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IDENTIFICAÇÃO DE GENES ASSOCIADOS À EPIFISIÓLISE PROXIMAL DO FÊMUR POR MEIO DA ANÁLISE DO TRANSCRIPTOMA DA CARTILAGEM EM FRANGOS DE CORTE

DISSERTAÇÃO DE MESTRADO

LUDMILA MUDRI HUL

GUARAPUAVA-PR

2020

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Dissertação apresentada a Universidade Estadual do Centro-Oeste, como parte das exigências do Programa de Pós-Graduação em Ciências Veterinárias, área de concentração em Saúde e Produção Sustentável, para obtenção do título de mestre.

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2020

“As criaturas que habitam esta terra em que vivemos, sejam elas seres humanos ou animais, estão aqui para contribuir, cada uma com sua maneira peculiar, para a beleza e a prosperidade do mundo.”

Dalai Lama

Dedico aos meus pais, minha irmãzinha, minha querida Yulie, e meus queridos Luigi e Thunder.

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RESUMO

Ludmila Mudri Hul. Identificação de genes associados à epifisiólise proximal do fêmur por meio da análise do transcriptoma da cartilagem em frangos de corte.

Resumo: Na atual indústria avícola**,** os problemas locomotores estão entre os principais, causando grandes perdas econômicas e afetando o bem-estar animal. Entre as anomalias ósseas, as mais frequentes no fêmur são a discondroplasia, a epifisiólise ou separação da cabeça do fêmur e a Condronecrose Bacteriana com Osteomielite (BCO), também descrita como Necrose da Cabeça do Fêmur (NCF). No presente trabalho, objetivou-se identificar os genes diferencialmente expressos na cartilagem articular de frangos de corte normais e acometidos por epifisiólise proximal do fêmur por meio da análise de RNA-Seq. Para isso, um total de oito frangos de corte comerciais com 35 dias de idade foram utilizados, sendo quatro normais e quatro afetados com epifisiólise na cabeça do fêmur. Após a extração do RNA da cartilagem articular (CA), foram construídas as bibliotecas de cDNA para o sequenciamento total do RNA com a plataforma Illumina, seguindo um protocolo paired-end. As sequências que passaram pelo controle de qualidade no programa Seqy-Clean foram mapeadas com o genoma de referência da galinha (*Gallus gallus*, assembly GRCg6a) usando a ferramenta *STAR*. Em seguida, os genes diferencialmente expressos (DE) entre o grupo normal e afetado foram determinados usando o pacote edgeR do R considerando False Discovery Rate (FDR) ≤0.05 . Na análise do transcriptoma, 12.169 genes apresentam-se expressos na cartilagem articular do fêmur. Destes, 107 genes foram DE, dos quais 9 foram menos expressos e 98 foram superexpressos no grupo afetado em relação ao grupo normal. Para validação, a expressão relativa de 10 genes foi avaliada. O RNA total do tecido da CA foi isolado de amostras de 10 frangos de corte normais e 10 afetados com epifisiólise. Para a análise de qPCR, um conjunto de 10 genes referência foi avaliado quanto a sua estabilidade para uso como genes normalizadores sendo que os genes constitutivos utilizados foram *RPL5 (Proteína Ribossomal L5)* e *RPLP1 (Subunidade Lateral do Stalk Proteína Ribossômica P1*), que foram considerados os mais estáveis. Dos 10 genes avaliados, 6 foram validados: *AVBD1, AVBD2, ANK1, ADA, EPX, RHAG,* sendo mais expressos em frangos de corte afetados pela epifisiólise. Na análise funcional, utilizando o conjunto de genes DE do transcriptoma, 79 processos

biológicos (PB) foram identificados na base DAVID, sendo agrupados em 12 macrobioprocessos com a ferramenta REViGO. Os principais PB encontrados estavam envolvidos na resposta ao estímulo biótico, transporte de gás, ativação celular, catabolismo de derivados de carboidratos, regulação de múltiplos organismos, sistema imunológico, contração muscular, processo de múltiplos organismos, citólise, leucócitos e adesão entre células. Entre os genes potencialmente desencadeadores da epifisiólise podem ser destacados: a proteína induzível pelo *interferon alfa 6 (IFI6), adenosina desaminase (ADA), cathelicidin-3 (CATH3), beta defensina aviária 1 (AVBD1), beta defesina aviária 2 (AvBD2), ankirina 1 (ANK 1), quimiotaxina derivada de células leucocitárias 2 (LECT2) e colágeno tipo XII cadeia alfa 1 (COL13A1)*. Desta forma, neste estudo foi gerado o primeiro transcriptoma da cartilagem articular de frangos possibilitando identificar um conjunto de genes candidatos a desencadear alterações na placa de crescimento do fêmur.

Palavras-chave: galinha, necrose de cabeça de fêmur, qPCR, RNA Seq.

ABSTRACT

Ludmila Mudri Hul. Identification of genes associated with proximal femoral epiphysiolysis through the cartilage transcriptome analysis in broilers.

Abstract: In the current poultry industry, locomotor problems are among the main ones, causing major economic losses and affecting animal welfare. Among bone disorders, the most frequent in the femur are dyschondroplasia, epiphysiolysis or separation of the femoral head and Bacterial Chondronecrosis with Osteomyelitis (BCO), also described as Femoral Head Necrosis (FHN). In the present study, the aim was to identify the differentially expressed (DE) genes in the articular cartilage between normal broilers and those affected by proximal epiphysiolysis of the femur through RNA-Seq analysis. For this, eight commercial broilers with 35 days of age, four normal and four affected with epiphysiolysis in the head of the femur were used. After RNA extraction from the articular cartilage (CA), cDNA libraries were prepared for mRNA sequencing with the Illumina platform, following a paired-end protocol. The sequences that passed the quality control in the Seqy-Clean program were mapped with the chicken reference genome (*Gallus gallus*, assembly GRCg6a) using the STAR tool. The DE genes between the normal and affected groups were determined using the edgeR package from R considering False Discovery Rate (FDR) ≤0.05.In the transcriptome analysis, 12,169 genes were expressed in the femoral articular cartilage. Of these, 107 genes were DE between normal and affected chickens, of which 9 were downregulated and 98 were upregulated in the affected broilers. For validation, the relative expression of 10 genes was evaluated. The total RNA of AC tissue was isolated from 10 normal and 10 epiphysiolysis-affected broilers . For the analysis of qPCR, a set of 10 constitutive genes was previously evaluated for their stability to be used as reference gene in this study. Thus, the reference genes used were *RPL5 (Ribosomal Protein L5)* and *RPLP1 (Lateral Subunit of the Stalk Ribosomal Protein P1),* the most stable genes within the tested candidates. From those 10 genes evaluated, 6 were validated: *AVBD1, AVBD2, ANK1, ADA, EPX, RHAG,* being upregulatedin broilers affected by epiphysiolysis. In the functional analysis, using the set of genes DE, 79 biological processes (PB) were identified in the DAVID database, being grouped into 12 macrobioprocesses with the REViGO tool. The main PB found were involved in the response to the biotic stimulus, gas transport, cell activation, catabolism of carbohydrate derivatives, regulation of multiple organisms, immune system, muscle contraction, multiple organism process, cytolysis, leukocytes, and adhesion between

cells. Among the genes potentially triggering epiphysiolysis, the *protein inducible by interferon-alpha 6 (IFI6), adenosine deaminase (ADA), cathelicidin-3 (CATH3), avian beta-defensin 1 (AVBD1), avian beta defense 2 (AvBD2) , ankirin 1 (ANK 1), chemotaxine derived from leukocyte cells 2 (LECT2) and collagen type XII alpha chain 1 (COL13A1)* can be highlighted. In this study, the first transcriptome of the articular cartilage of chickens was generated and, thus, it was possible to identify a set of candidate genes to trigger changes in the growth plate of the femur.

