turkeys (Silva et al., 2006, Oviedo-Rondón, 2007, Case et al., 2012).

The intense genetic selection for rapid growth in broilers also resulted in an increase in voluntary feed intake and growth rate. Once the occurrence of these myopathies in fast-growing broilers are higher, feed restriction might be used to control the early growth and the occurrence of metabolic diseases, thus reducing muscular

damage (De Jong et al., 2012; Sahraei, 2012; Butzen et al., 2013; Trocino et al., 2015). However, it is also unclear at what age, level, and duration the restriction is most effective.

To this, the present study intends to determine the occurrence of wooden breast (WB) and white striping (WS) myopathies in broiler breast fillets submitted to 70% quantitative feed restriction or qualitative restriction with diets based on low energy and protein at different age and their effects on meat quality and, in concomitance to ultrasound measurements, estimate the wooden breast myopathy in vivo.

MATERIAL AND METHODS

All procedures used in this study were approved by the Ethics and Research Committee of the Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil.

Birds husbandry

A total of 1,800 one-day-old, slow feathering Cobb x Cobb 500 male broiler chicks, vaccinated for Marek's disease at the hatchery, were randomly distributed into 72 pens (1.65 × 1.65 m; 9.2 birds/m^2).

The experiment was carried out at Federal University of Rio Grande do Sul experimental facilities. Environmental temperature was controlled to maintain bird comfort with the use of an infrared heating lamps. Ambient temperature was adjusted to 32ºC on the first day, being reduced by 1°C every two days until comfort temperature was reached, thermostatically controlled by the use of heaters, fans and foggers. Each pen had new rice hulls bedding and was equipped with one 15-kg capacity tube feeder and 3-nipple drinkers. Lighting was continuous until 7 d of age, with a 14L:10D cycle used

afterward. Birds had ad libitum access to water and mash feed. All pens were daily checked for sick and dead birds. Pen number, age and body weight of each dead bird were recorded in an excel sheet. General health status, weight, mortality and estimated cause of death were recorded. Water was supplied for ad libitum consumption.

Experimental Diets

Birds were individually weighted and neck-tagged with identification numbers and allocated into 9 treatments with 8 replications of 25 birds each in a completely randomized design. Broilers were fed pelleted diets formulated using Brazilian commercial nutrient and energy practical levels in a 5-phase feeding program composed by pre-starter (1 to 7 d), starter (8 to 21 d), grower I (22 to 35 d), grower II (36 to 42 d) and finisher (43 to 49 d) diets; however, the feed restrictions (qualitative and quantitative) was performed in a 3-phase program (pre-starter, starter and grower I), after feeding treatment intervals, the birds were fed ad libitum until the end of the trial.

The control diet had 3,030; 3,119 and 3,200 kcal/g AME and 1.31, 1.20 and 1.12 % dig. Lys in a 3-phase program. Four groups (1 to 7 d, 8 to 14 d, 15 to 21 d and 22 to 28 d) were submitted to a 70% quantitative feed restriction (**70% FR**) of ad libitum daily feed intake. Other 4 groups were fed in a qualitative restriction diets with low energy and protein (**Low EP**) (2,980; 3,070 and 3,149 kcal/kg AME and 1.10, 1.13 and 0.93%) in the same periods.

Broilers Measurements

Birds and feed were weekly weighted (at 7, 14, 21, 28, 35, 42 and 49 d of age) to determine body weight gain (**BWG**), feed conversion ratio (**FCR**) corrected for the weight of dead birds and feed intake (**FI**). At 49 d, 4 birds were randomly selected from each pen and processed for carcass and commercial cuts evaluation. Feed was removed 8 h prior to slaughter, which was done through a jugular vein cut after electrical stunning (44 mA per bird). Bleeding time was 180 s. Carcasses were scalded at 60ºC for 45s, feathers were mechanically plucked, and evisceration was manually performed. Eviscerated carcasses (without feet and neck, but with lungs) were statically chilled in slush ice for 3 h and then hung for 3 min to allow excess water dripping before individual carcass weighting. Carcass yield was expressed as a percentage of the live weight and breast meat yield was expressed as a percentage of the eviscerated carcass weight.

Ultrasound measurements and Breast muscle myopathies analyses

All birds were submitted to ultrasonography every week from 8 to 49 d (Logiq E, GE Healthcare, Little Chalfont, United Kingdom) with a probe (L8-18i-RS) placed on the skin surface parallel to the keel as well as cranial and caudal to the ribcage. The image frequency was 18 MHz with 64% brightness. Mean gray values were calculated from the obtained histogram (black to white scale from 0 to 255, respectively) using Adobe Photoshop® (Adobe Systems Inc, San Jose, CA, USA) to determine the breast muscle echo (Pillen et al., 2009). Breast depth was also taken through the images using Image ProPlus® software (Media Cybernetics Inc, Bathesda, MD, USA) according procedures described by Case et al. (2012).

Scores and the occurrence of wooden breast and white striping were performed on boneless and skinless pectoralis major muscles. Fillets were scored for white striping scores in: 0 – normal breast; 1 – visible lines with less than 1 mm; 2 – thick lines with more than 1 mm according to Kuttappan et al. (2012). In order to evaluate the wooden breast myopathy, breasts were scored in: 0 – normal breast; 1 – mild hardening in the upper; 2 – moderate hardening in the upper and lower part of the fillet; 3 – severe hardening; 4 – severe hardening with hemorrhagic lesions, increased volume, and presence of yellow fluid according to Vieira et al. (2017).

Meat Quality Analysis

Weekly, from 21 to 49 d, one bird from each pen was randomly taken to evaluate myopathies and PM was excised in order to proceed with meat quality analysis. Birds were sacrificed using the same methodology as to evaluate carcass yield shown above.

pH and Color measurements. pH measurements were done briefly using a pH meter with 0.01 precision coupled with a glass electrode in the homogenates of meat. Color was evaluated on the cranial surface of the PM with CR-400 Head chrome meter and expressed according to the Commision Internationale de l'Eclairage (CIE) in: lightness (**L***), redness (**a***), and yellowness (**b***).

Water holding capacity (WHC) and Cooking loss (CL). To measure the WHC, meat cubes weighing 2 g were laid for 5 min between two filter paper circles placed in between two glass plates with a 10 kg load on the top of the plates. Water loss was calculated as the difference between initial and final weights and results were expressed as the percentage of drip loss relative to initial sample weight. For CL, PM samples were weighted, placed into plastic bags and cooked in boiling water (82 to 85ºC) until 72ºC of

internal temperature. Once the samples reached room temperature, they were weighted, and the difference between initial and final weights corresponds to cooking loss.

Instrumental Texture Analysis. Texture of raw and cooked broiler breasts were measured using a Texture Analyzer (Model TA-XT-plus, Texture Technologies Corp, Hamilton, MA) with a 50 kg load cell. For raw samples, four replications (2 cranial and 2 medial) were taken from raw breast fillets placed with the muscle fibers longitudinally to quantify poultry firmness [Shear force (**MORS**) and Shear energy (**MORSE**)]. Three samples per breast used in cooking loss determination were cut in small slices (2x2x1 cm) and placed with the muscle fibers longitudinally oriented to the blades, with a Warner-Bratzler probe to determine cutting force (Work of shear (**WBSF**) and Firmness).

Statistical analysis

Data were analyzed using the GLM procedure of SAS Institute (2009; SAS Inst. Inc., Cary, NC). Significance was accepted at 5 %. Data were submitted to a one-way ANOVA and mean differences were separated using Tukey's HSD test (Tukey, 1991). Feed restriction was used as an independent variable. The obtained scores of WB afterwards were considered as independent variables to evaluate meat quality parameters and ultrasound measurements. Pearson's correlation analysis was conducted using the CORR procedures.

RESULTS

A decrease on BWG was observed ($P < 0.001$) on broilers during either quantitative and qualitative feed restrictions (70% FR and Low EP) periods when compared to those fed ad libitum; however, after restriction periods, the birds previous

197 restricted demonstrated to compensate their BWG and presented no differences on
198 performance from 1 to 49 d (Table 1).

199 No effects of weekly feed restriction periods were observed on the means of
200 carcass, breast yields and myopathies (WB and WS) according to Table 2. Birds
201 qualitative feed restricted from 1 to 7 d (Low EP 1 - 7 d) presented lower values of FCR
202 (1.582 g:g) and FI (5,995 g) when compared to other treatments.

203 The WB occurrence according to the treatments is presented on Figure 1. Normal
204 breasts (score 0) were observed only until 21 d, whereas the WB score 4 - when the fillets
205 present severe hardening with hemorrhagic lesions - was first seen at 28 d. The feed
206 restriction treatment with Low EP from 8-14 d had the lowest frequency of WB at 14 d,
207 when compared to other treatments. When comparing the periods from 22 to 28 d, both
208 quantitative and qualitative feed restrictions (70% FR and Low EP), there was no fillets
209 classified as score 4 of WB; however, after 28 d, all the feed restricted birds have the
210 same occurrence of breast myopathies when compared to ad libitum ones.

211 WB scores were considered as an independent variable to evaluate the ultrasound
212 measurements of echogenicity and depth of the breast fillet and meat quality parameters
213 according to the age of the broilers. As shown on Tables 3 and 4, the values of
214 echogenicity and depth of breast fillets increased according to the age and the severity of
215 WB. Fillets presenting WB score 4 had higher ($P < 0.05$) values of echogenicity at 28 d,
216 42 d and 49 d.

217 Analyzing the means of depth of breast, there is a difference between according
218 to the severity of WB from 21 d, being these values a tool to in vivo diagnose the wooden
219 breast myopathy from this age (Table 4).

220 Correlations between WB scores and depth were 0.58, 0.50, 0.42, and 0.16 at 28,
221 35, 42, and 49 d, respectively ($P < 0.001$), whereas those between WB score and
222 echogenicity were 0.43, 0.11, 0.39 and 0.34 at the same ages, respectively ($P < 0.05$).
223 Values of pH and color measurements of breast fillets are presented according to
224 the WB scores on Table 5. The WB scores had similar values for pH at 21, 28, 35, 42 and
225 49 d ($P > 0.05$); nevertheless, breast fillets scores of 0 and 1 were not observed after 35
226 d. At 49 d, breast fillets classified with score 4 to WB presented higher values ($P < 0.05$)
227 for L*, a* and b* values.
228 According to Table 6, values of cooking loss increased ($P < 0.05$) among the
229 severity of WB at 21, 28, 35, 42 and 49 d, and breast fillets with WB score 4 also presented
230 a decrease on water-holding capacity ($P < 0.001$) at 42 and 49 d, when compared to breast
231 fillets with WB scores 2 and 3. At 49 d, shear force (MORS) and shear energy (MORSE)
232 increased ($P < 0.05$) in raw breast fillets that were classified as WB score 4 when
233 compared with scores 2 and 3 (Table 7). Work of shear (WBSF) and firmness increased
234 on fillets classified with WB score 2 when compared to scores 0 and 1 at 21 d, and no
235 differences between the WB scores were observed at 28, 35, 42 and 49 d.
236
237 **DISCUSSION**
238
239 Once researches had already confirmed the correlation between growth rate and
240 the occurrence/severity of breast myopathies, the treatments of this present study aimed
241 to reduce the fast growing in a tentative to control those myopathies. As expected, our
242 results demonstrate a decrease on BWG ($P < 0.001$) while broilers were submitted to
243 both quantitative and qualitative feed restrictions (70% FR or Low EP) when compared
244 to those fed ad libitum; however, after restriction periods, birds demonstrated to

245 compensate their BWG. These findings are in agreement with Kuttappan et al. (2012a)
246 which found that the fillet degree of white striping increased from NORM to SEV as its
247 weight and yield increased ($P < 0.05$).

248 Radaelli et al. (2017), studied the effect of 80 % of feed restriction in broilers from
249 13 to 21 d and its influence on the occurrence of WB and WS and described significant
250 differences according to feeding system, age, and their interactions. Those authors found
251 a successful reduction on histological findings that lead to a decrease on the severity of
252 myopathies; however, the subsequent re-feeding period of restricted birds showed a
253 compensatory growth, as in agreement with the findings showed in this study.

254 The compensatory growth can also be seen as a result from an increase in feed
255 intake (Van der Klein et al., 2017). When 80% of restriction was applied from 13 to 21
256 d, Trocino and colleagues (2015) described that, during the re-alimentation period,
257 previous restricted birds showed a compensatory growth that allowed these birds to
258 reduce differences in final live weight and improved feed conversion rate. Bokkers et al.
259 (2004) showed that feed restriction in broilers increased the motivation to work for feed,
260 so a faster feed intake would have been expected. In the present study, no differences on
261 FI were observed after the restricted period, although there were no effects on the means
262 of carcass, breast yields and also, at the end, feed restricted birds had the same occurrence
263 of breast myopathies (WB and WS) when compared to ad libitum ones.

264 According to Griffin et al. (2018), the first documented case of score 4 of WB,
265 where the pectoralis major muscle is characterized as pale with severe hardening
266 accompanied to hemorrhagic lesions, were observed beginning on 30 d of age, in
267 accordance with the observations from this study, whereas the WB score 4 was first
268 described at 28 d. In a study conducted by Sihvo et al. (2017), at 24, 35 and 38 d over 60

269 % of the affected cases of WB were classified as WB1hard, with the breast fillets
270 markedly hardened consistency.

271 Breast fillets affected with WB are characterized by variable degrees of hardness
272 in the PM showing bulging and pale expansive areas, often accompanied with WS (Sihvo
273 et al., 2014; Cruz et al., 2017). Histologically, both WS and WB lesioned areas show
274 myodegeneration along with variable amount of interstitial connective tissue
275 accumulation (Kuttappan et al., 2011, 2013a; Sihvo et al., 2014; Tasoniero et al., 2016).
276 More recently, Velleman et al. (2018) reported that the primary feature of WB is the
277 fibrosis of muscle with the replacement of muscle fibers with extracellular matrix
278 proteins, such as collagen.

279 Pillen et al. (2009), when evaluating the muscular dystrophy in dogs, reported the
280 correlation between fibrous tissue and echo intensity using ultrasound measurements and
281 a significant correlation ($r = 0.87$) was found between the amount of interstitial fat and
282 muscle echo intensity. In order to predict in vivo the occurrence of WB, once this
283 myopathy is characterized as fibrosis and thicker fillets (Kuttappan et al., 2016), this study
284 intend to evaluate ultrasound measurements of echogenicity and depth of the breast fillet.
285 Our results demonstrate that fillets presenting WB score 4 had higher ($P < 0.05$) values
286 of echogenicity at 28 d, 42 d and 49 d, as well as the depth of breast at 21 d, confirming
287 that US images can be adequately utilized to predict the WB of broilers in vivo.