Key words: chicken, femur head necrosis, PCR, RNA Seq.

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1. INTRODUÇÃO

Nas últimas décadas houve intensa seleção para que frangos de corte tenham maior e mais rápido crescimento. Entretanto, com isso houve um impacto negativo, já que a qualidade da estrutura do esqueleto de aves de corte foi desfavorecida. Consequentemente os problemas locomotores aumentaram significativamente, causando um impacto negativo no bem-estar, eficiência alimentar, taxa de crescimento e outras características (COOK, 2000; HAVENSTEIN; FERKET; QURESHI, 2003; DURAIRAJ et al., 2009)

Estima-se que há mais de US$ 100 milhões de dólares em perdas econômicas anuais devido a esses problemas na última década (COOK, 2000). Atualmente, os distúrbios ósseos ainda estão entre as principais preocupações para a indústria avícola (LI et al., 2015). Dentre estas afecções que afetam a locomoção das aves, a condronecrose bacteriana com osteomielite (CBO) é a causa mais comum de claudicação, afetando cerca de 1,5% dos frangos abatidos aos 42 dias idade nos Estados Unidos e é uma importante causa de mortalidade em populações de frangos de corte (WIDEMAN et al., 2012; WIDEMAN; PRISBY, 2013). No Brasil, não há dados consistentes sobre a incidência deste problema. Esta afecção é um dos distúrbios mais importantes no sistema locomotor em frangos comerciais e vem sendo diagnosticada em todo o mundo (MCNAMEE; SMYTH, 2000). Esta patologia também é conhecida como necrose da cabeça do fêmur (WIDEMAN et al., 2012). No entanto, há poucos estudos sobre essa patologia, que ainda não tem sua genética e mecanismos envolvidos completamente compreendidos (ALMEIDA PAZ et al., 2009; OLKOWSKI et al., 2011; WIDEMAN et al., 2012).

A epifisiólise consiste na separação da placa de crescimento da cartilagem articular, o que é um fator de risco para infecção, podendo ocasionar a condronecrose bacteriana com osteomielite em frangos de corte (RIDDELL, 1983; PACKIALAKSHMI et al., 2015). Dessa forma, a epifisiólise pode ser uma etapa inicial da BCO, pois possivelmente ela seja iniciada por dano a colunas mal mineralizadas de condrócitos (células de cartilagem) nas placas de crescimento epifisárias e fisárias dos ossos da perna, seguido de colonização das fendas osteocondríticas por bactérias oportunistas (WIDEMAN et al., 2012; WIDEMAN; PRISBY, 2013).

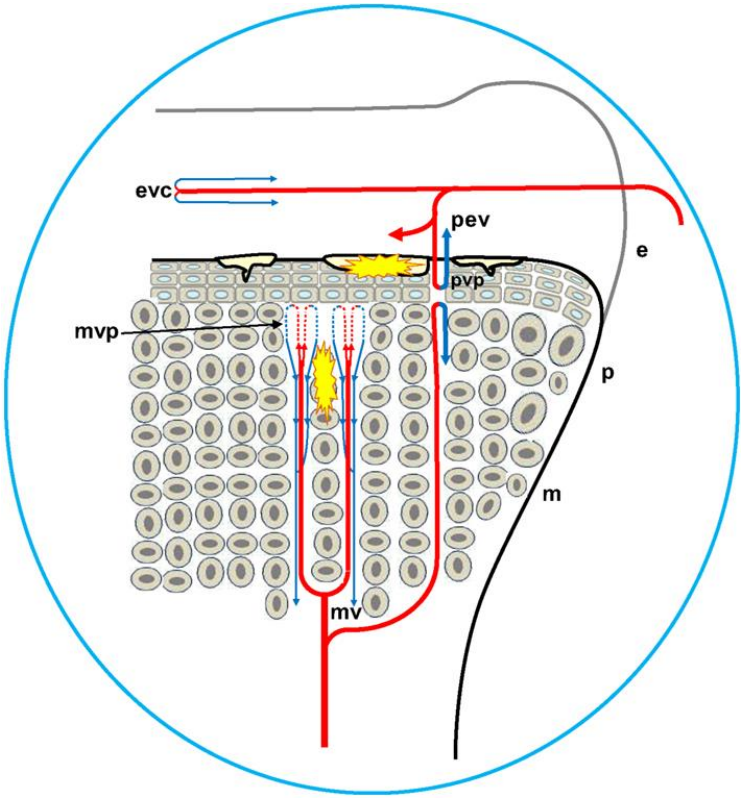

Figura representando a cabeça proximal do fêmur, com a formação de fendas osteocondróticas na delimitação entre a epífise (e) e a placa de crescimento (physis, p). Os vasos metafisários ascendentes (mv) penetram através dos canais entre longas colunas de células calcificantes na metáfise (m). O plexo vascular metafisário (mvp) é formado por curvas em gancho de cabelo nos capilares metafisários fenestrados que retornam como vênulas correndo pelo mesmo canal (setas azuis). Bactérias translocadas se espalham hematogenicamente e podem sair da corrente sanguínea através do endotélio fenestrado nas pontas do plexo vascular metafisário, aderindo diretamente à matriz da cartilagem, colonizam fendas osteocondróticas e zonas de necrose e formam embolia obstrutiva na vasculatura metafisária. Fonte: WIDEMAN; PRISBY, 2013.

Os componentes genéticos desempenham um papel considerável no desenvolvimento do sistema esquelético (COOK, 2000). De acordo com o banco de dados *chicken* QTLdb (base de dados de loci que controlam características quantitativas (QTL) em galinhas), QTL para características morfométricas, composição mineral e resistência de tíbia e fêmur foram mapeados em vários cromossomos da galinha, indicando regiões e genes importantes relacionadas ao sistema esquelético. No entanto, as vias funcionais, especialmente aquelas relacionadas com a epifisiólise e outros problemas de integridade óssea como a BCO, ainda são pouco conhecidas na galinha. Muitos dos estudos disponíveis sobre o tecido ósseo são realizados principalmente em humanos e roedores. Outro fato que deve ser levado em consideração é que as aves possuem uma

fisiologia óssea diferente dos mamíferos, sendo que sua remodelação óssea ocorre mais rapidamente que a dos mamíferos (JOHNSSON et al., 2015). Atualmente, não estão disponíveis estudos realizados com o transcriptoma da cartilagem de fêmur de frangos. Assim, para tentar esclarecer as vias envolvidas em alterações na placa de crescimento do fêmur, foram avaliados os transcriptomas da cartilagem da cabeça do fêmur de frangos normais e afetados por epifisiólise proximal do fêmur aos 35 dias de idade. Com isso, se visa obter alternativas para diminuir a incidência desse problema na produção avícola.