288 The increase on muscle echogenicity as seen in the present study is caused by the
289 disruption of normal muscle architecture by infiltration of fat and fibrous tissue
290 (Heckmatt et al., 1982; Reimers and Kellner, 1996; Walker et al., 2004; Pillen et al.,
291 2009). According to Serrano and Muñoz-Canóves (2010), fibrosis is the accumulation of
292 fibrous connective tissue due to muscle damage, necrosis, and the replacement of muscle

293 fibers with connective tissue. The muscle damage in WB is probably caused by the
294 increased size of PM muscle in the faster growing broiler line (Velleman et al., 2018).
295 Also, as a fast, easy and non-destructive technique, the application of real-time
296 ultrasound had already been successfully used in broilers to estimate in vivo carcass and
297 breast muscle weight and yields on broilers and turkeys (Silva et al., 2006, Oviedo-
298 Rondón, 2007, Case et al., 2012). This technique seems to be a valuable tool for assessing
299 breast meat yield development in broilers with high accuracy. Estimates of breast muscle
300 depth are strongly correlated to breast muscle yield and the genetic correlation between
301 breast muscle weight, predicted, using ultrasound muscle thickness, and carcass values is
302 high, ranging from 0.64 to 0.69 (Scheuermann et al., 2003; Case et al., 2012).
303 The extensive fibrosis observed in fillets affected with WB in the endomysial and
304 perimysial spaces will likely alter muscle function and, ultimately, meat quality, as
305 fibrillar collagen is a determinant of tissue flexibility and tenderness of meat (Velleman
306 et al., 2018). These undesirable appearance and hardness are increasing the occurrence of
307 lower quality fresh meat for the retail market and to some extent reduce the technological
308 properties of raw meat (Maiorano, 2017).
309 Although many researches had already demonstrated that wooden breast fillets
310 had higher pH (Tasoniero et al., 2016; Cai et al., 2017), in the present study the WB scores
311 had similar values for pH at 21, 28, 35, 42 and 49 d ($P > 0.05$); nevertheless, breast fillets
312 scores as 0 and 1 for this myopathy were not observed after 35 d. Trocino et al. (2015)
313 also did not record difference on WB-affected. At 49 d, breast fillets classified as score 4
314 to WB presented higher values ($P < 0.05$) for L*, a* and b* values, in an accordance with
315 other previous research (Dalle Zotte et al., 2014; Cai et al., 2017). This higher color values
316 may be due to the fact that, in severe cases of WB, a clear viscous fluid cover and/or
317 petechial multifocal lesions on the fillet surface is observed (Kuttappan et al., 2016).

318 According to our results, values of cooking loss increased among the age at 21,
319 28, 35, 42 and 49 and with the severity of WB, which agrees with other findings, once
320 the structure of the muscle tissue affected with this condition leads to the increased
321 cooking loss (Tijare et al., 2016; Cai et al., 2017). Breast fillets with WB score 4 also
322 presented a decrease on water-holding capacity at 42 and 49 d, when compared to breast
323 fillets with WB scores 2 and 3. Mundalal et al. (2014) reported that the presence of WS
324 and WB impair the quality of raw meat by reducing the water-holding abilities.
325 The instrumental texture analysis of shear force (MORS) and shear energy
326 (MORSE) increased at 49 d in raw breast fillets that were classified as WB score 4 when
327 compared with scores 2 and 3, in agreement with Chaterjee et al. (2016), which related a
328 strong positive relationship between MORS shear measurements and sensory descriptive
329 texture attributes of initial hardness and cohesiveness and MORS shear and consumer
330 liking attributes such as texture and tenderness acceptability (Xiong et al., 2016).
331 However, differences on WBSF and firmness were observed only at 21 d, where the fillets
332 classified with WB score 2 when compared to scores 0 and 1.
333 Under the experimental conditions used in this study, our results demonstrate that
334 the US images of depth and echo intensity can be adequately utilized to predict the WB
335 of broilers in vivo from 21 d of age. The growth performance was negatively affected
336 when broilers were submitted to both quantitative (70 % FR) or qualitative (Low EP) feed
337 restrictions; nevertheless, broilers had a compensatory performance after the restrictions
338 period. Broilers feed restricted from 22 to 28 d presented no fillets scored as 4 of WB.
339 The increased severity of WB affects the meat quality parameters resulting in breast fillets
340 with higher color, cooking loss, shear energy and shear force.
341
342

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Table 1. Body weight gain of broilers submitted to 70 % quantitative feed restriction or qualitative restriction with low energy and protein diets, g.

Treatments	1 to 7 d	8 to 14 d	15 to 21 d	22 to 28 d	29 to 35 d	36 to 42 d	43 to 49 d
Ad libitum	125[a]	332[a]	528[b]	759[b]	763[b]	831	684
70% FR[1] 1-7 d	73[b]	316[a]	506[bc]	727[b]	758[b]	813	688
70% FR 7-14 d	126[a]	180[c]	597[a]	732[b]	793[b]	834	698
70% FR 14-21 d	121[a]	323[a]	238[e]	872[a]	818[ab]	834	699
70% FR 21-28 d	122[a]	320[a]	503[bc]	385[d]	915[a]	845	741
Low EP[2] 1-7 d	74[b]	240[b]	490[bc]	702[b]	762[b]	816	731
Low EP 7-14 d	124[a]	135[d]	467[c]	664[b]	787[b]	835	745
Low EP 14-21 d	121[a]	322[a]	408[d]	690[b]	832[ab]	842	754
Low EP 21-28 d	124[a]	320[a]	506[bc]	529[c]	861[ab]	840	747
Mean	112	276	471	145	810	832	721
SEM	2.51	8.48	11.74	17.14	9.61	5.02	9.78
P-value	0.001	0.001	0.001	0.001	0.001	0.856	0.505

[a-d]Means with different superscript letter differ ($P < 0.05$) based on Tukey's honestly significant difference test.

[1]70% FR = 70 % of quantitative feed restriction

[2] Low EP = qualitative feed restriction with low energy and protein

449 **Table 2.** Effects of feed restriction on performance, carcass and breast yields from 1 to 49 d and
450 means of wooden breast and white striping scores.

Item	BW gain, g	FCR, g:g	FI, g	Carcass[3]	Breast fillets[4]	Tenders	WB score[5]	WS score[5]
Ad libitum	3,963	1.656[a]	6,565[a]	82.6	31.3	5.26	1.82	0.98
70% FR[1] 1-7 d	3,869	1.633[ab]	6,337[abc]	82.6	30.4	5.33	1.57	0.75
70% FR 7-14 d	3,938	1.645[a]	6,477[ab]	82.5	30.9	5.35	1.64	0.91
70% FR 14-21 d	3,842	1.642[ab]	6,306[abc]	82.7	30.6	5.33	1.64	0.91
70% FR 21-28 d	3,916	1.614[ab]	6,307[abc]	82.5	30.4	5.32	1.63	0.79
Low EP[2] 1-7 d	3,797	1.582[b]	5,995[c]	82.9	30.4	5.06	1.68	0.84
Low EP 7-14 d	3,750	1.633[ab]	6,125[bc]	83.0	30.5	5.27	1.38	0.75
Low EP 14-21 d	3,948	1.620[ab]	6,398[abc]	82.6	30.2	5.28	1.84	0.85
Low EP 21-28 d	3,822	1.650[a]	6,304[abc]	83.5	30.5	5.35	1.73	0.95
Mean	3,871	1.631	6,312	82.8	30.6	5.28	1.65	0.86
SEM	24.59	0.004	35.59	0.130	0.540	0.028	0.053	0.037
P-value	0.425	0.001	0.004	0.767	0.335	0.749	0.623	0.775

451 [a,d]Means with different superscript letter differ ($P < 0.05$) based on Tukey's honestly significant difference
452 test.
453 [11]70% FR = 70 % of quantitative feed restriction
454 [2] Low EP = qualitative feed restriction with low energy and protein
455 [3] Eviscerated carcasses as a percentage of body weight at 49 d.
456 [4] Skinless boneless pectoralis major as proportion of carcass.
457 [5] WS and WB scores from 7 to 49 d were square root transformed to present normal distribution of residual;
458 however, presented means were squared to facilitate the interpretation (n = 504).

459 **Table 3.** Means of echogenicity of breast according to the age and severity of wooden breast[1].

WB scores	Echo 21 d	Echo 28 d	Echo 35 d	Echo 42 d	Echo 49 d
0	82.6	.	.	.	.
1	84.6	91.3[b]	.	.	.
2	84.5	92.3[b]	102.0	103.1[b]	104.2[b]
3	.	96.9[ab]	103.5	104.8[b]	105.3[ab]
4	.	101.5[a]	.	110.6[a]	109.6[a]
Mean	84.2	94.5	102.6	104.5	105.7
SEM	0.866	0.970	0.975	0.722	0.780
P-value	0.6495	0.0039	0.424	0.0035	0.0395

460 [a-b] Means with different superscript letter differ ($P < 0.05$) based on Tukey's honestly significant
461 difference test.
462 [1] The absence of some echogenicity means occurred because the score was not observed

463 Table 4. Means of depth of breast according to the age and severity of wooden breast[1].

WB scores	Depth 21 d	Depth 28 d	Depth 35 d	Depth 42 d	Depth 49 d
0	17.1[c]	.	.	.	.
1	18.9[b]	17.9[c]	.	.	.
2	20.2[a]	19.8[b]	23.2[b]	28.3[b]	31.2
3	.	21.5[ab]	25.3[a]	30.0[ab]	31.9
4	.	21.9[a]	.	31.5[a]	32.2
Mean	19.0	20.3	24.2	29.2	31.7
SEM	0.240	0.234	0.274	0.356	0.355
P-value	0.0001	0.0001	0.0001	0.0031	0.497

464 [a-c]Means with different superscript letter differ ($P < 0.05$) based on Tukey's honestly significant
465 difference test.
466 [1]The absence of some depth means occurred because the score was not observed.

Table 5. Values of pH and color (L*- lightness, a* - redness, b* -yellowness) according to the age and severity of wooden breast[1].

Age	Item	WB Score					Mean	SEM	*P*-value
		0	1	2	3	4			
21 d	pH	5.8	5.9	5.9	.	.	5.8	0.012	0.4402
	L*	58.6	57.8	57.2	.	.	57.8	0.339	0.3279
	a*	12.9	13.8	13.8	.	.	13.6	0.161	0.0555
	b*	12.3	13.2	13.0	.	.	12.9	0.243	0.3466
28 d	pH	.	5.9	5.9	6.0	6.1	5.9	0.017	0.3959
	L*	.	52.6	54.0	54.9	52.5	54.0	0.341	0.0389
	a*	.	13.5^{b}	15.0^{ab}	15.1^{ab}	16.2^{a}	15.0	0.220	0.0001
	b*	.	11.7	10.8	11.0	10.9	11.2	0.233	0.0431
35 d	pH	.	.	6.0	6.0	.	6.0	0.017	0.9442
	L*	.	.	55.9^{b}	57.6^{a}	.	56.6	0.339	0.0135
	a*	.	.	13.6	13.0	.	13.4	0.217	0.1799
	b*	.	.	12.3	12.8	.	12.5	0.275	0.3377
42 d	pH	.	.	6.2	6.2	6.3	6.2	0.021	0.9631
	L*	.	.	52.8^{b}	54.1^{b}	56.4^{a}	53.7	0.316	0.0001
	a*	.	.	14.3^{b}	14.6^{b}	16.6^{a}	14.7	0.191	0.0013
	b*	.	.	11.0	11.3	12.2	11.4	0.216	0.0524
49 d	pH	.	.	5.9	6.0	5.9	5.9	0.020	0.2484
	L*	.	.	54.1^{b}	55.1^{b}	58.1^{a}	55.0	0.427	0.0097
	a*	.	.	14.1^{b}	14.2^{b}	16.3^{a}	14.5	0.193	0.0001
	b*	.	.	11.4^{b}	12.4^{ab}	13.5^{a}	12.1	0.246	0.0116

[a-b] Means with different superscript letter differ ($P < 0.05$) based on Tukey's honestly significant difference test.

L = lightness, a* = redness, and b* = yellowness.

[1]The absence of some means occurred because the score was not observed.

473 **Table 6.** Means of meat quality according to the age and severity of wooden breast[1].

Age	Item	WB score					Mean	SEM	*P*-value
		0	1	2	3	4			
21 d	CL, %[2]	1.1[b]	1.3[b]	1.7[a]	.	.	1.4	0.052	0.0001
	WHC, %[3]	92.4	91.8	93.6	.	.	92.6	0.401	0.1488
28 d	CL, %[1]	.	1.0[b]	1.8[a]	2.0[a]	2.3[a]	1.9	0.072	0.0002
	WHC, %[2]	.	93.1	93.2	92.4	94.3	93.2	0.216	0.0638
35 d	CL, %[1]	.	.	3.8	4.1	.	3.9	0.131	0.2212
	WHC, %[2]	.	.	90.9	89.9	.	90.4	0.519	0.3020
42 d	CL, %[1]	.	.	5.8[b]	6.3[b]	7.6[a]	6.2	0.130	0.0002
	WHC, %[2]	.	.	92.8[a]	89.4[b]	84.0[c]	90.8	0.552	0.0001
49 d	CL, %[1]	.	.	5.4[b]	6.2[ab]	7.7[a]	6.0	0.293	0.0508
	WHC, %[2]	.	.	93.4[a]	93.8[a]	87.8[b]	92.8	0.430	0.0001

474 [a-c] Means with different superscript letter differ ($P < 0.05$) based on Tukey's honestly significant
475 difference test.
476 [1] The absence of some means occurred because the score was not observed.
477 [2] CL = cooking loss.
478 [3] WHC = water-holding capacity.

Table 7. Results of MORS (N) and MORSE (N.mm) cranial and medial on pectoralis major muscle according to the severity of wooden breast[1].

Age	Item	WB Score					Mean	SEM	*P*-value
		0	1	2	3	4			
21 d	MORS (N) cranial	8.6	8.3	8.2	.	.	8.3	0.139	0.5045
	MORSE (N.mm) cranial	9.8	9.5	9.9	.	.	9.7	0.173	0.6438
	MORS (N) medial	45.7	45.5	43.2	.	.	44.6	0.835	0.3941
	MORSE (N.mm) medial	47.1	47.5	48.0	.	.	47.6	0.677	0.8935
28 d	MORS (N) cranial	.	11.7	11.4	11.2	10.8	11.3	0.150	0.5342
	MORSE (N.mm) cranial	.	11.7	12.0	11.9	11.4	12.0	0.191	0.4176
	MORS (N) medial	.	98.5^{a}	89.2^{ab}	85.5^{b}	77.5^{b}	87.5	1.321	0.0030
	MORSE (N.mm) medial	.	90.5	89.4	87.9	85.7	88.8	0.793	0.0166
35 d	MORS (N) cranial	.	.	11.3	10.7	.	11.1	0.172	0.1402
	MORSE (N.mm) cranial	.	.	12.6	12.2	.	12.4	0.171	0.3241
	MORS (N) medial	.	.	111.6	112.0	.	111.8	1.587	0.8907
	MORSE (N.mm) medial	.	.	115.6	116.3	.	115.9	1.366	0.8216
42 d	MORS (N) cranial	.	.	12.9	12.3	13.6	12.8	0.213	0.1530
	MORSE (N.mm) cranial	.	.	15.5	14.9	16.5	15.4	0.249	0.1424
	MORS (N) medial	.	.	122.2	119.9	118.9	121.2	1.146	0.1730
	MORSE (N.mm) medial	.	.	126.2^{b}	129.4^{ab}	136.4^{a}	129.4	1.576	0.0139
49 d	MORS (N) cranial	.	.	14.8	14.9	15.1	14.9	0.346	0.9286
	MORSE (N.mm) cranial	.	.	16.7^{b}	17.6^{b}	20.5^{a}	17.7	0.339	0.0003
	MORS (N) medial	.	.	131.8^{b}	135.9^{b}	156.2^{a}	137.6	3.110	0.0138
	MORSE (N.mm) medial	.	.	142.8^{b}	143.1^{b}	161.8^{a}	146.8	2.884	0.0256

[a-b] Means with different superscript letter differ ($P < 0.05$) based on Tukey's honestly significant difference test.

[1] The absence of some means occurred because the score was not observe.

484 **Table 8.** Work of shear and firmness on pectoralis major muscle according to the age and severity of wooden breast[1].

Age	Item	WB score					Mean	SEM	*P*-value
		0	1	2	3	4			
21 d	Work of Shear (N/mm.sec)	7.7[b]	9.7[ab]	11.6[a]	.	.	10.0	0.383	0.0002
	Firmness (N/mm)	1.0[b]	1.3[b]	1.7[a]	.	.	1.4	0.0	0.0001
28 d	Work of Shear (N/mm.sec)	.	12.0	13.5	12.1	12.1			0.4808
	Firmness (N/mm)	.	2.0	2.0	1.6	1.4			0.0518
35 d	Work of Shear (N/mm.sec)	.	.	11.1	10.3	.	10.8	0.4466	0.4155
	Firmness (N/mm)	.	.	1.5	1.3	.	1.5	0.0729	0.1998
42 d	Work of Shear (N/mm.sec)	.	.	13.6	11.9	10.6	12.7	0.4832	0.0965
	Firmness (N/mm)	.	.	1.8	1.7	1.5	1.7	0.0677	0.2688
49 d	Work of Shear (N/mm.sec)	.	.	12.3	11.7	10.2	11.8	0.3319	0.1100
	Firmness (N/mm)	.	.	1.3	1.2	1.2	1.2	0.0574	0.3946

485 [a-b]Means with different superscript letter differ ($P < 0.05$) based on Tukey's honestly significant difference test.
486 [1]The absence of some means occurred because the score was not observed

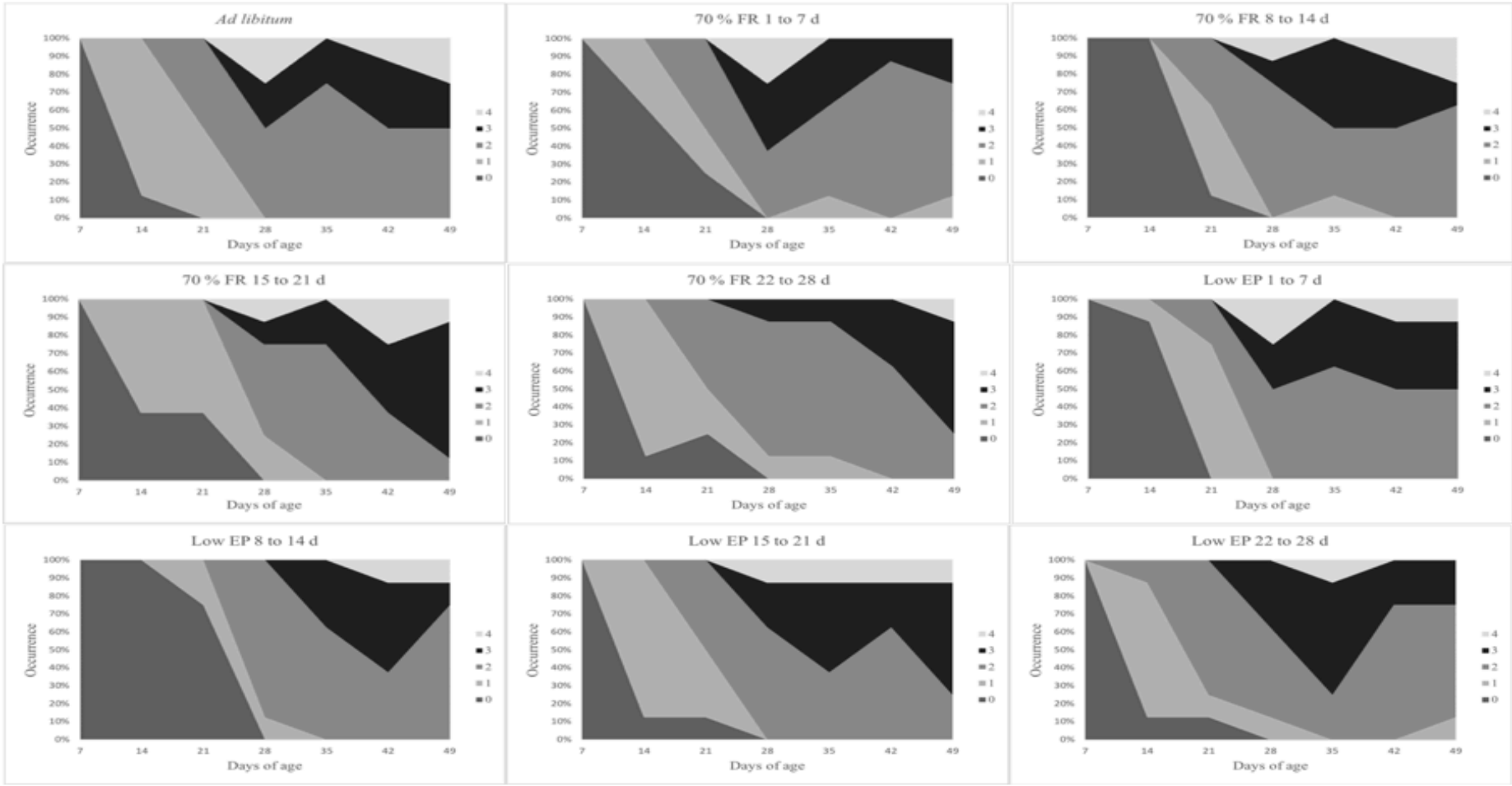

Figure 1. Wooden breast occurrence in breast fillets of broilers from 7 to 49 d. Wooden breast scores were: normal breast (score 0), mild hardening in the upper (score 1), moderate hardening in the upper and/or lower part of the fillet (score 2), severe hardening (score 3), and severe hardening with hemorrhagic lesions, increased volume, and presence of yellow fluid (score 4).

6. ARTIGO CIENTÍFICO 2

IMMUNOLOGY, HEALTH, AND DISEASE

WOODEN BREAST AND MUSCLE CHARACTERIZATION

Muscle cellular characterization of wooden breast myopathy on broilers at 21 and 49 d of age

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ABSTRACT

This study was conducted to evaluate the pectoralis major on muscle cellular characterization according to the severity of wooden breast (WB) on male Cobb 500 broilers slaughtered at 21 and 49 d of age. For each day, eighteen birds were randomly selected in a processing plant and blood samples was performed to analyze the serum enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine kinase (CK) and lactate dehydrogenase (LDH) and their butterfly fillets were collected, weighted and scored for WB. The scale used to score ranged from 0 (normal) to 4 (severe hardening with hemorrhagic lesions, increased volume, and presence of yellow fluid). A tissue sample from each pectoralis major was fixed in formalin, processed for histology and histomorphometric evaluated for muscle fiber diameter (minimum and mean), density (fibers/cm^2), thickness of endomysium (EMT) and perimysium (PMT), microvessels density/mm^2 (MD) and the partial muscle volume (Vv, %). Major histological changes on samples scored as 4 of WB consisted of variable fiber size (degeneration and regeneration), including giant ones and proliferative connective tissue (fibrosis) in the overall section. Morphometric evaluations showed a decrease on muscle volume, fiber and microvessels densities ($P < 0.001$) and an increase on endomysium and perimysium thickness ($P < 0.001$). Greater serum enzymes levels ($P < 0.001$) were also found according to the age and severity of WB. The results from this study showed that although WB fillets had greater diameters, these fibers are being degenerated and replaced by connective tissue, leading to a reduction on the microvessels density and biochemical changes, that can interfere on the muscle fiber regeneration.

Key words: broilers, microvessels supply, fibrosis, histomorphometric analysis, serum enzymes.

INTRODUCTION

Selection criteria for fast-growing bird have created conditions favoring disease susceptibility and modifying skeletal-muscle cell structure, metabolism and meat quality; however, with higher growth rate and muscle size, the incidence of pectoral myopathies has increased (Barbut et al., 2008; Kuttappan et al., 2013).

Breast fillets affected with wooden breast (**WB)** are characterized by variable degrees of hardness in the pectoralis major showing bulging and pale expansive areas, often accompanied with white striping (Sihvo et al., 2014; Cruz et al., 2017). Histologically, WB lesioned areas show myodegeneration along with variable amount of interstitial connective tissue accumulation (Kuttappan et al., 2011, 2013; Sihvo et al., 2014; Tasoniero et al., 2016).

According to Sósnicki and Wilson (1991), the width of the muscle fiber exceeds that of the connective tissue, leading to a loss of muscle integrity or focal myopathy in turkeys selected for rapid growth and an increase in the number of giant fibers in pectoralis muscle from fast-growing birds (Dransfield and Sósnicki, 1999). More recently, Velleman et al. (2018) reported that the primary feature of WB is the fibrosis of muscle with the replacement of muscle fibers with extracellular matrix proteins, such as collagen.

In a study conducted by Sihvo et al. (2017), the WB fibrotic lesions were first observed in broilers at 18 d of age and, as the bird ages, the incidence of WB increased as did the severity of the fibrosis, directly associated with increased accumulation of inflammatory cells. Also, without adequate circulation satellite cell function and muscle regeneration will be hindered, thereby affecting posthatch muscle growth (Luque et al., 1995; Christon et al., 2007; Rhoades et al., 2009; Clark and Velleman, 2016).

According to Clark and Velleman (2016), spatial differences in hyperplastic and hypertrophic growth are required to produce the pectoralis major shape, which may cause variations in muscle fiber size, fiber bundle organization, perimysial and endomysial connective tissue spacing, and circulatory supply availability to the muscle.

So, this study was intended to evaluate the pectoralis major muscle cellular characterization according to the severity of WB on male Cobb 500 broilers slaughtered at 21 and 49 d of age using morphometric and biochemical analysis.

MATERIALS AND METHODS

Birds and Sample Collection

Male Cobb 500 commercial broiler chickens aged 21 and 49 d were obtained during two slaughtering in a processing plant. Average body weights were 632.89 ± 0.2 kg and 3.98 ± 0.2 kg. Of 216 carcasses, thirty-six (16.67 %) boneless, skinless pectoralis major muscles were selected, 18 per slaughter day, in the deboning area for further analysis.

In order to evaluate the wooden breast myopathy, breasts were graded into 4 scores as follows: 0 – normal breast; 1 – mild hardening in the upper; 2 – moderate hardening in the upper and lower part of the fillet; 3 – severe hardening; and 4 – severe hardening with hemorrhagic lesions, increased volume, and presence of yellow fluid according to Vieira et al (2017).

Carcass and fillets (pectoralis major and minor) were also weighted and the pectoralis major dimensions — length and cranial width — were measured using caliper rule (Mehaffey et al., 2006; Kuttappan et al., 2017).

Muscle Structure Measurements

After macroscopic classification, samples of pectoralis major muscle were obtained from the anteroventral position, cut into transversal section, fixed in buffered 10% formalin, dehydrated with ethanol in increasing concentration (30-100%), diaphanized in xylol, and included in paraffin. From each sample, three semi-serial cross-sections (3 μm) were cut (Microtome Leica DM500; Leica Biosystems Nussloch GmbH, Germany) and stained with hematoxylin-eosin. Microscopy were performed using a light microscope (Leica DM500; Leica Biosystems Nussloch GmbH, Germany) with an image capture system (ICC50HD) and LAS EZ software for image capture.

A total of 360 images were captured and submitted to morphometric analysis of muscle fiber diameter (minimum and mean), density (fibers/cm^2), thickness of endomysium (EMT) and perimysium (PMT) and the microvessels density/mm^2 (MD) defined as the total number of microvessels divided by the total area of the section analyzed (Hoving-Bolink, 2000; Macrae et al., 2006; An et al., 2010; Radaelli et al., 2017) that were estimated using the software Image ProPlus®. The partial volume of muscle tissue (Vv, %) was estimated by point-counting stereology using the M42 testing system (Weibel et al., 1979; Wilson et al., 1990).

Serum Analysis

Blood collection for serology (between 1.5 and 3 mL from each bird) was performed upon bleeding after electronarcosis. Samples were stored in disposable capped vacuum tubes (Vacutainer) without anticoagulant. Samples were kept at room temperature for 30 to 60 min then centrifuged (3,000 × g for 10 min at 4ºC), and the serum was stored at

−20◦ C until analyzed. The obtained serum was used to estimate plasma activities of creatine kinase (**CK**) (Randox, Crumlin, County Antrim, UK), alanine aminotransferase (**ALT**) and lactate dehydrogenase (**LDH**) (Sigma Aldrich, Poole, Dorset, UK), as well aspartate aminotransaminase (**AST**) (Thermo Electron Corporation, Runcorn, Warrington, UK).

***Statistical* Analysis**

Data were analyzed using the GLM procedure of SAS Institute (2009; SAS Inst. Inc., Cary, NC) considering the WB scores and age at slaughter (21 and 49 d). Significance was accepted at $P < 0.05$. Data were submitted to a one-way ANOVA and mean differences were separated using Tukey's HSD test (Tukey, 1991).

RESULTS

From a total of thirty-six pectoralis major muscles evaluated in the present study, 83.33 % of the fillets were classified as WB-affected (13.89, 36.11, 13.89 and 19.44 % as scores 1 to 4, respectively). At 21 d, no fillets were scored as 3 and 4 for WB; however, and at the same time, at 49 d, no fillets were scored as normal and score 1 for WB.

As expected, carcass ($P < 0.05$) and fillets (pectoralis major and minor; $P < 0.001$) weights were increased according to the severity of WB and also higher values of fillet length and width (107.83 to 202.14 mm; 48.67 to 67.86 mm from score 0 to 4, respectively).

Histological observations on the pectoralis major muscle showed the WB evolution according to the severity of this myopathy. At 21 d, a normal fillet (score 0) showed an

150 organized skeletal muscle covered by fibrous connective tissue, although the presence of
151 some hyper-eosinophilic fibers (Figure 1A). At 49 d, fillets classified as score 4 of WB,
152 macroscopically characterized by severe hardening with hemorrhagic lesions, increased
153 volume, and presence of yellow fluid, histologically shows different sizes of fibers,
154 including giant ones. Several fibers with degeneration and infiltrated by inflammatory
155 cells and proliferative connective tissue (fibrosis) in the overall section (Figure 2B).
156 According to results obtained from the morphometric analysis, there were
157 significant differences between the age and severity of WB (Table 1). The minimum fiber
158 diameter of the myofiber increased according to the age and severity of WB (11.35 to
159 20.35 µm scores 0 and 4; $P < 0.001$). Although there were no significant differences on
160 the mean fiber diameter at 49 d, we can observe an increase between each WB score
161 severity (43.29 µm on score 2 to 58.65 µm on score 4). That happened after the significant
162 decrease seen among the severity of this condition on broilers at 21 d of age (29.36 µm
163 to 24.58 µm, scores 0 and 2, respectively; $P < 0.001$).
164 Despite the fibers diameters enlargement, there was a decrease ($P < 0.001$) on the
165 density of muscle fibers (fibers/cm^2) in fillets WB-affected with the score 4 when
166 compared to normal ones (1,820.42 and 630.27).
167 These differences on the diameter and the density of muscle fibers are explained by
168 the increase in the thickness of endomysium (6.01 and 12.57 µm ($P < 0.001$)) and
169 perimysium (from 54.56 to 95.62 µm ($P < 0.001$)) between the normal fillets (score 0)
170 and WB hardly affected (WB score 4) and the decrease on the muscle partial volume (Vv)
171 to 51.58 when compared to 64.36 % on normal fillets ($P < 0.001$)) (Figure 2A).
172 As seen of Figure 2B, the microvessels density was affected by the age and severity
173 of WB, with higher values (155.66 mm^2) on normal fillets when compared to severe ones
174 (119.05 mm^2) ($P < 0.001$).