A avicultura também teve muitas mudanças, focando no melhoramento genético dos animais, buscando animais com melhor ganho de peso, crescimento mais rápido e eficiente. A redução de perdas na produção de frangos e a melhora na conversão alimentar das aves está diretamente ligada a sustentabilidade da indústria avícola, gerando um menor impacto para o meio ambiente com a redução de resíduos e diminuição no consumo de insumo pelas aves. No entanto, nos dias atuais nota-se uma maior preocupação com bem-estar e desenvolvimento sustentável, adaptando o manejo e buscando novas metodologias de criação, nos quais sejam beneficiados tanto os animais, com o bem-estar, quanto o criador/mercado, com o lucro oriundo dessa atividade (COOK, 2000; LEFF, 2001; HAVENSTEIN; FERKET; QURESHI, 2003; FEIDEN, 2005; MAZOYER; ROUDART, 2010; LI et al., 2015). Desta forma, este trabalho teve como objetivo identificar genes associados com a manifestação da epifisiólise na cartilagem articular proximal do fêmur em frangos de corte. Para isso, foram gerados os transcriptomas da cartilagem articular de animais normais e afetados por epifisiólise proximal do fêmur, identificados um conjunto de genes diferencialmente expressos associados a epifisiólise proximal do fêmur utilizando a técnica de RNA-Seq, que foram validados, por meio da técnica de qPCR. Para a validação por qPCR, foram escolhidos os genes mais estáveis dentre os genes avaliados para serem utilizados como referência nessa análise. Além disso, foram identificadas rotas metabólicas envolvidas no desenvolvimento de epifisiólise na cartilagem proximal do fêmur em frangos.

2. REVISÃO BIBLIOGRÁFICA

2.1 AVICULTURA E O MELHORAMENTO GENÉTICO

A produção de frangos vem sendo uma atividade de destaque no Brasil nas últimas décadas, mostrando um intenso crescimento tanto na produção quanto no consumo de carne. Isso pode ser observado em uma escala mundial também, com uma tendência ao contínuo crescimento no consumo e produção de carne de frango no mundo. Um dos principais motivos desse aumento no consumo de carne de aves se deve à ausência de obstáculos culturais ou religiosos, e as propriedades dietéticas e nutricionais, sendo uma carne com baixo teor de gordura, baixos níveis de sódio e colesterol (BARROETA et al., 2006; VALCESCHINI, 2006; CAVANI et al., 2009; GIVENS, 2009; ABPA, 2018). As mudanças no estilo de vida dos consumidores fizeram com que os mesmos procurassem produtos de rápido preparo e processados, um nicho que há tempos vem sendo explorado pela indústria avícola, resultando em fortes investimentos na área de processamento, aumentando a disponibilidade de variadas refeições prontas (CAVANI et al., 2009).

A contínua evolução técnica da avicultura brasileira nas áreas de genética, nutrição e manejo tem possibilitado a obtenção de produtos de baixo custo, alta qualidade e de comprovada competitividade mundial (CARRIJO et al., 2005; PETRACCI; CAVANI, 2012). Analisando a produção nos últimos anos, nota-se um aumento significativo, quando comparando dados do ano de 2006 com uma produção de 9,34 milhões de toneladas de carne e a produção de 2017, com 13.056 milhões de toneladas. As regiões Sul, Sudeste e Centro Oeste do país se destacam como as maiores produtoras de frangos de corte e exportadoras deste produto. O estado do Paraná lidera as exportações com 37,2%, seguido de Santa Catarina com 22,95% e Rio Grande do Sul com 17,58% demonstrando a importância do frango de corte para a economia brasileira (ABPA, 2018).

A crescente demanda por carne de frango resultou em pressão sobre os criadores, nutricionistas e produtores para produzir animais com maior taxa de crescimento, melhor eficiência alimentar, maior tamanho do músculo do peito e redução da gordura abdominal. Atualmente os animais são comercializados em cerca de metade do tempo e

cerca do dobro do peso corporal quando comparado com os de 50 anos atrás (BARBUT et al., 2008).

Desde o final da década de 1940 as empresas vêm se especializando na aplicação da seleção genética de frangos para consumo de carne (HAVENSTEIN et al., 1994). Com isso houve um aumento considerável na taxa de crescimento, melhora na conversão alimentar e diminuição na idade de abate para frangos de corte comerciais, o que levou ao desenvolvimento da indústria de frangos e sua capacidade para produzir carne de frango com um valor mais acessível (HAVENSTEIN; FERKET; QURESHI, 2003). Contudo, este tipo de seleção colocou mais pressão sobre a ave em crescimento, resultando em modificações no organismo das mesmas (LE BIHAN, 2003; BARBUT et al., 2008).

2.2 PROBLEMAS LOCOMOTORES

Devido ao intenso melhoramento genético que os frangos de corte foram submetidos no decorrer do tempo, houve um rápido aumento no ganho de peso e deposição de carne na carcaça, levando ao aparecimento de problemas indesejáveis para a produção, como os problemas locomotores (LEDUR et al., 2007). Apesar de saber que o desenvolvimento destas afecções seja multifatorial, o crescimento rápido e o aumento da massa muscular na região do peito causam sobrecarga aos ossos das pernas e são fatores importantes a serem considerados. O aumento da massa muscular na região do peito de frangos de corte de crescimento rápido também causou uma mudança no centro de gravidade para frente resultando em aves com padrão de andar diferente, sendo cansativo para as aves (CORR et al., 2003; FALCONE; OTTONI, 2007).

Os problemas locomotores têm contribuído para a redução na produtividade em função do aumento de condenações de carcaças inteiras. A incidência de distúrbios locomotores afeta em torno de 6 % de animais em lotes comerciais, causando grande impacto na avicultura mundial, ocasionando prejuízos econômicos para a avicultura (PAZ, 2008). Além disso, há a preocupação com o bem-estar das aves, uma vez que a ave que apresenta dificuldade para se locomover, vai menos ao comedouro e bebedouro, diminuindo a ingestão de alimento e água (CORDEIRO, 2009; BERNARDI, 2011).

Diversos transtornos locomotores afetam as aves, como a discondroplasia tibial, osteoporose, raquitismo, espondilolistese, síndrome da perna torta, necrose da cabeça

do fêmur, pododermatite, doença articular degenerativa, rotação da tíbia e a síndrome dos dedos tortos, além de agentes infecciosos que causam enfermidades, tais como: doença de Marek, micoplasmose, artrite viral e salmoneloses (LIMA et al., 2007). A Condronecrose Bacteriana com Osteomielite (BCO), também conhecida como Necrose da Cabeça do Fêmur (NCF), apresenta alta incidência em frangos de corte, sendo considerada a causa mais comum dos problemas locomotores (BRADSHAW; SAGE, 2001). A epifisiólise, por sua vez, consiste na separação da placa de crescimento da cartilagem articular, sendo um fator de risco para infecção, podendo desencadear a BCO em frangos de corte (RIDDELL, 1983; PACKIALAKSHMI et al., 2015).

2.3 TECIDO CARTILAGINOSO

O tecido cartilaginoso consiste em uma forma especializada de tecido conjuntivo de consistência semirrígida, avascular, com muita matriz extracelular esparsamente povoada por células (condrócitos) (CAMANHO, 2001; JUNQUEIRA; CARNEIRO, 2008). A matriz cartilaginosa é composta por 48% a 62% de colágeno tipo II e 22 a 38% de proteoglicanos (CAMANHO, 2001). Outros tipos de colágenos como V, VI, IX, X e XI, e outros colágenos secundários fazem parte da composição da matriz cartilaginosa em menor concentração (MANDELBAUM et al., 1998). Esse tecido possui um importante papel na fisiologia do animal, desempenhando diversas funções essenciais para o organismo, como: suporte de tecidos moles, revestimento de superfícies articulares, absorvendo choques e facilitando o deslizamento, e sendo importantes na formação e crescimento dos ossos longos (JUNQUEIRA; CARNEIRO, 2008).