Serum enzymes levels are estimated to assess tissue damages, and greater levels of AST, CK and LDH were found according to the severity of WB in broilers slaughtered at 21 and 49 d (Table 2). When comparing the CK levels, a specific enzyme for skeletal muscle damage, there was an increase ($P < 0.001$) from the normal muscle (4,378 U/L) to the score 4 of WB-affected (56,229 U/L).

DISCUSSION

The broiler selection focused on the improvement of growth rate and, specially, breast yield leading to pectoralis major muscle wider, thicker and larger. The results from the present study showed that the fillets scored as 4 for WB had higher fillets diameters (weight, length and width ($P < 0.001$)). Also, muscle hypertrophy is an essential factor for an increase in muscle volume; however, these improvements are associated with the increase on breast myopathies (Guernec et al., 2003; Scheuermann et al., 2004; Petracci et al., 2013; Kuttappan et al., 2017).

As previously described, fillets WB-affected showed correlations between the macroscopic and the muscle structure measurements, once the affected areas had variations in shape and diameter of the fibers (Sihvo et al., 2014; Radaelli et al., 2017; Sihvo et al., 2017; Soglia et al., 2017). According to our results, the diameter of the myofiber increased according to the severity of WB, that is possibly due to the presence of the abnormal giant fibers. This increase on muscles fiber size are typically present in the pectoralis major of fast-growing broilers, however, normal muscles had no differences on the density of fibers as presented in this study.

Previous studies had already demonstrated the close relationship between muscle fiber characteristics (including muscle fiber density and diameter) and tenderness of meat,

that being: thinner muscle, more density, better tenderness (Sifre et al., 2005; Zhao et al., 2011); these variably sized muscle fibers and the extensive fibrosis observed on WB muscle are altering the muscle flexibility and leading to the macroscopically hardness characterized on this condition.

In agreement with our study, the WB myopathy has been associated with an increased muscle hypertrophy of fast-growing chickens, which brings about reduced microvessels density adjacent to the myofiber, this affecting regeneration, degeneration and necrosis of skeletal muscles (Velleman, 2015; Radaelli et al., 2017).

The replacement of muscle fibers with connective tissue, fibrosis, is induced by the necrosis process with chronic muscle fiber damage and corresponding inflammation. Macrophages are recruited to sites of muscle damage and phagocytose necrotic fibers. In healthy muscle, the necrotic fibers are removed, and the muscle fibers are regenerated. Fibrosis results from repeated cycles of necrosis and regeneration with the excessive accumulation of fibrillar collagen and could directly interfere with the satellite-cell mediated muscle fiber regeneration (Velleman et al., 2018).

An increased on the thickness of endomysium and perimysium between the normal fillets and the WB-affected are in agreement with Soglia et al (2017) that revealed an accumulation of endomysial and perimysial fibrotic tissue and a subsequent increase in the space between muscle fibers. Also, these authors reported that the main causes and/or consequences associated with the increase of interstitial connective tissue (resulting in fibrosis) might be attributed to a reduced microcirculation and subsequent impaired muscle fiber metabolism and oxygen supply leading to ischemia (Sósnicki and Wilson, 1991; Kuttappan et al., 2013; Petracci et al., 2015; Trocino et al., 2015; Soglia et al., 2017) and hypoxia (Hoving-Bolink et al., 2000; Joiner et al., 2014).

Our results demonstrate a decrease on microvessels density according to the severity of WB and this potentially insufficient size or density of the microvessels network to provide sufficient oxygen for the muscle has been under discussion once the selection for higher muscle fibers diameters and growth rate had increased (Soglia et al., 2017). Also, the loss of vasculature supply in fast-growing broilers leads to the accumulation of lactic acid, leading to a decrease on muscle pH and further damage the muscle (Velleman et al., 2018).

The extensive fibrosis observed in WB muscle in the endomysial and perimysial spaces will likely alter muscle function, and this damage occurring in the muscle tissue could be reflected on the serum biochemical profiles (Hoffman and Solter, 2008; Kuttappan et al., 2013; Velleman et al., 2018). In accordance, our results demonstrate that the occurrence and severity of WB increased the serum enzymes concentrations in broilers slaughtered at 21 and 49 d.

The results from this study shows the morphological changes and evolution of the WB myopathy according to the age and severity of this condition, with the degeneration of muscle fibers and proliferation of connective tissue, leading to a reduction on the microvessels density and biochemical changes, that could interfere on the muscle fiber regeneration.

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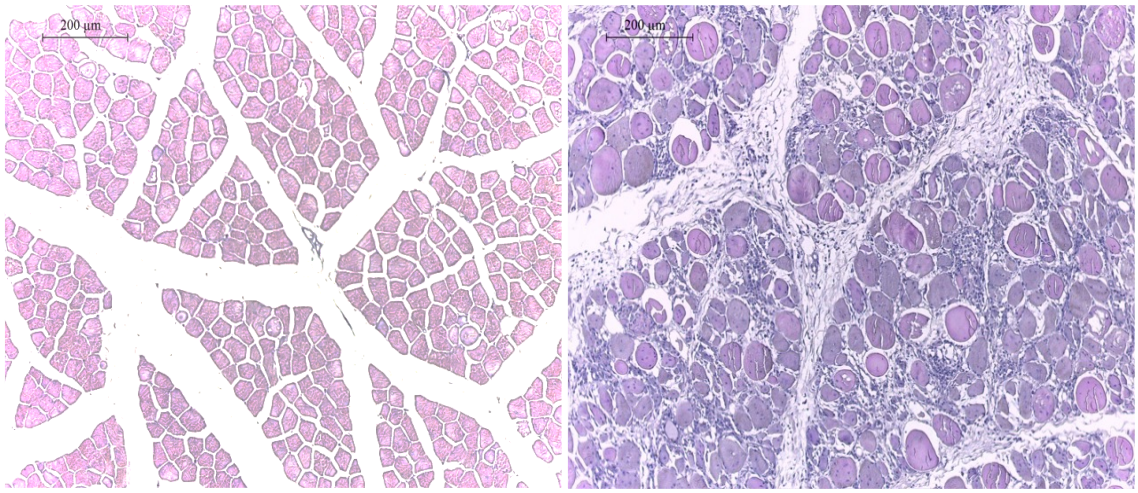

Figure 1. Histological representative micrographs samples taken from pectoralis major muscle in broilers at 21 d and 49 d. A) At 21 d, a normal (unaffected) muscle with the presence of some hyper-eosinophilic fibers. B) At 49 d, a score 4 of WB section showing a proliferative collagen infiltration with variable fiber size (degeneration and regeneration). HE.

353 **Table 1.** Histomorphometric measurements of breast fillets according to the age and severity of
354 wooden breast.

Age, d	Item	WB score[1]					Mean	SEM	*P*-value
		0	1	2	3	4			
21	Minimum fiber diameter, µm	11.35[a]	11.96[ab]	12.44[b]	.	.	11.94	0.16	0.001
	Mean fiber diameter, µm	29.36[a]	27.56[b]	24.58[c]	.	.	27.27	0.16	0.01
	Density, fibers/cm^2	1,820.42[a]	1,644.14[a]	1,350.57[b]	.	.	1588.73	34.80	0.001
	EMT[2], µm	6.01[a]	5.93[a]	7.33[b]	.	.	6.50	0.09	0.001
	PMT[3], µm	54.56[a]	61.76[a]	79.09[b]	.	.	66.10	1.36	0.001
	Vv[4], %	64.37[a]	60.16[b]	60.03[b]	.	.	61.75	0.55	0.007
	MD[5], mm^2	155.66[a]	145.68[ab]	138.71[b]	.	.	146.77	2.50	0.014
49	Minimum fiber diameter, µm	.	.	17.74[a]	19.95[b]	20.35[b]	19.37	0.22	0.001
	Mean fiber diameter, µm	.	.	43.29	52.23	58.65	51.74	3.66	0.206
	Density, fibers/cm^2	.	.	694.96[a]	616.80[ab]	578.30[b]	630.27	16.61	0.011
	EMT[2], µm	.	.	10.94[a]	11.39[a]	12.57[b]	11.70	0.08	0.001
	PMT[3], µm	.	.	76.74[a]	90.23[b]	95.62[b]	87.97	1.70	0.001
	Vv[4], %	.	.	62.16[a]	56.17[b]	51.58[b]	56.53	1.01	0.001
	MD[5], mm^2	.	.	148.96[a]	121.91[b]	119.05[b]	129.74	2.44	0.001

355 [a,d] Means with different superscript letter differ ($P < 0.05$) based on Tukey's honestly significant difference
356 test.
357 [1]Absence of means are due to no recorded values
358 [2]EMT = Thickness of endomysium in µm.
359 [3]PMT = Thickness of perimysium in µm.
360 [4]Vv = partial volume of muscle tissue in %.
361 [5]MD = microvessels density in mm^2.

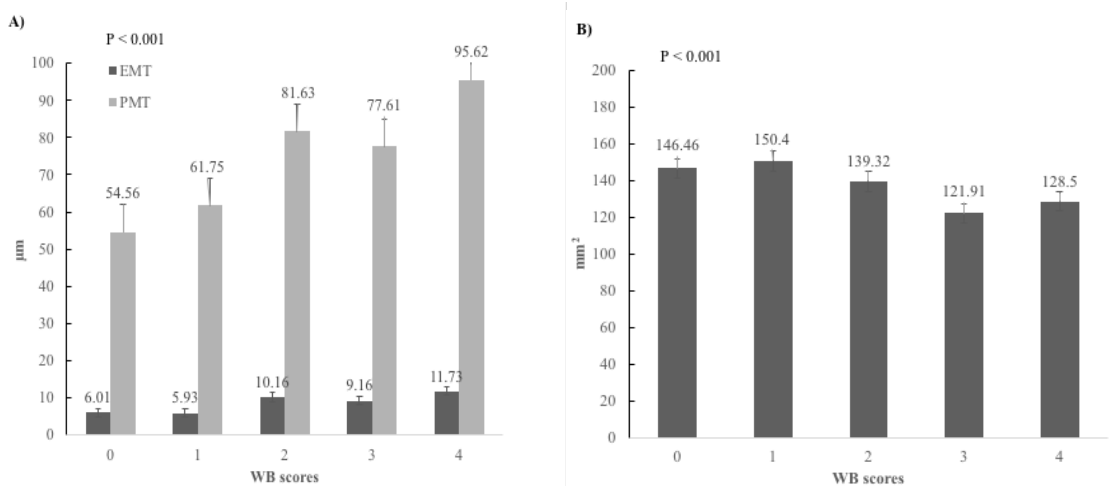

Figure 2. A) Size distribution (µm) of thickness of endomysium (EMT) and perimysium (PMT) according to the WB scores (0 to 4). B) Microvessels density (mm^2) according to the WB scores (0 to 4).

Table 2. Serum enzymes concentration according to the age and severity of wooden breast.

Age, d	Enzyme, U/L	WB score[1] 0	1	2	3	4	Mean	SEM	*P*-value
21	ALT[2]	0.00	0.20	1.86	.	.	0.77	0.66	0.455
	AST[3]	199.83	202.00	240.00	.	.	216.05	9.29	0.116
	CK[4]	4,378[a]	5,201[a]	8,072[b]	.	.	6,043	553.87	0.003
	LDH[5]	3,123	3,206	3,032	.	.	3,110	134.13	0.885
49	ALT	.	.	3.83	10.20	5.00	6.05	1.44	0.201
	AST	.	.	688[a]	1,267[b]	1,758[b]	1,265	138.55	0.001
	CK	.	.	25,162[a]	39,395[b]	56,229[c]	41,197	3,724	0.001
	LDH	.	.	6,180[a]	8,470[ab]	26,393[c]	15,214	3,586	0.020

[a,c] Means with different superscript letter differ ($P < 0.05$) based on Tukey's honestly significant difference test.

[1]Absence of means are due to no recorded values.

[2]ALT = Alanine aminotransferase.

[3]AST = Aspartate aminotransferase.

[4]LDH = Lactate dehydrogenase.

[5]CK = Creatine kinase**.**

7. ARTIGO CIENTÍFICO 3

MOLECULAR AND CELLULAR BIOLOGY

WOODEN BREAST CHARACTERIZATION

Characterization of myogenic stem cell populations and collagen infiltration in broiler chickens affected by wooden breast

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ABSTRACT

Satellite cells (SC) function is to repair muscle tissue after injury, a process called regenerative myogenesis. Since the SC are regulated by their surrounding niche, which includes multiple cell types, the aim of this study was to evaluate the myogenic stem cell population in broilers breasts muscle growing according to the wooden breast (WB) myopathy score. To this, a total of 48 birds were slaughtered at 25 and 43 d of age (24 birds per slaughter day) and the pectoralis major muscles were excised, weighed and visually scored on a 3-point scale (0 = none; 1 = mild; 2 = severe) for WB. In order characterize the myogenic stem cell population, PM samples were cryosectioned and evaluated for MyoD+, Myf-5+, Pax7+, macrophages and type-1 collagen using immunofluorescence microscopy with specific antibodies. Fillets WB-affected were 66.67 % at 25 d and 75 % at 43 d, and at both ages, fillets with score 2 of WB presented higher values ($P < 0.05$) for macrophage density and Type-1 collagen proliferation. At 43 d, higher densities for MyoD+, Myf-5+: MyoD+, Myf-5+: MyoD+: Pax7+ were presented on pectoralis major muscles scored as 1 of WB, when the palpable hardness was present in less than half the total fillet surface area. In conclusion, our results demonstrate that there is a macrophage proliferation with Type-1 collagen deposition in fillets WB-affected and the myogenic stem cell population decreased according to the age and severity of wooden breast.

Key words: breast myopathy, broilers, connective tissue, muscle regeneration.

INTRODUCTION

The poultry industry has made substantial genetic improvements in growth rate and breast meat yield. However, these increases have changed both the cell structure and cell biology of the pectoralis major muscle (Velleman and Clark, 2015). These alterations lead to an increase in skeletal muscle myopathies such as Wooden Breast (**WB**) that is characterized by a pale, hardened breast muscle with hemorrhagic lesions (Sihvo et al., 2014). Because of this specific hardness of the pectoralis major muscle committed with WB, this condition is negatively impacting the poultry processing industry, once is only detected at slaughter and the industry is facing great economic losses due to customer complaints about fillets affected (Tijare et al., 2016).