Ha três diferenciações de cartilagens para atender diferentes necessidades funcionais. A cartilagem hialina é a mais comum, cuja matriz possui delicadas fibrilas constituídas principalmente de colágeno tipo II; a cartilagem elástica, possui poucas fibras de colágeno tipo II e abundantes fibras elásticas e a cartilagem fibrosa, que apresenta matriz constituída preponderantemente por fibras de colágeno tipo I (JUNQUEIRA; CARNEIRO, 2008).

Os condrócitos apresentam-se em disposição zonal, a matriz é composta de 68 a 85% de água, característica que atribui elasticidade ao tecido cartilaginoso (MANDELBAUM et al., 1998; BITTENCOURT, 2008). Macroscopicamente, a cartilagem articular é brilhante, lisa, branca e semirrígida (SCHILLER, A. L.; TEITELBAUM, 2002). Microscopicamente, a cartilagem articular divide-se em várias zonas. A zona superficial

apresenta células fusiformes com estrutura citoplasmática semelhante ao fibroblasto. Tanto os condrócitos quanto as fibras de colágenos estão organizados paralelamente à superfície articular. A zona intermediária apresenta condrócitos arredondados, apresentando prolongamentos citoplasmáticos, e se dispõe em filas alongadas e irregulares. As fibras colágenas se organizam de modo perpendicular à superfície articular. Na zona mais profunda os condrócitos tendem a hipertrofiar e degenerar. No microscópio eletrônico observa se um acúmulo de glicogênio e material lipídico no citoplasma. Há o acúmulo de cristais de hidroxiapatita, levando à calcificação da matriz cartilaginosa (HIROHATA, K. MORIMOTO, K. KIMURA, 1981). Durante o período de crescimento esta região é substituída por tecido ósseo, enquanto que as células da zona intermediária se dividem por mitose, permitindo o aumento da epífise óssea (HAM, 1977). As fibras de colágeno, na zona mais profunda, estão firmemente inseridas no osso subcondral, dando estabilidade à cartilagem articular (BENTLEY et al., 2001).

Por não possuir vasos sanguíneos, o tecido cartilaginoso é nutrido por capilares do conjuntivo envolvente (pericôndrio) ou através do líquido sinovial das cavidades articulares. O tecido cartilaginoso também não possui vasos linfáticos e nervos (LANZER; KOMENDA, 1990; JUNQUEIRA; CARNEIRO, 2008). Quando lesionada, a cartilagem regenera-se com dificuldade e, na maioria das vezes, de modo incompleto. Quando ocorre uma lesão, células derivadas do pericôndrio invadem a área da fratura e dão origem a tecido cartilaginoso que repara a lesão. Em alguns casos, há formação de uma cicatriz e tecido conjuntivo denso (JUNQUEIRA; CARNEIRO, 2008).

2.4 CONDRONECROSE BACTERIANA COM OSTEOMIELITE (CBO)

A Condronecrose Bacteriana com Osteomielite, também descrita como NCF, é uma anomalia óssea que afeta a região proximal do fêmur, causando degeneração da epífise femoral levando a perdas significativas na produção. O conceito de necrose se refere ao estado de morte de um tecido, onde primeiramente ocorre a falta de vascularização no osso e em seguida há um processo patológico e desordenado de morte celular (CAPONI, 2009). Os fatores que desencadeiam esta patologia nas aves ainda não estão claros, mas fatores ambientais como manejo, idade, nutrição, e genética, como crescimento rápido já foram relacionados com a susceptibilidade da doença (KEALY, 1987; WIDEMAN et al., 2013). O rápido crescimento muscular pode influenciar

o aparecimento da NCF, pois o frango de corte vai de 40g a 4kg de peso vivo em até 8 semanas de idade, afetando a integridade estrutural do esqueleto (WIDEMAN; PRISBY, 2013). Nesta patologia, observa-se alteração no tecido ósseo e degeneração da cartilagem e da epífise femoral, onde ocorre a angiogênese (ALMEIDA PAZ et al., 2009). Uma das supostas causas iniciais da BCO é a epifisiólise, a qual consiste na separação da placa de crescimento da cartilagem articular, sendo um fator de risco para infecção, podendo ocasionar a condronecrose bacteriana com osteomielite em frangos de corte (RIDDELL, 1983; PACKIALAKSHMI et al., 2015).

A NCF ocorre na parte proximal da cabeça do fêmur, iniciando com a degeneração da cartilagem articular e da placa de crescimento, ocorre à separação da cartilagem do osso, comumente entre a placa de crescimento e a região metafisária óssea. Em seguida, devido a desproteção do osso, tem início a degeneração óssea, o que pode promover maior incidência de outras patologias locomotoras, tornando-se uma porta de entrada para vírus e bactérias. Os animais apresentam sérios problemas para se locomover, afetando-os de forma uni ou bilateral (KEALY, 1987). Os animais apresentam grande dificuldade para beber e se alimentar de forma correta, sendo que o caso se agrava com o decorrer da idade e com o aumento de peso do animal, também resultando na redução do bem-estar das aves (THORP et al., 1993; GONZALES; MENDONÇA JÚNIOR, 2006). Já foram isoladas das lesões de NCF várias bactérias oportunistas como *Staphylococcus* spp, *Escherichia coli* e *Enterococcus* spp., sugerindo que as lesões encontradas nos ossos favorecem a sobrevivência destas bactérias aumentando a associação com a progressão da NCF (JIANG et al., 2015).

2.5 ESTUDOS DO TRANSCRIPTOMA

O transcriptoma é o conjunto completo de transcritos de uma célula, podendo proporcionar conhecimento dos mecanismos moleculares dos processos biológicos que estão ocorrendo em uma célula em determinado momento (LI et al., 2012). Um transcrito de RNA pode estar sujeito a uma ampla gama de diferentes processos regulatórios, logo a regulação do transcriptoma é fundamental para processos fisiológicos, patológicos e de desenvolvimento, pois o perfil de expressão do mRNA determinará as características das células ou dos tecidos em seu estado específico. Os perfis de um transcriptoma em resposta a estímulos biológicos e fisiológicos fornecem informações e respostas valiosas para a interpretação dos elementos funcionais do genoma, revelando mecanismos

moleculares das células, e para a compreensão do desenvolvimento de processos biológicos fundamentais (BLENCOWE; AHMAD; LEE, 2009; WANG et al., 2011). O desenvolvimento de métodos de sequenciamento de alto rendimento para a análise de diversos conjuntos de RNA, também conhecido como “RNA-Seq”, proporcionou um grande avanço nessa direção (BLENCOWE; AHMAD; LEE, 2009).

O RNA-seq é uma técnica recente, capaz de sequenciar o que está sendo expresso em um determinado tecido, em um momento especifico, possibilitando a análise do transcriptoma (TANG et al., 2010; WANG et al., 2011). Depois da extração total do RNA, seleciona-se o mRNA (RNA mensageiro) através da cadeia poli (A), que é fragmentado e convertido a uma biblioteca de cDNA, fornecendo de maneira simples e abrangente a composição do transcriptoma (WANG et al., 2011). Através do sequenciamento do mRNA é possível descobrir novos genes, quantificar a expressão gênica, facilitando a anotação e montagens de novos transcritos, a identificação de sítios de splicing e a identificação polimorfismos (SUNKARA; JIANG; ZHANG, 2012; TRAPNELL et al., 2012). Com isso, a partir da comparação de transcriptomas é possível compreender como as mudanças na atividade celular podem interferir em uma determinada doença (OKAZAKI et al.,2002).