If selection for larger diameter fibers is altering the vascular nature of the stem cell niche, regeneration of the breast muscle in response to damage is suppressed, resulting in the development of myopathies (Velleman, 2015). Skeletal muscle has the ability to regenerate new muscle fibers after it has been damaged by injury or as a consequence of diseases (Carlson, 1973; Tedesco et al., 2010). To this, myogenic satellite cells (**SC**) are responsible for postnatal muscle fiber hypertrophy and muscle regeneration following injury (Mauro, 1961). Once satellite cells are activated to proliferate, these cells begin to adhere and migrate, align, and eventually fuse with either existing myotubes of other satellite cells.

Since a high proportion of necrotic or hypercontracted myofibers exists with the WB myopathy, Velleman and Clark (2015) had demonstrated that satellite cell-mediated regeneration of WB-affected muscles is occurring as indicated by the elevated expression of myogenic transcriptional regulatory factors regulating satellite cell proliferation and differentiation.

We have recently demonstrated that broilers affected by severe WB had a greater total population myogenic stem cell types (Myf-5+, Pax7+, or Myf-5+: Pax7+) and a concurrent increase in the mitotic activity (Myf-5+: BrdU+, and Pax7+: BrdU+, and Myf-5+: Pax7+: BrdU+) (Meloche et al., 2018). The objective of this study was to provide new information and evaluate the myogenic stem cell population in broiler breast muscle growing according to the Wooden Breast (WB) myopathy score.

MATERIALS AND METHODS

Birds and Husbandry

The Institutional Animal Care and Use Committee at Auburn University approved the use of live birds in this experimental protocol (PRN 2016-2829). As previous described by Meloche et al. (2018), four hundred eighty-one-day old Yield Plus × Ross 708 (Aviagen Inc. Huntsville) male broiler chicks were obtained from a commercial hatchery. Chicks were vaccinated for Marek's disease, Newcastle disease, and infectious bronchitis at the hatchery. Until 6 d of age, chicks were housed in groups of 8 in raised pens (0.03 m^2/bird) located in a solid-sided research facility with thermostatically controlled ventilation. Each pen was equipped with individual feeders, two water nipples, and clean wood shaving litter. At 7 d of age, all chicks were weighed and the upper and lower 12% of the BW range were excluded from the experiment. The remaining 360 chicks were identified using wing bands and allocated by weight into the individual-housing pens (0.20 m^2/bird). All birds consumed feed and fresh water on an ad libitum basis and diets were corn-soybean meal-based as described in Table 1. Ambient temperature was set at 33°C at placement and reduced to maintain comfort with

advancing bird age. Birds were exposed to a 23L:1D photoperiod from placement to 7 d of age, followed by an 18L:6D photoperiod for the remainder of the experiment. Light intensity was set at 30 lux from 1 to 7 d of age, 10 lux from 8 to 14 d of age, 5 lux from 15 to 24 d of age, and 3 lux from 25 to 48 d of age. Light intensity settings were verified at bird level (30 cm) using a photometric sensor with National Institute of Standards and Technology-traceable calibration (403125, Extech Instruments, Waltham, Mass) for each intensity adjustment.

Wooden Breast Scores and Muscle Sample Collection

On days 25 and 43 post-hatch, a total of 48 birds (24 per slaughter day) were weighted and insensibility by CO_2 asphyxiation followed and immediately euthanized by cervical dislocation and the PM muscles were excised, weighed and visually scored on a 3-point scale (0 = none; 1 = mild; 2 = severe) for WB. All fillets were scored by the same evaluator. The defect was considered "mild" if palpable hardness was present in less than half the total fillet surface area, but was considered "severe" if it exceeded this limit.

For the cryohistological analyses, samples were taken from the anteroventral area of the pectoralis major muscles and processed according to procedures adapted from Hutton et al. (2014).

Cryohistological Analysis

Samples collected from the left PM were coated in talc, snap frozen in liquid nitrogen, and subsequently stored at −80°C. After being held at −20°C for 24 hours, samples were embedded in frozen section compound (VWR International, Westchester,

PA) and cryosectioned using a Leica CM 1850 cryomicrotome. Serial cross-sections (5-μm thickness) were cut from each PM sample, mounted on positively charged glass slides (VWR International), and stored at 4°C prior to immunofluorescence staining.

Immunofluorescence Staining

Cryosections were fixed and stained according to the methods of Day et al. (2009) and Hutton et al. (2014) with minor modifications as described below. All procedures were conducted at room temperature in unless otherwise noted. Slides were rehydrated in phosphate buffered saline (**PBS**, pH 7.4; Invitrogen, Carlsbad, CA) for 10 min. and then fixed in paraformaldehyde (4 % in PBS; VWR International) for 10 min, and then rinsed twice in PBS. Cryosections were then exposed to 0.5 % Triton X-100 (VWR International) to permeabilize the cell membranes. Tissue sections were incubated in a blocking solution of 10% horse serum (Sigma-Aldrich), 2 % bovine serum albumin (VWR International), and 0.2 % Triton X-100 in PBS for 30 minutes to block non-specific antibody binding. Antibody reactions were conducted in a dark and humidified box. Primary antibodies diluted with blocking solution were allowed to react with tissue samples for 1 hour, followed by rinsing 3 times in PBS, for 5 minutes each rinse. Likewise, secondary antibodies diluted with blocking solution were allowed to react with tissue for 30 min followed by rinsing 3 times for 5 minutes per rinse in PBS. All slides were briefly exposed to 4',6-diamidino-phenylindole (**DAPI**; 1 μg/mL; VWR International) and immediately rinsed twice in PBS. Slides were mounted using Fluoro-gel Mounting Media (VWR International), covered with thin glass coverslips (VWR International) and allowed to dry at 4°C overnight. All cryosections were imaged within 72 h of immunofluorescence staining.

Primary and Secondary Antibodies

Control cryosections processed in the manner described above, but without adding either primary or secondary antibodies, were used to ensure that no fluorescence signal beyond natural autofluorescence was observed for the selected combination of antibodies described below.

Primary antibodies were as follows: rabbit IgG Type-1 Collagen (1:1.500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA); mouse IgG1 Monocyte/Macrophage (1:750 dilution; Santa Cruz Biotechnology, Santa Cruz, CA); mouse IgG2b MyoD (1:2.000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA); rabbit IgG Myf-5 (1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) and mouse IgG2b Pax7 (1:10 dilution). Secondary antibodies (Invitrogen) used at a 1:1000 dilution to detect the primary antibodies were as follows: AlexaFluor 488 Goat anti-rabbit IgG (H+L), AlexaFluor 546 Goat anti-mouse IgG, Alexa Fluor 633 Goat anti-mouse IgG2b, AlexaFluor 546 Goat anti-mouse IgG2b and AlexaFluor 633 Goat anti-mouse IgG1.

Fluorescence Photomicroscopy and Image Analysis

Immunofluorescence-stained cryohistological slides were imaged at 200X magnification using an inverted fluorescence microscope (Nikon Eclipse, Ti-U; Nikon Instruments, Inc. Mellville, NY) equipped with a UV light source (Nikon Intensilight). Images were captured and analyzed using an Evolve 512 EMCCF camera (Photometrics, Tuscon, AZ) and Elements Imaging software (Nikon Instruments, Inc.). A representative image was captured from each slide, Myf-5+, Pax7+, MyoD+, (Myf-5+: Pax7+), (Myf-5+: MyoD+), (Pax7+: MyoD+), (Myf-5+: Pax7+: MyoD+) and Macrophage cells were

enumerated to determine a macrophage density. The total number of DAPI-stained nuclei was counted in each image as a measure of nuclear density. After the cell measurements, the percentage of the collagen in each slide was estimated using the binary component in NIS Elements (MURPHY et al., 2011 with modifications).

Statistical Analyses

Statistical analysis was performed using the GLIMMIX and MIXED procedures of PC SAS (v9.4, SAS Inst. Inc., Cary, NC). For all data analysis, WB score served as the fixed effect and the Satterthwaite adjustment was used to correct degrees of freedom with individual bird serving as the experimental unit. Bird BW and PM weight were tested as possible covariates for all independent variables and were found to be insignificant resulting in exclusion from the model. The MIXED procedure of SAS was used to analyze variance for all continuous data such as MSC and macrophage densities, while GLIMMIX was used to analyze all proportional data. Proportional data were analyzed using the events/experiments syntax with a binomial distribution and R-side covariance structure. Where applicable, residual variance was visually assessed to ensure normality and nonnormal data were transformed prior to analysis. All treatment means were separated using the PDIFF option and considered significantly different when $P \leq 0.05$. Tendencies for differences among treatment means were declared when $0.06 \geq P \leq 0.10$.

RESULTS

Wooden Breast Scores

Since the severity of the WB condition is associated with the increase of BW and weight of PM muscles, at 43 d of age we could observe that 75 % (18 from a total of 24) of the PM muscles were classified with some score (1 or 2) of WB. However, we also observed that 66.67 % of the birds slaughtered at 25 d of age presented WB condition, with only 16.67 % of the PM muscles affected with score 2 of WB.

When evaluating the fillets consider as normal to WB, there was a decrease according to the slaughter day, with 8 PM muscles (33.33 %) at 25 d compared to only 6 (25 %) PM muscles at 43 d of age.

Collagen, Macrophage and Myogenic stem cell population

At 25 d of age, there was an increase ($P = 0.023$) on macrophage density (255.50, 320.31, and 414.93 mm^2) and collagen proportion (1.39, 1.14, and 5.98 %) according to the severity of WB (Table 2). However, no differences on density or relative density were found on the myogenic stem cell populations in relation to the WB score.

At 43 d of age, there was an increase on collagen proportion (0.41, 1.23, and 5.30 %) and also an increase on macrophage density (297.11, 359.06, and 470.06 mm^2) according to the severity of WB as observed on Figure 1.

In relation to the myogenic stem cell population and the severity of WB at 43 d, tissues from broilers with severe WB had increased ($P < 0.05$) density number of Pax7

(13.50, 26.25 and 52.87 mm^2), relative density of total Pax7+ (2.67, 3.19, and 7.63 %), and also myogenic Pax7+ (5.63, 5.12, and 18.30 %, respectively).

In contrast, PM muscles classified as mild WB at 43d had higher values of MyoD+ density (3.75, 30.63, and 9.00 mm^2), Myf-5+: MyoD+ (58.13, 177.13, and 57.37 mm^2), and Myf-5+: MyoD+: Pax7+ (30.00, 72.37, and 41.87 mm^2, respectively), when compared with normal and severe score of WB, respectively. And an increase on MyoD+ (0.74, 3.73, 1.29 %) and non-myogenic DAPI (52.71, 37.57, and 58.29 %, respectively).

DISCUSSION

At 25 d of age, 16.67 % of the PM muscles were classified as severe WB. This data agrees with findings by Griffin et al. (2017), which documented the first case of severe WB on day 23, characterized as a PM muscle that was extremely pale in color with a ridge-like bulge on the caudal end. Similarly, Mutryn et al. (2015) reported that lesions seen as early as at 3-wk-of-age and can affect a high proportion of birds in a flock.

In response to damage and regenerate the muscle fiber, SCs will re-enter the cell cycle and upon being activated they will express muscle-specific transcriptional regulatory factors in a sequential pattern, with myogenic determination factor 1 (MyoD1) being expressed in proliferating cells, followed by myogenic expression as the cells enter differentiation (Cornelison and Wold, 1997; Cooper at al., 1999; Kästner et al., 2000; Yablonka-Reuveni and Paterson, 2001; Velleman and Clark, 2015).

The remarkably macroscopic characteristic of WB is the hardened diffusely or on focally extensive areas (Sihvo et al., 2014) and its due to an extensive fibrillar collagen deposition and fibrosis. Because the collagen fibers make meat less tender, detailed information about the growth of interstitial collagen fibers is important for evaluating

240 different qualities of chicken meat at various growth stages (Nakamura et al. 1975;
241 Torrescano et al. 2003; Das et al., 2010).

242 Our study demonstrates an increase on a specific regenerative collagen Type-1 at
243 35 (5.98 %) and 43 d of age (5.30 %) of PM muscles affected with mild WB. Once the
244 skeletal muscle turns mature, collagen provides the tissue with tensile strength for
245 structural support and resistance to tension and stretch, since the collagen is the most
246 abundant extracellular matrix protein (Velleman and McFarland, 1999).

247 In growth-selected birds, the breast muscle is selected for larger diameter fibers
248 altering the available space for capillaries (Velleman et al., 2003) and this reduction
249 further decreases pH through the retention of lactic acid that enhance the muscle damage
250 process. However, if sufficient vascularization is not presented, satellite cells
251 regeneration is inhibited by the lack of blood supply needed and fibrosis of the muscle
252 occurs (Markley et al., 1978; Velleman et al., 2015). Daughtry et al. (2017) demonstrated
253 that SC number and their functional capacity decrease with time and hypertrophy, arguing
254 that SCs have a finite number of divisions.

255 Corroborating with Daughtry et al. (2017) that found that larger muscle fibers in
256 larger birds contain more myonuclei, possess less satellite cells and exhibit greater
257 nyonuclear domain, our results indicate that the proliferative capacity of SC declines with
258 hypertrophy, once the myogenic stem cell population decreased with the severity of WB.
259 However, our findings presented a higher density of Pax7+, that gives rise to degenerated
260 muscle after injury (Murphy et al., 2011), Myf-5+: MyoD+, Myf-5+: MyoD+: Pax7+ and
261 MyoD+ relative density on 42 d at degree 1 of WB suggesting this prolife as an acute
262 process.

263 Those differences in satellite cell type populations can affect the ability of satellite
264 cells to proliferate and differentiate during muscle growth and regeneration (Velleman et

al., 2015). However, the full expression of the genetic potential for growth and meat yields of the modern broiler can only be fulfilled by adequate nutrition (Cruz et al., 2017). Daughtry et al. (2017) reported that the rapid cell division may come at a price, since the SCs may lose their ability to function with age or by hypertrophy.

Velleman and Clark (2015) also had a significant increase in myogenic expression (MyoD, Myogenin, Decorin, TGF-β, and Myostatin) in wooden breast-affected PM muscles, supporting that satellite-cell mediated regeneration is occurring in PM muscle with the WB myopathy. The increase expression of decorin in WB-affected muscles founded for Velleman and Clark (2015) also supports the increased collagen crosslinking that our results demonstrate according to the severity of this myopathy.

Previous results had already demonstrated that WB muscles clearly exhibit severe histological lesions, including fiber degeneration and regeneration, fibrosis, and lipidosis (Sihvo et al., 2014; De Brot et al., 2016; Soglia et al., 2016, Soglia et al., 2017). Altogether, our results showed that there is a macrophage proliferation with Type-1 collagen infiltration in WB affected. Also, the myogenic stem cell population decreased according to the age and severity of wooden breast.

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Table 1. Ingredient composition of diets fed to male Yield Plus × Ross 708 broilers from 1 to 40 d of age.