2.6 PCR QUANTITATIVA (qPCR) E GENES REFERÊNCIA

A PCR em tempo real é uma das técnicas mais utilizadas para quantificar a expressão gênica devido à sua sensibilidade metodológica e reprodutibilidade. No método da quantificação relativa, a expressão de um gene alvo é quantificada por meio da normalização com um ou mais genes de referência. Contudo a expressão dos genes endógenos pode ser influenciada por condições ambientais ou patológicas, podendo distorcer seriamente a interpretação de resultados quantitativos (JOHANSSON et al., 2007).

Os genes referência, também conhecidos como endógenos, são usados em estudos de expressão gênica para auxiliar no controle da variação na quantidade inicial de material estudado, qualidade do RNA e as diferenças na síntese de cDNA, medindo se os resultados encontrados se referem ou não aos tratamentos estudados (JOHANSSON et al., 2007; WEI et al., 2014). Logo, conhecer o comportamento desses genes em cada delineamento experimental é fundamental para obtenção de resultados confiáveis (DHEDA et al., 2004; REBOUÇAS et al., 2013). Dentre os genes de referência,

os mais conhecidos são *GAPDH (gliceraldeído 3-fosfato desidrogenase), PGK (fosfoglicerato quinase), UBQ (ubiquitina), RPL19 (proteína ribossômica L19), 18S rRNA (RNA ribossomal 18S), β-actina e β -tubulina* (REBOUÇAS et al., 2013). Esses genes têm sido utilizados em diversas espécies para estudos com diferentes tecidos. Os genes *GAPDH* e 18S *rRNA* foram utilizados como genes de referência individuais para a normalização de dados de qPCR em múltiplos estudos em frangos de corte (HONG et al., 2006; RICHARDS; POCH; MCMURTRY, 2006; ADAMS et al., 2009; ZHANG et al., 2012a; HRABIA; LEŚNIAK; SECHMAN, 2013). Mesmo sendo genes relativamente estáveis, os genes de referência podem ter sua estabilidade alterada dependendo do tecido, idade, tratamento e outras condições. Com isso, se torna indispensável testar a estabilidade de vários genes antes de usá-los como referência (UDDIN et al., 2011a; WANG et al., 2012, 2015). A seleção de um ou mais genes de referência estável é crítica para o desempenho confiável dos experimentos (TANG et al., 2010).

2.7 AVICULTURA E SUSTENTABILIDADE NOS DIAS ATUAIS

A aplicação da visão mecanicista e reducionista aos sistemas naturais, à agricultura e produção animal, proporcionaram um aumento nos ganhos de produtividade, redução de preços e *superavits* na produção de alimentos. Contudo, também ocorreram impactos negativos como degradação do solo, desperdício e uso exagerado de água, poluição do ambiente, dependência de insumos externos e perda da diversidade genética (FEIDEN, 2005). O conceito de sustentabilidade surge do reconhecimento da função de suporte da natureza, condição e potencial do processo de produção (SACHS, 1982). Mas em muitas discussões, o conceito de sustentabilidade pode ter significados ou interpretações diferentes, sendo necessário uniformizar esse entendimento (FEIDEN, 2005). Para isso é importante compreender um significado que seja de concordância geral (GLIESSMANN, 2001).

Em 1960, evidencia-se a crise ambiental, decorrente da irracionalidade ecológica dos padrões dominantes de produção e consumo, marcando os limites de crescimento econômico. Com isso, tem início o questionamento teórico político para que se valorize a natureza e internalize as externalidades socioambientais ao sistema econômico (SACHS, 1982; LEFF, 2001). Posteriormente, surgiram novos paradigmas da economia

ecológica, no qual se visou integrar o processo econômico com a dinâmica ecológica populacional (COSTANZA, 1996).

A degradação ambiental é um sintoma da crise de civilização, onde o desenvolvimento da tecnologia está acima da natureza. Pela problemática ambiental ter se tornado uma questão política, há uma emersão dos conflitos socioambientais provenientes de princípios ético, direitos culturais e lutas por apropriação da natureza, indo além dos custos ecológicos para assegurar um crescimento sustentável. Há uma busca pela conciliação do meio ambiente e o crescimento econômico, no qual há crescimento econômico como um processo sustentável, assegurando equilíbrioecológico e igualdade social, buscando valorizar os recursos naturais e culturais (LEFF, 2001).

A avicultura teve muitas mudanças, focando no melhoramento genético dos animais, buscando animais com melhor conversão alimentar e crescimento mais rápido. Essas melhoras também influenciaram na quantidade de agua necessária para produção, onde, para produzir 1kg de carne de frango há a necessidade de aproximadamente 4,5 L de agua, sendo bem menos que a quantidade de água necessária para produzir 1kg de carne suína (aproximadamente 6 L de água) e carne bovina (aproximadamente 15,4 L) (PIMENTEL et al., 2004). Essas melhorias no desempenho do frango de corte, redução da idade de abate e menor consumo de água para produção de carne estão diretamente ligadas a sustentabilidade da indústria avícola, gerando um menor impacto para o meio ambiente.

2.8 BEM ESTAR ANIMAL

Nos dias atuais nota-se uma maior preocupação com bem-estar e desenvolvimento sustentável, adaptando o manejo e buscando novas metodologias de criação, nos quais sejam beneficiados tanto os animais, com o bem-estar, quanto o criador/mercado, com o lucro oriundo dessa atividade (COOK, 2000; LEFF, 2001; HAVENSTEIN; FERKET; QURESHI, 2003; FEIDEN, 2005; MAZOYER; ROUDART, 2010; LI et al., 2015).

O bem-estar animal tem ganhado destaque nas últimas décadas, sendo que principalmente nos países europeus tem-se tido uma exigência maior com relação ao tema. Por ser um tema amplo, há uma variação muito grande com relação a sua interpretação, no qual há uma diversidade no contexto de acordo com diferentes autores. Nota-se uma preocupação com os impactos que as mudanças e implementação de um

manejo voltado ao bem-estar causa na criação e produtores, a razão da preocupação com os animais de produção, que muitas vezes são vistos apenas como uma fonte de renda e quais alterações podem ser feitas para que o animal tenha bem-estar sem prejudicar o produtor, buscando uma harmonia entre ambos (HELLMEISTER FILHO et al., 2003).

Pelo fato de terem sido domesticados, serem criados em cativeiro e servindo de alguma maneira, há um consenso de que os mesmos merecem níveis mínimos de bem-estar (FRASER, A. F., BROOM, 1990). Nos países desenvolvidos, cresce a preocupação com os maus tratos aos animais nas áreas urbanas e conforto dos animais utilizados na pesquisa e na produção animal. Apesar de ser em menor intensidade, a preocupação com a qualidade de vida dos animais vem aumentando no Brasil (HÖTZEL; MACHADO FILHO, 2004).

O debate pelo bem-estar animal se deu início em 1964, com o livro Animal Machines, escrito por Ruth Harrison, que introduziu o debate sobre a ética da produção animal, denunciando os maus tratos a que os animais de confinamento recebiam na Grã-Bretanha. Em 1965, o Comitê Brambell, recém-criado e reconhecendo as dificuldades encontradas pelos animais na agricultura moderna, apresentou as cinco liberdades que todo animal deveria possuir. Essas cinco liberdades eram constituídas pelo direito de virar-se; cuidar-se corporalmente; levantar-se; deitar-se e estirar seus membros. O debate que se seguiu levou a uma visão um tanto quanto simples dos problemas da agropecuária e do bem-estar animal, não promovendo o entendimento entre as partes interessadas (FRASER, 2001). Posteriormente, as cinco liberdades foram modificadas, sendo constituídas por liberdade para expressar comportamento da espécie, estar livre de fome e sede; livre de desconforto; livre de dor, doença e injúria; livre de medo e estresse (AUTRAN; ALENCAR; VIANA, 2017).

Há dois lados a serem considerados: em um lado apresenta-se uma visão inteiramente negativa da agricultura animal, divulgada por grupos de defesa dos direitos dos animais, e do outro, uma visão totalmente positiva da mesma, difundida por organizações de criadores de animais e pela indústria. Ambas são visões simplistas e extremas, com isso há uma camuflagem da complexidade das diferentes realidades da agricultura, as quais também levantam temas e preocupações genuínas que devem ser consideradas por estudiosos da área de bem-estar animal (FRASER, 2001).

Alguns dos principais fatores que podem influenciar o bem-estar na criação de animais de produção estão relacionadas ao manejo. Em sistema intensivos, algumas

vezes os animais são submetidos a formas de transporte e manejo pré-abate inadequados e a mutilações realizadas no manejo de rotina. Deve-se atentar a qualidade dos cuidados por parte dos humanos e a seleção genética para alta produção que influenciam a qualidade de vida, saúde e longevidade dos animais (HÖTZEL; MACHADO FILHO, 2004). Devido a isso, boa parte das pesquisas aplicadas ao bem-estar animal estão situadas nos efeitos do ambiente, como alojamento e manejo, incluindo a relação humano- animal - na fisiologia, produtividade e comportamento dos animais durante as diversas fases da criação até o abate (HÖTZEL; MACHADO FILHO, 2004).

Já, alguns problemas de bem-estar estão relacionados ao sistema de criação confinado, devido ao ambiente relativamente pouco complexo e espaço insuficiente onde os animais não estão aptos a desenvolver o padrão comportamental próprio da sua espécie. Com isso, o estresse social decorrente das altas densidades e falta de espaço, acabam causando estresse nos confinamentos. Nesse cenário, nota-se um aumento na incidência de bicagem das penas e canibalismo em aves (WECHSLER; HUBER-EICHER, 1998) e comportamentos anormais redirecionados a objetos e partes das baias em suínos (LAWRENCE; TERLOUW, 1993) que podem ser acompanhados de estresse fisiológico (DE JONGE et al., 1996; DE JONG et al., 2000; DE JONG et al2002).

O bem-estar animal pode ser definido como “estado de harmonia entre o animal e seu ambiente, caracterizado por condições físicas e fisiológicas ótimas e alta qualidade de vida do animal” (HURNIK, J.F.; PHILLIPS, C.; PIGGINGS, 1992). Mas há autores que propõe que bem-estar não é um atributo dado pelo homem aos animais, mas uma qualidade inerente a estes. Sendo assim, refere-se ao estado de um indivíduo do ponto de vista de suas tentativas de adaptação ao ambiente, a quanto tem de ser feito para o animal conseguir adaptar-se ao ambiente e ao grau de sucesso com que isto está acontecendo (BROOM, 1991; MENCH, 1993). Logo, o bem-estar pode variar entre muito ruim e muito bom e pode ser avaliado cientificamente a partir do estado biológico do animal e de suas preferências (BROOM, 1991)

O bem-estar também pode ser avaliado através da produtividade, sucesso reprodutivo, taxa de mortalidade, comportamentos anômalos, severidade de danos físicos, atividade adrenal, grau de imunossupressão ou incidência de doenças (BROOM, 1991; MENCH, 1993). No entanto, deve-se destacar que o sofrimento normalmente está relacionado com o bem-estar, mas a ausência de sofrimento não é, necessariamente, sinônimo de bem-estar (BROOM, 1991).

Há alguns anos vem se utilizado o melhoramento genético para melhorar a eficiência produtiva dos animais, com isso houve consequências como diminuição na imunidade e os problemas locomotores. Mas com o aumento da atenção dos consumidores ao bem-estar animal, a seleção genética também se volta para aumentar o bem-estar animal, identificando genes responsáveis por certas alterações e tentando inibi-los das populações, beneficiando tanto os animais, quanto os produtores (HELLMEISTER FILHO et al., 2003; HÖTZEL; MACHADO FILHO, 2004).

Com isso, o estudo dos genes candidatos é muito importante, pois através do mesmo, é possível determinar associações entre variações genéticas com algum distúrbio e identificar quais os genes responsáveis por essa condição. Dessa forma, o presente trabalho teve como objetivos: gerar os transcriptomas da cartilagem articular de frangos normais e afetados por epifisiólise proximal do fêmur, identificar um conjunto de genes diferencialmente expressos associados à epifisiólise proximal do fêmur, identificar genes referência para uso em análise de PCR quantitativo de cartilagem de frangos visando a validação de resultados obtidos pelo RNA-Seq e identificar rotas metabólicas envolvidas no desenvolvimento de epifisiólise em frangos.

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3. CAPÍTULO I

Artigo submetido a Revista PlosOne

Reference genes for proximal femoral epiphysiolysis expression studies in broilers cartilage

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Abstract

The use of reference genes is required for relative quantification in gene expression analysis and the stability of these genes can be variable depending on the experimental design. Therefore, it is indispensable to test the reliability of endogenous genes previously to their use. This study evaluated 10 candidate reference genes to select the most stable genes to be used as reference in gene expression studies with the femoral cartilage of normal and epiphysiolysis-affected broilers. The femur articular cartilage of 29 male broilers with 35 days of age was collected, frozen and further submitted to RNA extraction and quantitative PCR (qPCR) analysis. The candidate reference genes evaluated were B2M, GAPDH, HMBS, HPRT1, MRPS27, MRPS30, RPL30, RPL4, RPL5, and RPLP1. For the gene stability evaluation, three software were used: GeNorm, BestKeeper and NormFinder, and a global ranking was generated using the function RankAggreg. In this study, the RPLP1 and RPL5 were the most reliable endogenous genes being recommended for expression studies with femur cartilage in broilers with epiphysiolysis and possible other femur anomalies.
Keywords: Endogenous genes, Gene expression, qPCR, Poultry.

Introduction

The use of gene expression analysis intends to clarify biological processes involved with several conditions in living organisms enabling the identification of diagnostic markers as therapeutic targets in the treatment of the diseases (1). The quantitative PCR (qPCR) is a fast, easy-to-use technique that provides simultaneous measurement of gene expression in many different samples for a limited number of genes (2). In qPCR, fluorescent dyes are used to combine the amplification and detection steps of the PCR reaction in a single tube (3,4). In addition, qPCR has been widely used for validating RNA-seq results due to its high sensitivity and precision (5). When comparing to other techniques, it has advantages such as sensitivity, real-time detection of reaction progress, rapid results and accuracy in the measurement of the analyzed material (5,6). Although the qPCR is a highly sensitive technique (7), its use must be standardized, especially considering the correct choice of reference genes to avoid mistaken results. The use of stable reference genes ensures the normalization in input RNA levels between samples, avoiding errors in the quantification (8). Therefore, knowing the expression profile of these genes in each experimental design is crucial to obtain reliable results (7,9).

A valid reference gene should have its expression constant between different experimental conditions, tissues or physiological state of the tissue or organism (8). In relative quantification analyses, the use of reference genes is required to normalize the gene expression and to obtain the fold-change through mathematical algorithms (10–12). Some of the most well-known reference genes are *GAPDH (glyceraldehyde 3-phosphate dehydrogenase), PGK (phosphoglycerate kinase), UBQ (ubiquitin), RPL19 (ribosomal protein L19), 18S rRNA (ribosomal RNA 18S), β-actin and β-tubulin* (7), which are used in several studies with many species. For instance, *GAPDH* and *18S rRNA* were used as individual reference genes for normalization of qPCR data in multiple studies in chickens (13–17).