Ingredient (%)	Starter 1 to 10 d	Grower 1 11 to 14 d	Grower 2 15 to 25 d		Finisher 26 to 40 d
			Diet 1	Diet 2	
Corn	52.07	58.86	62.33	62.33	62.07
Soybean Meal	36.27	25.64	23.54	23.54	25.51
Corn Gluten Meal	2.00	5.00	4.00	4.00	2.00
Peanut Meal	2.00	3.00	3.00	3.00	2.00
Poultry Fat[1]	3.15	2.98	2.63	2.63	4.53
Dicalcium Phosphate	2.02	1.86	1.88	1.88	1.64
Calcium Carbonate	1.05	0.98	0.99	0.99	0.88
Sodium Chloride	0.44	0.29	0.24	0.24	0.32
DL-Methionine	0.33	0.30	0.26	0.26	0.28
L-Lys·HCl	0.23	0.38	0.35	---	0.23
L-Thr	0.10	0.11	0.10	0.10	0.07
L-Val	0.00	0.02	0.01	0.01	---
Sodium Bicarbonate	0.00	0.23	0.29	0.29	0.19
Vitamin premix[2]	0.10	0.10	0.10	0.10	0.10
Mineral premix[3]	0.10	0.10	0.10	0.10	0.10
Choline Chloride[4]	0.07	0.10	0.11	0.11	0.08
TBCC[5]	0.02	0.02	0.02	0.02	0.02
Salinomycin	0.05	0.05	0.05	0.05	---
Sand	---	---	---	0.35	---

[1]Poultry fat was added at 0.75% in the mixer, with the remainder spray-added post-pelleting.

[2]Vitamin premix provided the following per kilogram of diet: Vitamin A (Vitamin A acetate), 18,739 IU; Vitamin D (cholecalciferol), 6,614 IU; Vitamin E (DL-alpha tocopheryl acetate), 66 IU; Vitamin B12 (cyanocobalamin), 0.03 mg; D-biotin (biotin), 0.18 mg; menadione (menadione sodium bisulfate complex), 4 mg; thiamine (thiamine mononitrate), 5.5 mg; riboflavin (riboflavin), 22 mg; D-pantothenic acid (calcium pantothenate), 31 mg; pyridoxine (pyridoxine hydrochloride), 7.7 mg; niacin (niacinamide), 88 mg; folacin (folic acid), 2.6 mg.

[3]Mineral premix includes per kg of diet: Mn (manganese sulfate), 120 mg; Zn (zinc sulfate), 100 mg; Fe (iron sulfate monohydrate), 30 mg; Cu (tri-basic copper chloride), 8 mg; I (stabilized ethylenediamine dihydriodide), 1.4 mg; Se (sodium selenite), 0.3 mg.

[4]Choline chloride-60 (Balchem Corporation, New Hampton, NY)

[5]Tribasic copper chloride (Micronutrients, Inc., Indianapolis, IN).

Table 2. Effect of Wooden Breast score on collagen proportion and satellite cell population density (per mm^2) and relative density (%) in the Pectoralis major from broilers at 25 d of age.

Population[2]	Wooden Breast Score[1]				
	Normal (0)	Mild (1)	Severe (2)	SEM	*P-value*
	n = 8	n = 8	n = 8		
Collagen Proportion (%)	1.39[a]	1.14[ab]	5.98[c]	0.01	0.005
Density (per mm^2)					
Macrophage	255.50[a]	320.31[ab]	414.93[b]	37.53	0.023
DAPI	412.69	521.44	547.55	107.92	0.650
Myf-5+	106.63	84.75	139.37	19.46	0.161
MyoD+	16.38	16.50	12.00	6.44	0.855
Pax7+	60.25	60.63	78.75	13.85	0.567
Myf-5+: MyoD+	37.38	39.50	35.00	13.31	0.972
Myf-5+: Pax7+	11.25	17.25	7.50	6.50	0.573
MyoD+: Pax7+	6.75	12.75	15.75	5.06	0.454
Myf-5+: MyoD+: Pax7+	18.00	21.00	45.50	9.59	0.109
Relative Density (%)					
DAPI+, % of Total (Non-myogenic)	61.69	67.46	62.14	0.05	0.644
Macrophage, % of Total DAPI+	53.17[a]	41.80[a]	77.48[b]	0.09	0.042
MyoD+SC, % of Total DAPI+	60.00	67.68	73.66	0.06	0.462
Myf-5+, % of Total DAPI+	15.94	0.96	15.82	0.03	0.459
MyoD+, % of Total DAPI+	2.45	2.14	1.36	0.01	0.487
Pax7+, % of Total DAPI+	9.01	7.84	8.94	0.01	0.825
MyoD: Myf-5+, % of Total DAPI+	5.58	5.11	3.97	0.02	0.825
Myf-5+: Pax7+, % of Total DAPI+	1.68	2.23	0.85	0.01	0.569
MyoD+: Pax7+, % of Total DAPI+	1.01	1.65	1.79	0.01	0.649
MyoD+:Myf-5+: Pax7+, % of Total DAPI+	2.69	2.72	5.16	0.01	0.173
MyoD+, % of Total Myogenic	6.38	6.55	3.59	0.02	0.537
Myf-5+, % of Total Myogenic	41.59	33.65	41.78	0.05	0.374
Pax7+, % of Total Myogenic	23.50	24.07	23.60	0.06	0.997
MyoD+:Myf-5+, % of Total Myogenic	14.58	15.68	10.49	0.04	0.587
Myf-5+: Pax7+, % of Total Myogenic	4.39	6.85	2.25	0.02	0.416
MyoD+: Pax7+, % of Total Myogenic	2.63	5.06	4.72	0.02	0.593
MyoD+: Myf-5+: Pax7+, % of Total Myogenic	7.02	8.34	13.64	0.03	0.104

[1]The *Pectoralis major* muscles of each bird were visually assessed and scored on a 3-point scale (0 = none; 1 = mild; 2 = severe) for Wooden Breast.

[2]Total = Every cell positive for the specified immunofluorescence target, regardless of the status of other targets; Non-myogenic = not positive for Myf-5, Pax7, MyoD, or any combination thereof; Myogenic = positive for Myf-5, Pax7, MyoD, or any combination thereof.

[a-b]Means within the same row that do not share a common superscript are significantly different ($P < 0.05$).

Table 3. Effect of Wooden Breast score on collagen proportion and satellite cell population density (per mm^2) and relative density (%) in the Pectoralis major from broilers at 43 d of age.

Population[2]	Wooden Breast score[1]			SEM	*P-value*
	Normal (0)	Mild (1)	Severe (2)		
	n = 8	n = 8	n = 8		
Collagen Proportion (%)	0.41^{a}	1.23^{ab}	5.30^{c}	0.01	0.021
Density (per mm^2)					
Macrophage	297.11^{a}	359.06^{ab}	470.06^{bc}	49.51	0.074
DAPI	267.43	308.41	403.76	56.25	0.236
Myf-5+	123.62	187.12	121.38	22.83	0.092
MyoD+	3.75^{a}	30.63^{b}	9.00^{ac}	7.06	0.032
Pax7+	13.50^{a}	26.25^{ab}	52.87^{bc}	11.43	0.067
Myf-5+: MyoD+	58.13^{a}	177.13^{b}	57.37^{a}	36.38	0.046
Myf-5+: Pax7+	0.75	7.50	3.75	2.79	0.252
MyoD+: Pax7+	10.50	12.00	3.00	4.08	0.269
Myf-5+: MyoD+: Pax7+	30.00^{a}	72.37^{b}	41.87^{a}	11.63	0.048
Relative Density (%)					
DAPI+, % of Total (Non-myogenic)	52.71^{a}	37.57^{a}	58.29^{b}	0.06	0.049
Macrophage, % of Total DAPI+	38.34	39.98	60.84	0.14	0.422
MyoD+SC, % of Total DAPI+	52.36	47.33	55.48	0.14	0.877
Myf-5+, % of Total DAPI+	24.37	22.80	17.52	0.04	0.272
MyoD+, % of Total DAPI+	0.74^{b}	3.73^{a}	1.29^{b}	0.01	0.033
Pax7+, % of Total DAPI+	2.67^{a}	3.19^{b}	7.63^{b}	0.01	0.042
Myf-5+: MyoD+, % of Total DAPI+	11.46	21.58	8.28	0.05	0.115
Myf-5+: Pax7+, % of Total DAPI+	0.15	0.92	0.54	0.01	0.451
MyoD+: Pax7+, % of Total DAPI+	2.07	1.46	0.43	0.01	0.121
Myf-5+: MyoD+: Pax7+, % of Total DAPI+	5.91	8.82	6.05	0.01	0.243
Myf-5+, % of Total Myogenic	51.54	36.51	42.02	0.06	0.174
MyoD+, % of Total Myogenic	1.56	5.98	3.12	0.01	0.105
Pax7+, % of Total Myogenic	5.63^{a}	5.12^{a}	18.30^{b}	0.03	0.029
Myf-5+: MyoD+, % of Total Myogenic	24.23	34.56	19.86	0.06	0.191
Myf-5+: Pax7+, % of Total Myogenic	0.31	1.46	1.29	0.01	0.702
MyoD+: Pax7+, % of Total Myogenic	4.38	2.34	1.04	0.01	0.085
Myf-5+: MyoD+: Pax7+, % of Total Myogenic	12.51	14.12	14.50	0.03	0.864

[1]The *Pectoralis major* muscles of each bird were visually assessed and scored on a 3-point scale (0 = none; 1 = mild; 2 = severe) for Wooden Breast.

[2]Total = Every cell positive for the specified immunofluorescence target, regardless of the status of other targets; Non-myogenic = not positive for Myf-5, Pax7, MyoD, or any combination thereof; Myogenic = positive for Myf-5, Pax7, MyoD, or any combination thereof.

[a-b]Means within the same row that do not share a common superscript are significantly different ($P < 0.05$).

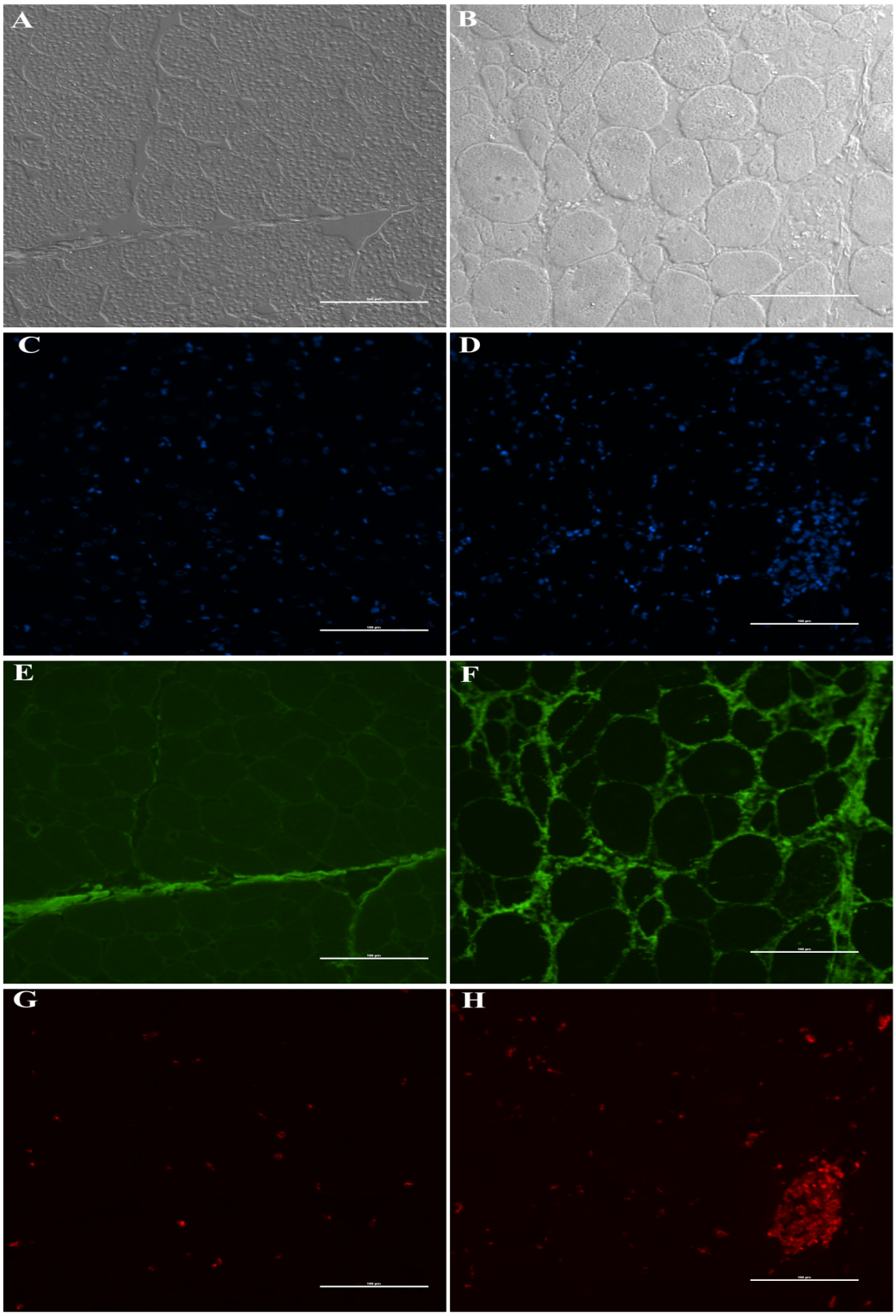

Figure 1. SC derived from PM muscle from broiler slaughtered at 43 d and unaffected and affected with WB score 2. Identification of SCs isolated with DIC (A – unaffected, B – WB affected), DAPI (C – unaffected, D – WB affected), Type – 1 Collagen infiltration (E – unaffected, F – WB affected) and Macrophage accumulation on degenerative fiber (G – unaffected, H – WB affected). Scale bar = 100 µm.

8. CONSIDERAÇÕES FINAIS

No presente trabalho foram estudados o processo de crescimento, degeneração e regeneração celular do músculo *Pectoralis major* de frangos de corte de rápido crescimento acometidos com miopatias peitorais, o efeito das restrições alimentares quantitativas e qualitativas sobre a ocorrência das miopatias *wooden breast* e *white striping* em frangos de corte abatidos em diferentes idades e as consequências na qualidade da carne, e, sobretudo, a validação de uma metodologia para diagnóstico *in vivo* da miopatia *wooden breast* através de análises de imagens ultrassonográficas. Os resultados provenientes destas pesquisas foram suficientes para a elaboração de três artigos científicos, com os quais pode-se concluir que:

- Programas de restrições alimentares tanto quantitativas (70% consumo) quanto qualitativas (hipoproteica e hipoenergética) aplicadas sobre as fases inicial e crescimento (até 28 dias de idade) não afetaram o desempenho zootécnico dos animais, confirmando o efeito do crescimento compensatório nestas fases dos animais.
- Ambas restrições alimentares (quantitativas e qualitativas) aplicadas entre 22 e 28 dias de idade dos animais apresentaram uma redução do grau de severidade de miopatias, considerando que não foram encontrados peitos classificados com o grau 4 de WB. Entretanto, ao fim do período experimental, aos 49 dias, não houve efeitos na redução da ocorrência das miopatias peitorais WB e WS.
- As imagens de ultrassonografia do peito podem ser utilizadas para diagnóstico *in vivo* da miopatia WB quando correlacionadas as medidas de ecogenicidade e profundidade do peito nos frangos de corte avaliados aos 21 dias de idade.
- Com o aumento da severidade de WB, filés de peito apresentaram alterações na qualidade da carne, como aumento da perda por cocção, das coordenadas cromáticas L*, a* e b* e valores de força de deformação.
- Músculo *Pectoralis major* de frangos de corte acometidos com WB possuem, histologicamente, fibras musculares com variabilidade de tamanho e presença de células gigantes, com extensa proliferação de tecido conjuntivo.
- Ao analisar as características histomorfométricas do músculo *Pectoralis major* de frangos de corte acometidos com a miopatia WB aos 21 e 49 dias de idade, pode-se concluir que, apesar do aumento no diâmetro das fibras musculares de animais classificados como de escore 4 para WB, houve uma redução na densidade (fibras/cm^2)

e volume do tecido muscular, e uma redução na densidade de pequenos vasos, diminuindo consequentemente o aporte vascular. Também foi verificado aumento dos marcadores bioquímicos de lesão muscular de acordo com a idade e severidade desta condição.