The stability of reference genes can be altered depending on the tissue, age, treatment and other conditions, which makes it indispensable to test the stability of several genes before using those as reference (18–20). The selection of stable reference genes is critical for the reliable performance of qPCR experiments (21).

The epiphysiolysis or femur head separation (FHS) consists of the separation of the growth plate from the articular cartilage (22). This condition is a risk factor for infection and may cause bacterial chondronecrosis with osteomyelitis (BCO) in broilers (22,23). BCO is also named as femur head necrosis (FHN) and occurs in the proximal femoral head, beginning with the degeneration of the articular cartilage and the growth plate. The bone degeneration begins due to bone deprotection, which may promote a high incidence of other locomotor pathologies, becoming a gateway for viruses and bacteria. Animals have serious problems with movement, affecting them unilaterally or bilaterally (24).

Hence, expression studies are required to clarify the genetic mechanism involved with femur pathologies. Although some reference genes were described for bones (PALUDO et al., 2016; PEIXOTO et al., 2020), it is important to elucidate those for the cartilage as well, since both tissues are involved in the development of those disorders. However, studies on candidate reference genes for chicken cartilage are not available to date. Therefore, to obtain stable genes to be used as reference in expression studies related to bone/cartilage disorders in broilers, the present study evaluated 10 endogenous candidate genes in the articular cartilage of normal and affected chickens with 35 days of age.

Material and Methods

Animals and sample collection

The Embrapa Swine and Poultry National Research Center Ethical Committee of Animal Use (CEUA/CNPSA) approved this study under the protocol number 012/2012. The samples used were previously collected from chickens at 35 days of age, as described in detail by Peixoto et al. (2020) (27). Briefly, 29 male broilers from a commercial line (14 normal and 15 with lameness) were selected and sent to the Embrapa Swine and Poultry National Research Center, in Concórdia, SC, Brazil for sampling. The femur proximal head was classified based on the clinical examination of the separation of the growth plate (GP) from the articular cartilage (AC), according to Wideman et al. (2012) (25). The normal (control) group (CG) was characterized by good adhesion between the GP and the AC, and the epiphysiolysis-affected group (AG) presented epiphysiolysis and, consequently, separation between the GP and AC. From the 29 broilers, the AC of eight normal (with average body weight of 2,336.75 ± 233.43 g) and 8 affected broilers (2,189.62 ± 444.23g) were collected, stored in liquid nitrogen and transferred to a freezer at -80ºC until the samples were processed.

RNA extraction

Eight samples of the cartilaginous tissue from each group were ground in a mortar with liquid nitrogen. Then, Trizol reagent (Invitrogen, Carlsbad, CA, USA) was added for total RNA extraction, following the manufacturer's recommendations. To each 100mg of tissue, 1mL of Trizol was added and samples were vortexed and incubated for five minutes at room temperature. Then, 200µL of chloroform were added, the tubes were vigorously homogenized for 15 seconds and incubated at room temperature for five minutes. Centrifugation was performed at 16,000 xg at 4ºC for fifteen minutes. The aqueous phase (containing the RNA) was separated into a new microtube, and 500µL of isopropanol were added, gently homogenized by inversion and incubated for 10 minutes at room temperature. Samples were centrifuged for 13,000 xg for 10 minutes at 4 °C for RNA pellet formation. Subsequently, the supernatant was discarded and 1mL 75% Alcohol was added, homogenized and centrifuged at 10,500 xg for five minutes at 4 ºC. After discarding the supernatant, the remained pellet was dried for 15 to 20 minutes at room temperature and resuspended in 40µL of ultrapure water. To assure the samples quality and purity, the RNA clean up protocol was performed using the Qiagen Rneasy kit (Qiagen, Hilden, NRW, Germany), following the manufacturer´s instructions. The Biodrop (Biodrop, Cambridge, UK) spectrophotometer equipment was used to quantify the extracted samples, and samples with 260/280nm ratio above 1.8 were considered pure. To evaluate the integrity of each extracted sample, a 1.0% agarose gel was run and electrophoresed for 90 min.

Complementary DNA (cDNA) synthesis

The cDNA synthesis was performed using the SuperScript III First-Strand Synthesis SuperMix kit (Invitrogen, Carlsbad, CA, USA.), following the manufacturer's recommendations. Approximately 3 µg of total RNA were mixed with 1µL of 50µM OligoDT primer, and added 1 µL of Annealing buffer, completing the reaction up to 10µL. The samples were incubated in a thermocycler at 65 °C for five minutes and

chilled on ice for one minute. In sequence, 10 µL of the 2X First-Strand Reaction Enzyme Mix and 2µL of the SuperScript III/RNAseOUT Enzyme Mix (Invitrogen, Carlsbad, CA, USA) were added in the initial reaction for each sample. Subsequently, samples were incubated at 50°C for 50 minutes followed by 85 °C for five minutes in the thermocycler. After, the samples were stored in a freezer -20°C for subsequent quantitative Polymerase Chain Reaction (qPCR).

Real-time qPCR

In order to perform the qPCR analyses, primers for 10 genes: *B2M, GAPDH, HMBS, HPRT1, MRPS27, MRPS30, RPL30, RPL4, RPL5* and *RPLP1* were designed using NCBI Primer-BLAST (Table 1). These genes were selected based on previous studies in chicken (8, 9, 18, 19, 20). For the quantification analyses, the reactions were prepared using 7.5 µL of Master Mix SYBR Green 2x, 0,166 µM of forward primer, 0,166 µM of reverse primer, 2 µl of cDNA at the 1:10 dilution and ultrapure water to complete 15 µL. The reactions were distributed in 96-well plates of Microamp Fast 96-Well Reaction Plate (Applied Biosystems, Foster City, CA, USA) and then submitted to the QuantStudio 6 Real-Time PCR equipment (Applied Biosystems, Foster City, CA, USA) with a temperature cycling of 95ºC for 10 minutes, 40 cycles of 95ºC for 15 seconds and 60ºC for one minute. The melting curve was performed with the cycling of 95ºC for 15 seconds, 60ºC for 1 minute and 95ºC for 15 seconds to evaluate the amplification specificities. Reactions were performed in duplicates to calculate the Ct (threshold cycle) mean, standard deviation and coefficient of variation between the two replicates.

Stability Evaluation of the Candidate Reference Genes

To evaluate the candidate reference genes from this study, three algorithms were used to identify the most stable expressed genes: geNorm (28), NormFinder (29) and BestKeeper (30). The BestKeeper is an Excel-based software that classifies genes through an index (gene power) composed of Ct, fold-change (FC), standard deviation (SD) and coefficient of variation (CV) values (30). The most consistent genes present SD values of Cts less than 1 and SD of FC less than 2. The authors recommend not using genes with SD of Cts above 1.5 (30). The GeNorm is a program that calculates a measurement of stability of the internal control gene (M) for each combination of two control genes tested, obtaining a transformed expression rate, and then calculates a standard deviation of those combinations of paired genes. From the lowest values of M, the two most stable genes are determined and values smaller than 1.5 indicate stable genes (28). The NormFinder is a program that automatically calculates the stability value based on intra- and inter-group variation of the tested genes, considering their co-regulation, classifying genes according to their expression stability and similarity. Lower stability values indicate better or more stable genes to be used as normalizers. The author suggested using data transformed into $\log_2$ (29). When comparing with GeNorm, Bestkeeper has the advantage of using both constitutive and target genes in the analysis, while GeNorm analyzes only reference genes (30).