- Visando o aprofundamento do processo de regeneração muscular, foram analisadas a população das células miogênicas MyoD, Myf-5 e Pax7 ativas e não ativas. Animais levemente acometidos com WB, ou seja, com o filé do peito classificado macroscopicamente como menos de 50 % de dureza, apresentaram maiores valores de células miogênicas quando comparados aos filés classificados com maior severidade desta miopatia.
- A detecção de colágeno Tipo I com proliferação de macrófagos nos filés de peito classificados em grau severo de WB justifica a dureza verificada macroscopicamente no tecido.

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ANEXO

Normas para publicação de artigos no periódico *Journal of Poultry Science*

I. Scope and General Information

A. Scope

Poultry Science publishes the results of fundamental and applied research concerning poultry, poultry products, and avian species in general. Submitted manuscripts shall provide new facts or confirmatory data. Papers dealing with experimental design, teaching, extension endeavors, or those of historical or biographical interest may also be appropriate. Opinions or views expressed in papers published by Poultry Science are those of the author(s) and do not necessarily represent the opinion of the Poultry Science Association or the editor-in-chief.

B. Submission

All manuscripts are submitted and reviewed via the journal's Scholar One Manuscripts submission site at https://mc04.manuscriptcentral.com/ps. New authors should create an account prior to submitting a manuscript for consideration.

C. Contact information for journal staff

For information on the scientific content of the journal, contact the editor-in-chief, Dr. Robert L. Taylor, Division Director and Professor of Animal & Nutritional Sciences, West Virginia University, G038 Agricultural Science Building, P.O. Box 6108, Morgantown, WV 26506-6108; contact at PS-Editor@mail.wvu.edu.

For assistance with ScholarOne Manuscripts and manuscript submission, contact David Busboom at david.busboom@poultryscience.org.

For assistance with article proofs, copyright forms, and published manuscripts, contact Matthew Marusak at poultry.science@oup.com.

For assistance with invoicing, contact Jnls.Cust.Serv@oup.com.

D. Types of Articles

i.) Full-Length Articles

The journal emphasizes the importance of good scientific writing and clarity in presentation of the concepts, apparatus, and sufficient background information that would be required for thorough understanding by scientists in other disciplines. The results of experiments published in Poultry Science must be replicated, either by replicating treatments within experiments or by repeating experiments. Care should be taken to ensure that experiments are adequately replicated.

ii.) Review Papers

Review papers are accepted only if they provide new knowledge or a high-caliber synthesis of important knowledge. Reviews are not exempt from pages charges. All Poultry Science guidelines for style and form apply.

iii.) Research Notes

Research Notes are short notes giving the results of complete experiments but are less comprehensive than full-length articles. Preliminary or progress reports will not be accepted. Research Notes will be published as a subsection of the scientific section in which they were reviewed and are limited to five printed pages including tables and figures. Manuscripts should be prepared according to the guidelines for full-length articles. Symposium chair must decide whether or not the symposium is to be published and will inform the editor-in-chief of this decision at the January meeting. If the decision is not to publish the symposium, the individual authors retain the right to submit their papers for consideration for the journal as ordinary manuscripts. If publication is decided upon, all manuscript style and form guidelines of the journal shall be followed.

iv.) Symposium Papers

If you are interested in publishing a symposium in Poultry Science, please contact the editor-in-chief for full guidelines.

v.) Invited Papers

Invited papers are subject to review, and all manuscript style and form guidelines of the journal shall be followed. Invited papers are exempt from page charges.

vi.) Invited Reviews

Invited Reviews will be approximately 10 published pages and in review format. Nominations or suggestions for potential timely reviews are welcomed and should be sent directly to the editor-in-chief.

vii.) Contemporary Issues

Contemporary Issues will address critical issues facing poultry scientists and the poultry industry. As such, submissions to this section should be of interest to any poultry scientist, to the industry, to instructors and faculty teaching contemporary issues classes, and to undergraduate and graduate students. The section will consist of short papers (approximately 2 published pages) written in essay format and will include an abstract, appropriate subheadings, and references.

viii.) Book Reviews

A limited number of book reviews will publish in Poultry Science. Book reviews shall be prepared in accordance to the style and form requirements of the journal, and they are subject to editorial revision. No page charges will be assessed.

ix.) Letters to the Editor

The purpose of letters will be to discuss, critique, or expand on scientific points made in articles recently published in Poultry Science. Introduction of unpublished data will not be allowed, nor will material based on conjecture or speculation. Letters must be received within 6 months of an article's publication. Letters will be limited to 400 words and 5 references. The author(s) of the original paper(s) will be provided a copy of the letter and offered the opportunity to submit for consideration a reply within 30 days. Replies will have the same page restrictions and format as letters, and the titles shall end with "—Reply." Letters and replies will be published together. Letters and replies shall

follow appropriate Poultry Science formatting and may be edited by the editor-in-chief and a technical editor. If multiple letters on the same topic are received, a representative letter concerning a specific article may be published. Letters and replies will be published as space permits.

II. Journal Policies

A. Peer review process

All submissions to the journal are initially reviewed the editorial office. At this stage, manuscripts may be rejected without peer review if it is felt that they are not relevant to the journal's scope or do not conform to manuscript formatting requirements. This fast rejection process means that authors are given a quick decision and do not need to wait for the review process.

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editor. Failure to return the manuscript within 6 weeks will lock the author out of re-submitting the revision.

Rejected manuscripts can be resubmitted only with an invitation from the section editor or editor-in-chief. Revised versions of previously rejected manuscripts are treated as new submissions.

B. Pre-submission language editing

If your first language is not English, to ensure that the academic content of your paper is fully understood by journal editors and reviewers, we recommend using an external language editing service. Language editing is optional and does not guarantee that your manuscript will be accepted for publication. For further information on this service, please click here. Several specialist language editing companies offer similar services and you can also use any of these. Authors are liable for all costs associated with such services.

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Authors' corrections to Supplementary Data are made only in exceptional circumstances (for example major errors that compromise the conclusion of the study). Because the Supplementary Data is part of the original paper and hence the published record, the information cannot be updated if new data have become available or interpretations have changed.

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E. Care and use of animals

Authors must make it clear that experiments were conducted in a manner that avoided unnecessary discomfort to the animals by the use of proper management and laboratory techniques. Experiments shall be conducted in accordance with the principles and specific guidelines presented in Guide for the Care and Use of Agricultural Animals in Research and Teaching, 3rd edition, 2010 (found here.); and, if applicable, Guide for the Care and Use of Laboratory Animals (United States Department of Human Health and Services, National Institutes of Health, Publication Number ISBN 0-309-05377-3, 1996); or Guide to the Care and Use of Experimental Animals, 2nd ed. Volume 1, 1993 (Canadian Council on Animal Care). Methods of killing experimental animals must be described in the text. In describing surgical procedures, the type and dosage of the anesthetic agent must be specified. Intra-abdominal and intrathoracic invasive surgery requires anesthesia. This includes caponization. The editor-in- chief of Poultry Science may refuse to publish manuscripts that are not compatible with these guides. If rejected solely on that basis, however, the paper may be resubmitted for reconsideration when accompanied by a written verification that a committee on animal care in research has approved the experimental design and procedures involved.

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i.) General

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ii.) Preparing the manuscript file

Manuscripts should be typed double-spaced, with lines and pages numbered consecutively, using Times New Roman font at 12 points. All special characters (e.g., Greek, math, symbols) should be inserted using the symbols palette available in this font. Complex math should be entered using MathType from Design Science (www.dessci.com). Tables and figures should be placed in separate sections at the end of the manuscript (not placed within the text).

iii.) Headings

• Major headings: Major headings are centered (except ABSTRACT), all capitals, boldface, and consist of ABSTRACT, INTRODUCTION, MATERIALS AND METHODS, RESULTS, DISCUSSION (or RESULTS AND DISCUSSION), ACKNOWLEDGMENTS (optional), APPENDIX (optional), and REFERENCES.

• First subheadings: First subheadings are placed on a separate line, begin at the left margin, the first letter of all important words is capitalized, and the headings are boldface and italic. Text that follows a first subheading should be in a new paragraph.

• Second subheadings: Second subheadings begin the first line of a paragraph. They are indented, boldface, italic, and followed by a period. The first letter of each important word should be capitalized. The text follows immediately after the final period of the subheading.

iv.) Title page

The title page shall begin with a running head (short title) of not more than 45 characters. The running head is centered, is in all capital letters, and shall appear on the top of the title page. No abbreviations should be used.

The title of the paper must be in boldface; the first letter of the article title and proper names are capitalized, and the remainder of the title is lowercase. The title must not have abbreviations.

Under the title, names of authors should be typed (first name or initial, middle initial, last name). Affiliations will be footnoted using the following symbols: *, †, ‡, §, #, ||‖, and be placed below the author names. Do not give authors' titles, positions, or degrees. Numbered footnotes may be used to provide supplementary information, such as present address, acknowledgment of grants, and experiment station or journal series number. The corresponding author should be indicated with a numbered footnote (e.g., Corresponding author: name@university.edu).

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v.) Abbreviations

Author-derived abbreviations should be defined at first use in the abstract and again in the body of the manuscript. The abbreviation will be shown in bold type at first

use in the body of the manuscript. Refer to the Miscellaneous Usage Notes for more information on abbreviations.

vi.) Abstract

The Abstract disseminates scientific information through abstracting journals and through convenience for the readers. The Abstract, consisting of not more than 325 words, appears at the beginning of the manuscript with the word ABSTRACT without a following period. It must summarize the major objectives, methods, results, conclusions, and practical applications of the research. The Abstract must consist of complete sentences and use of abbreviations should be limited. References to other work and footnotes are not permitted. The Abstract and Key Words must be on a separate sheet of paper.

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The Abstract shall be followed by a maximum of five key words or phrases to be used for subject indexing. These should include important words from the title and the running head and should be singular, not plural, terms (e.g., broiler, not broilers). Key words should be formatted as follows: Key words: . . .

viii.) Introduction

The Introduction, while brief, should provide the reader with information necessary for understanding research presented in the paper. Previous work on the topic should be summarized, and the objectives of the current research must be clearly stated.

ix.) Materials and methods

All sources of products, equipment, and chemicals used in the experiments must be specified parenthetically at first mention in text, tables, and figures [i.e., (model 123, ABC Corp., Provo, UT)]. Model and catalog numbers should be included. Information shall include the full corporate name (including division, branch, or other subordinate part of the corporation, if applicable), city, and state (country if outside the United States), or Web address. Street addresses need not be given unless the reader would not be able to determine the full address for mailing purposes easily by consulting standard references.

Age, sex, breed, and strain or genetic stock of animals used in the experiments shall be specified. Animal care guidelines should be referenced if appropriate.

Papers must contain analyzed values for those dietary ingredients that are crucial to the experiment. Papers dealing with the effects of feed additives or graded levels of a specific nutrient must give analyzed values for the relevant additive or nutrient in the diet(s). If products were used that contain different potentially active compounds, then analyzed values for these compounds must be given for the diet(s). Exceptions can only be made if appropriate methods are not available. In other papers, authors should state whether experimental diets meet or exceed the National Research Council (1994) requirements as appropriate. If not, crude protein and metabolizable energy levels should be stated. For layer diets, calcium and phosphorus contents should also be specified.

When describing the composition of diets and vitamin premixes, the concentration of vitamins A and E should be expressed as IU/kg on the basis of the following equivalents:

Vitamin A
1 IU = 0.3 μg of all-trans retinol
1 IU = 0.344 μg of retinyl acetate
1 IU = 0.552 μg of retinyl palmitate 1 IU = 0.60 μg of β-carotene

Vitamin E
1 IU = 1 mg of dl-α-tocopheryl acetate
1 IU = 0.91 mg of dl-α-tocopherol
1 IU = 0.67 mg of d-α-tocopherol
In the instance of vitamin D3, cholecalciferol is the acceptable term on the basis that 1 IU of vitamin D3 = 0.025 μg of cholecalciferol.

The sources of vitamins A and E must be specified in parentheses immediately following the stated concentrations.

• Statistical analysis: Biology should be emphasized, but the use of incorrect or inadequate statistical methods to analyze and interpret biological data is not acceptable. Consultation with a statistician is recommended. Statistical methods commonly used in the animal sciences need not be described in detail, but adequate references should be provided. The statistical model, classes, blocks, and experimental unit must be designated. Any restrictions used in estimating parameters should be defined. Reference

to a statistical package without reporting the sources of variation (classes) and other salient features of the analysis, such as covariance or orthogonal contrasts, is not sufficient. A statement of the results of statistical analysis should justify the interpretations and conclusions. When possible, results of similar experiments should be pooled statistically. Do not report a number of similar experiments separately.

The experimental unit is the smallest unit to which an individual treatment is imposed. For group-fed animals, the group of animals in the pen is the experimental unit; therefore, groups must be replicated. Repeated chemical analyses of the same sample usually do not constitute independent experimental units. Measurements on the same experimental unit over time also are not independent and must not be considered as independent experimental units. For analysis of time effects, use time-sequence analysis.

• Usual assumptions are that errors in the statistical models are normally and independently distributed with constant variance. Most standard methods are robust to deviations from these assumptions, but occasionally data transformations or other techniques are helpful. For example, it is recommended that percentage data between 0 and 20 and between 80 and 100 be subjected to arc sin transformation prior to analysis. Most statistical procedures are based on the assumption that experimental units have been assigned to treatments at random. If animals are stratified by ancestry or weight or if some other initial measurement should be accounted for, they should include a blocking factor, or the initial measurement should be included as a covariate.

• A parameter [mean (μ), variance (σ2)], which defines or describes a population, is estimated by a statistic (x, s2). The term parameter is not appropriate to describe a variable, observation, trait, characteristic, or measurement taken in an experiment.

• Standard designs are adequately described by name and size (e.g., “a randomized complete block design with 6 treatments in 5 blocks”). For a factorial set of treatments, an adequate description might be as follows: “Total sulfur amino acids at 0.70 or 0.80% of the diet and Lys at 1.10, 1.20, or 1.30% of the diet were used in a 2 × 3 factorial arrangement in 5 randomized complete blocks consisting of initial BW.” Note that a factorial arrangement is not a design; the term “design” refers to the method of grouping

experimental units into homogeneous groups or blocks (i.e., the way in which the randomization is restricted).

• Standard deviation refers to the variability in a sample or a population. The standard error (calculated from error variance) is the estimated sampling error of a statistic such as the sample mean. When a standard deviation or standard error is given, the number of degrees of freedom on which it rests should be specified. When any statistical value (as mean or difference of 2 means) is mentioned, its standard error or confidence limit should be given. The fact that differences are not "statistically significant" is no reason for omitting standard errors. They are of value when results from several experiments are combined in the future. They also are useful to the reader as measures of efficiency of experimental techniques. A value attached by "±" to a number implies that the second value is its standard error (not its standard deviation). Adequate re-porting may require only 1) the number of observations, 2) arithmetic treatment means, and 3) an estimate of experimental error. The pooled standard error of the mean is the preferred estimate of experimental error. Standard errors need not be presented separately for each mean unless the means are based on different numbers of observations or the heterogeneity of the error variance is to be emphasized. Presenting individual standard errors clutters the presentation and can mislead readers.

• For more complex experiments, tables of subclass means and tables of analyses of variance or covariance may be included. When the analysis of variance contains several error terms, such as in split-plot and repeated measures designs, the text should indicate clearly which mean square was used for the denominator of each F statistic. Unbalanced factorial data can present special problems. Accordingly, it is well to state how the computing was done and how the parameters were estimated. Approximations should be accompanied by cautions concerning possible biases.

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unstructured or not related. Least squares means are the correct means to use for all data, but arithmetic means are identical to least squares means unless the design is unbalanced or contains missing values or an adjustment is being made for a covariate. In factorial treatment arrangements, means for main effects should be presented when important interactions are not present. However, means for individual treatment combinations also should be provided in table or text so that future researchers may combine data from several experiments to detect important interactions. An interaction may not be detected in a given experiment because of a limitation in the number of observations.

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Results and Discussion sections may be combined, or they may appear in separate sections. If separate, the Results section shall contain only the results and summary of the author's experiments; there should be no literature comparisons. Those comparisons should appear in the Discussion section. Manuscripts reporting sequence data must have GenBank accession numbers prior to submitting. One of the hallmarks for experimental evidence is repeatability. Care should be taken to ensure that experiments are adequately replicated. The results of experiments must be replicated, either by replicating treatments within experiments or by repeating experiments.

v.) Acknowledgements

An Acknowledgments section, if desired, shall follow the Discussion section. Acknowledgments of individuals should include affiliations but not titles, such as Dr., Mr., or Ms. Affiliations shall include institution, city, and state.

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• In the References section, references shall first be listed alphabetically by author(s)’ last name(s), and then chronologically. The year of publication follows the authors’ names. As with text citations, two or more publications by the same author or set of authors in the same year shall be differentiated by adding lowercase letters after the date. The dates for papers with the same first author that would be abbreviated in the text as et al., even though the second and subsequent authors differ, shall also be differentiated by letters. All authors’ names must appear in the Reference section. Journals shall be abbreviated according to the conventional ISO abbreviations given in journals database of the National Library of Medicine. One-word titles must be spelled out. Inclusive page numbers must be provided. Sample references are given below. Consult recent issues of Poultry Science for examples not included below.

• Article:

Bagley, L. G., and V. L. Christensen. 1991. Hatchability and physiology of turkey embryos incubated at sea level with in- creased eggshell permeability. Poult. Sci. 70:1412–1418.

Bagley, L. G., V. L. Christensen, and R. P. Gildersleeve. 1990. Hematological indices of turkey embryos incubated at high altitude as affected by oxygen and shell permeability. Poult. Sci. 69:2035– 2039.

Witter, R. L., and I. M. Gimeno. 2006. Susceptibility of adult chickens, with and without prior vaccination, to challenge with Marek’s disease virus. Avian Dis. 50:354–365. doi:10.1637/7498-010306R.1

• Book:

Metcalfe, J., M. K. Stock, and R. L. Ingermann. 1984. The effects of oxygen on growth and development of the chick embryo. Pages 205- 219 in Respiration and Metabolism of Embryonic Vertebrates. R. S. Seymour, ed. Dr. W. Junk, Dordrecht, the Netherlands.

National Research Council. 1994. Nutrient Requirements of Poultry. 9th rev. ed. Natl. Acad. Press, Washington, DC.

• Federal Register:

Department of Agriculture, Plant and Animal Health Inspection Service. 2004. Blood and tissue collection at slaughtering and rendering establishments, final rule. 9CFR part 71. Fed. Regis. 69:10137–10151.

• Other:

Choct, M., and R. J. Hughes. 1996. Long-chain hydrocarbons as a marker for digestibility studies in poultry. Proc. Aust. Poult. Sci. Symp. 8:186. (Abstr.)

Dyro, F. M. 2005. Arsenic. WebMD. Accessed Feb. 2006. http:// www.emedicine.com/neuro/topic20.htm.

El Halawani, M. E., and I. Rosenboim. 2004. Method to enhance reproductive performance in poultry. Univ. Minnesota, as- signee. US Pat. No. 6,766,767.

Hruby, M., J. C. Remus, and E. E. M. Pierson. 2004. Nutritional strategies to meet the challenge of feeding poultry without antibiotic growth promotants. Proc. 2nd Mid-Atlantic Nutr. Conf., Timonium, MD. Univ. Maryland, College Park.

Luzuriaga, D. A. 1999. Application of computer vision and electronic nose technologies for quality assessment of color and odor of shrimp and salmon. PhD Diss. Univ. Florida, Gainesville.

Peak, S. D., and J. Brake. 2000. The influence of feeding program on broiler breeder male mortality. Poult. Sci. 79(Suppl. 1):2. (Abstr.)

B. Tables

Tables must be created using the MS Word table feature and inserted in the manuscript after the references section. When possible, tables should be organized to fit across the page without running broadside. Be aware of the dimensions of the printed page when planning tables (use of more than 15 columns will create layout problems). Place the table number and title on the same line above the table. The table title does not require a period. Do not use vertical lines and use few horizontal lines. Use of bold and italic typefaces in the table should be done sparingly; you must define such use in a footnote. Each table must be on a separate page. To facilitate placement of all tables into the manuscript file

(just after the references) authors should use “section breaks” rather than “page breaks” at the end of the manuscript (before the tables) and between tables.

Units of measure for each variable must be indicated. Papers with several tables must use consistent format. All columns must have appropriate headings. Abbreviations not found on the inside front cover of the journal must be defined in each table and must match those used in the text. Footnotes to tables should be marked by superscript numbers. Each footnote should begin a new line. Superscript letters shall be used for the separation of means in the body of the table and explanatory footnotes must be provided [i.e., “Means within a row lacking a common superscript differ ($P < 0.05$).”]; other significant P-values may be specified. Comparison of means within rows and columns should be indicated by different series of superscripts (e.g., a,b, . . . in rows; x–z . . . in columns) The first alphabetical letter in the series (e.g., a or A) shall be used to indicate the largest mean. Lowercase super- scripts indicate $P \leq 0.05$. Uppercase letters indicate $P \leq 1.1$ or less.

Probability values may be indicated as follows: $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, and $\dagger P \leq 0.10$. Consult a recent issue of Poultry Science for examples of tables.
Generally, results should be presented to the significant figure of the instrument used to collect the data. For example, results should not be presented to 5 digits when the instrument used only reads to 2 digits.

C. Miscellaneous usage notes

i.) Abbreviations

• Abbreviations shall not be used in the title, key words, or to begin sentences, except when they are widely known throughout science (e.g., DNA, RNA) or are terms better known by abbreviation (e.g., IgG, CD). A helpful criterion for use of abbreviation is whether it has been accepted into thesauri and indexes widely used for searching major bibliographic databases in the scientific field. Abbreviations may be used in heads within the paper, if they have been first defined within the text. The inside back cover of every issue of the journal lists abbreviations that can be used without definition. The list is subject to revision at any time, so authors should always consult the most recent issue of the journal for relevant information. Abbreviations are allowed when they help the flow of the manuscript; however, excessive use of abbreviations can confuse the reader. The

suitability of abbreviations will be evaluated by the reviewers and editors during the review process and by the technical editor during editing. As a rule, author-derived abbreviations should be in all capital letters. Terms used less than three times must be spelled out in full rather than abbreviated. All terms are to be spelled out in full with the abbreviation following in bold type in parentheses the first time they are mentioned in the main body of the text. Abbreviations shall be used consistently thereafter, rather than the full term.

• The abstract, text, each table, and each figure must be understood independently of each other. Therefore, abbreviations shall be defined within each of these units of the manuscript.

• Plural abbreviations do not require "s." Chemical symbols and three-letter abbreviations for amino acids do not need definition. Units of measure, except those in the standard Poultry Science abbreviation list, should be abbreviated as listed in the CRC Handbook for Chemistry and Physics (CRC Press, 2000 Corporate Blvd., Boca Raton, FL, 33431) and do not need to be defined.

• The following abbreviations may be used without definition in Poultry Science:
A adenine
ADG average daily gain
ADFI average daily feed intake
AME apparent metabolizable energy
AMEn nitrogen-corrected apparent metabolizable energy ANOVA analysis of variance
B cell bursal-derived, bursal-equivalent derived cell
bp base pairs
BSA bovine serum albumin
BW body weight
C cytosine
cDNA complementary DNA
cfu colony-forming units CI confidence interval
CP crude protein
cpm counts per minute CV coefficient of variation d day
df degrees of freedom

DM dry matter

DNA deoxyribonucleic acid

EDTA ethylenediaminetetraacetate

ELISA enzyme-linked immunosorbent antibody assay EST expressed sequence tag

g gram

g gravity

G guanine

GAT glutamic acid-alanine-tyrosine G:F gain-to-feed ratio

GLM general linear model

h hour

HEPES N-2-hydroxyethyl piperazine-N′-ethane-sulfonic acid

HPLC high-performance (high-pressure) liquid chromatography ICU international chick units

Ig immunoglobulin

IL interleukin

IU international units

kb kilobase pairs

kDa kilodalton

L liter*

L:D hours light:hours darkness in a photoperiod (e.g., 23L:1D)

m meter

µ micro

M molar

MAS marker-assisted selection

ME metabolizable energy

MEn nitrogen-corrected metabolizable energy

MHC major histocompatibility complex

mRNA messenger ribonucleic acid

min minute

mo month

MS mean square

n number of observations

N normal

NAD nicotinamide adenine dinucleotide

NADH reduced nicotinamide adenine dinucleotide NRC National Research Council
NS not significant
PAGE polyacrylamide gel electrophoresis
PBS phosphate-buffered saline
PCR polymerase chain reaction
pfu plaque-forming units
QTL quantitative trait loci
r correlation coefficient
r2 coefficient of determination, simple
R2 coefficient of determination, multiple
RH relative humidity
RIA radioimmunoassay
rpm revolutions per minute
s second
SD standard deviation
SDS sodium dodecyl sulphate
SE standard error
SEM standard error of the mean
SRBC sheep red blood cells
SNP single nucleotide polymorphism
T thymine
TBA thiobarbituric acid
T cell thymic-derived cell
TME true metabolizable energy
TMEn nitrogen-corrected true metabolizable energy Tris tris(hydroxymethyl)aminomethane
TSAA total sulfur amino acids
U uridine
USDA United States Department of Agriculture
UV ultraviolet
vol/vol volume to volume
vs. versus
wt/vol weight to volume
wt/wt weight to weight

wk week
yr year

*Also capitalized with any combination, e.g., mL.

ii.) International words and phrases:

Non-English words in common usage (defined n recent editions of standard dictionaries) will not appear in italics (e.g., in vitro, in vivo, in situ, a priori). However, genus and species of plants, animals, or bacteria and viruses should be italicized. Authors must indicate accent marks and other diacriticals on international names and institutions. German nouns shall begin with capital letters.

iii.) Capitalization:

Breed and variety names are to be capitalized (e.g., Single Comb White Leghorn).

iv.) Number style:

Numbers less than 1 shall be written with preceding zeros (e.g., 0.75). All numbers shall be written as digits. Measures must be in the metric system; however, US equivalents may be given in parentheses. Poultry Science requires that measures of energy be given in calories rather than joules, but the equivalent in joules may be shown in parentheses or in a footnote to tables. Units of measure not preceded by numbers must be writ- ten out rather than abbreviated (e.g., lysine content was measured in milligrams per kilogram of diet) unless used parenthetically. Measures of variation must be defined in the Abstract and in the body of the paper at first use. Units of measure for feed conversion or feed efficiency shall be provided (i.e., g:g).

v.) Nucleotide sequences:

Nucleotide sequence data must relate to poultry or poultry pathogens and must complement biological data published in the same or a companion paper. If sequences are excessively long, it is suggested that the most relevant sections of the data be published in Poultry Science and the remaining sequences be submitted to one of the sequence databases. Acceptance for publication is contingent on the submission of sequence data to one of the databases. The following statement should appear as a footnote to the title on the title page of the manuscript. “The nucleotide sequence data

reported in this paper have been submitted to Embank Submission (Mail Stop K710, Los Alamos National Laboratories, Los Alamos, NM 87545) nucleotide sequence database and have been assigned the accession number XNNNNN." Publication of the description of molecular clones is assumed by the editors to place them in the public sector. Therefore, they shall be made available to other scientists for research purposes.

Nucleotide sequences must be submitted as camera- ready figures no larger than 21.6 × 27.9 cm in standard (portrait) orientation. Abbreviations should follow Poultry Science guidelines.

vi.) Gene and protein nomenclature:

Authors are required to use only approved gene and protein names and symbols. For poultry, full gene names should not be italicized. Gene symbols should be in uppercase letters and should be in italics. A protein symbol should be in the same format as its gene except the protein symbol should not be in italics.

vii.) General usage:

• Note that "and/or" is not permitted; choose the more appropriate meaning or use "x or y or both."

• Use the slant line only when it means "per" with numbered units of measure or "divided by" in equations. Use only one slant line in a given expression (e.g., g/d per chick). The slant line may not be used to indicate ratios or mixtures.

• Use "to" instead of a hyphen to indicate a range. Insert spaces around all signs (except slant lines) of operation (=, –, +, ×, >, or <, etc.) when these signs occur between two items.

• Items in a series should be separated by commas (e.g., a, b, and c).

• Restrict the use of "while" and "since" to meanings related to time.

• Appropriate substitutes include "and," "but," or "whereas" for "while" and "because" or "al- though" for "since."

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• Commas should be used in numbers greater than 999.

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D. Figures/illustrations

For information on how to submit figure files, please see the Oxford Journals page on figures. You can also send queries about figure files to poultry.science@oup.com.

E. Supplementary data

Submit all material to be considered as Supplementary Material online at the same time as the main manuscript. Ensure that the supplementary material is referred to in the main manuscript at an appropriate point in the text. Supplementary material will be available online only and will not be copyedited, so ensure that it is clearly and succinctly presented, and that the style conforms to the rest of the paper. Also ensure that the presentation will work on any Internet browser. It is not recommended for the files to be more than 2 MB each, although exceptions can be made at the editorial office's discretion.

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