After performing each analysis, the stability values obtained in each tool were used to generate a ranking with the most stable genes using RankAggreg (31). This is a package from the R environment that calculates the Spearman distance between two genes based on the Monte Carlo algorithm (32,33). All of these analyses were

performed using the endoGenes automatized pipeline available at https://github.com/hanielcedraz/endoGenes.

Table 1. Primers for the candidate reference genes used for qPCR analysis in the femur head's cartilage of broilers.

Gene/ Ensembl ID	Functions	Primer Sequences (5'-3')
HMBS[1] Hydroxymethylbilane synthase *ENSGALG00000042939*	Heme synthesis, porphyrin metabolism, Third enzyme of the biosynthetic pathway of the Hemegroup	F: ACTAGTTCACTTCGGCGAGC R: CTCAGGAGCTGACCTATGCG
RPL5[2] Ribosomal Protein L5 *ENSGALG00000005922*	Responsible for the synthesis of proteins in the cell, structural constituent of ribosome, 5S rRNA binding	F: AATATAACGCCTGATGGGATGG R: CTTGACTTCTCTCTTGGGTTTCT
MRPS27[2] Mitochondrial Ribosomal Protein S27 *ENSGALG00000015002*	Mitochondrial ribosome binding, rRNA binding, tRNA binding	F: GCTCCCAGCTCTATGGTTATG R: ATCACCTGCAAGGCTCTATTT
MRPS30[2] Mitochondrial ribosomal protein S30 ENSGALG00000014874	Structural constituent of ribosome, RNA binding.	F: CCTGAATCCCGAGGTTAACTATT R: GAGGTGCGGCTTATCATCTATC
RPL4[3] Ribosomal Protein L4 *ENSGALG00000007711*	poly(U) RNA binding, rRNA binding, structural constituent of ribosome	F: TGTTTGCCCCAACCAAGACT R: CTCCTCAATGCGGTGACCTT
HPRT1[4] Hypoxanthine-guanine phosphoribosyltransferase *ENSGALG00000006098*	Purine synthesis in the salvage pathway	F: TGGGGATGACCTCTCAACCT R: TCCAACAAAGTCTGGCCGAT
GAPDH[4] Glyceraldehyde-3-Phosphate Dehydrogenase ENSGALG00000014442	Transcription, RNA transport, DNA replication, and apoptosis.	F: TGGGAAGCTTACTGGAATGG R: ATCAGCAGCAGCCTTCACTAC
RPLP1[4] Ribosomal protein lateral stalk subunit P1 *ENSGALG00000030878*	Protein kinase activator activity, ribonucleoprotein complex binding, structural constituent of ribosome.	F: CCCTCATTCTCCACGACGACZ R:CCAGAGCCTTAGCAAAGAGAC
RPL30[3] Ribosomal Protein L30 *ENSGALG00000029897*	Antimicrobial humoral immune response mediated by an antimicrobial peptide, cytoplasmic translation, defense response to Gram-negative bacterium, the killing of cells of another organism.	F: ATGATTCGGCAAGGCAAAGC R: GTCAGAGTCACCTGGGTCAA
B2M[4] Beta-2-microglobulin *ENSGALG00000002160*	Encodes a major histocompatibility complex (MHC) class I heavy chain protein in the surface of nucleated cells	F: CTTCCACCCACCCAGGATCA R: ACTCGGGATCCCACTTGAAGAC

[1]Paludo et al. [18] (34), [2] Nascimento et al. [15] (35), [3] Petry et al. [18] (36), [4] Marciano et al. [20] (37).

Results

The total RNA average concentration was 195.92 ng/μL for the normal and 172.01 ng/μL for the epiphysiolysis-affected broilers. Regarding RNA quality, the mean A260/280 ratio was 2.07 for normal and 2.08 for the affected chickens. These values

demonstrate good samples' quality. The Ct mean (± SD) values of the candidate reference genes ranged from approximately 18.51 ± 0.65 to 28.68 ± 0.59 (Fig 1, Table 2). One gene, *B2M* globulin, had no amplification and was removed from the analyses. According to the melting curve analysis, all genes showed specific amplification (Fig 2).

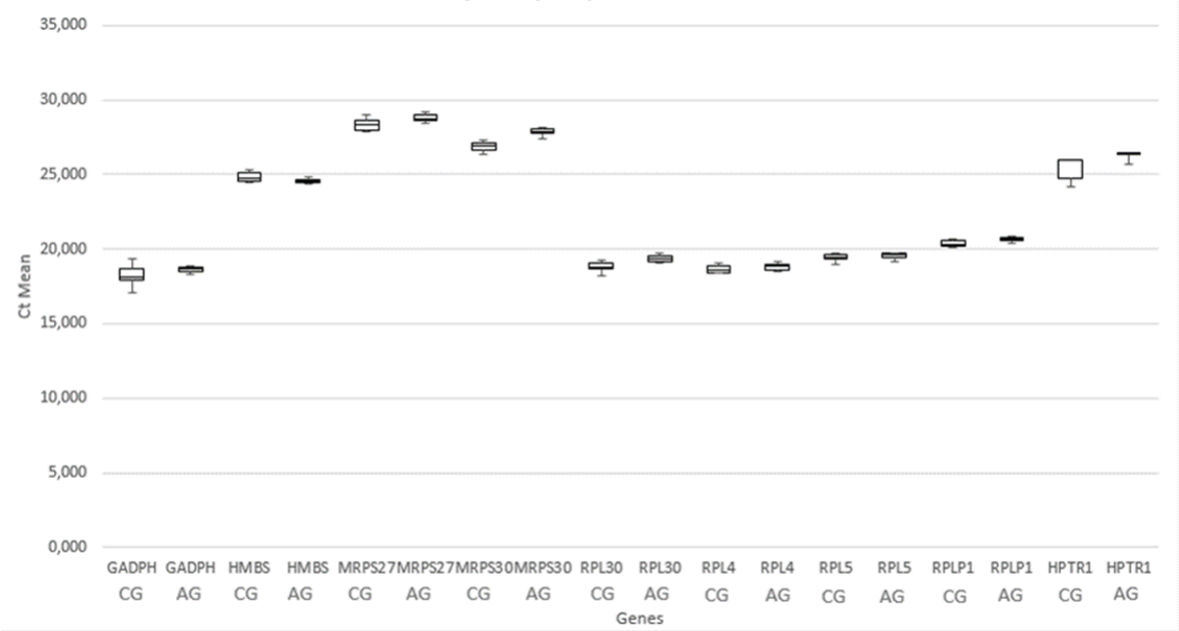

Figure 1. Cycle threshold (Ct) variation of the candidate reference genes in normal and proximal femoral epiphysiolysis-affected broilers. CG: control group and AG: affected group

Table 2. Ct means of the nine candidate reference genes.

	Ct Mean ± SD		
Gene	**CG**	**AG**	**General mean**
RPLP1	20.36 ± 0.25	20.77 ± 0.39	20.56 ± 0.32
RPL5	19.45 ± 0.23	19.64 ± 0.44	19.54 ± 0.33
RPL4	18.66 ± 0.25	18.99 ± 0.68	18.82 ± 0.46
RPL30	18.82 ±0.31	19.50 ± 0.56	19.16 ± 0.43
MRPS27	28.44 ± 0.63	28.90 ± 0.47	28.67 ± 0.55
GAPDH	18.25 ± 0.70	18.77 ± 0.52	18.51 ± 0.61
HMBS	24.82 ± 0.30	24.60 ± 0.48	24.71 ± 0.39
MRPS30	26.88 ± 0.34	27.98 ± 0.50	27.43 ± 0.42
HPRT1	25.90 ± 1.44	26.77 ± 0.96	26.33 ± 1.20

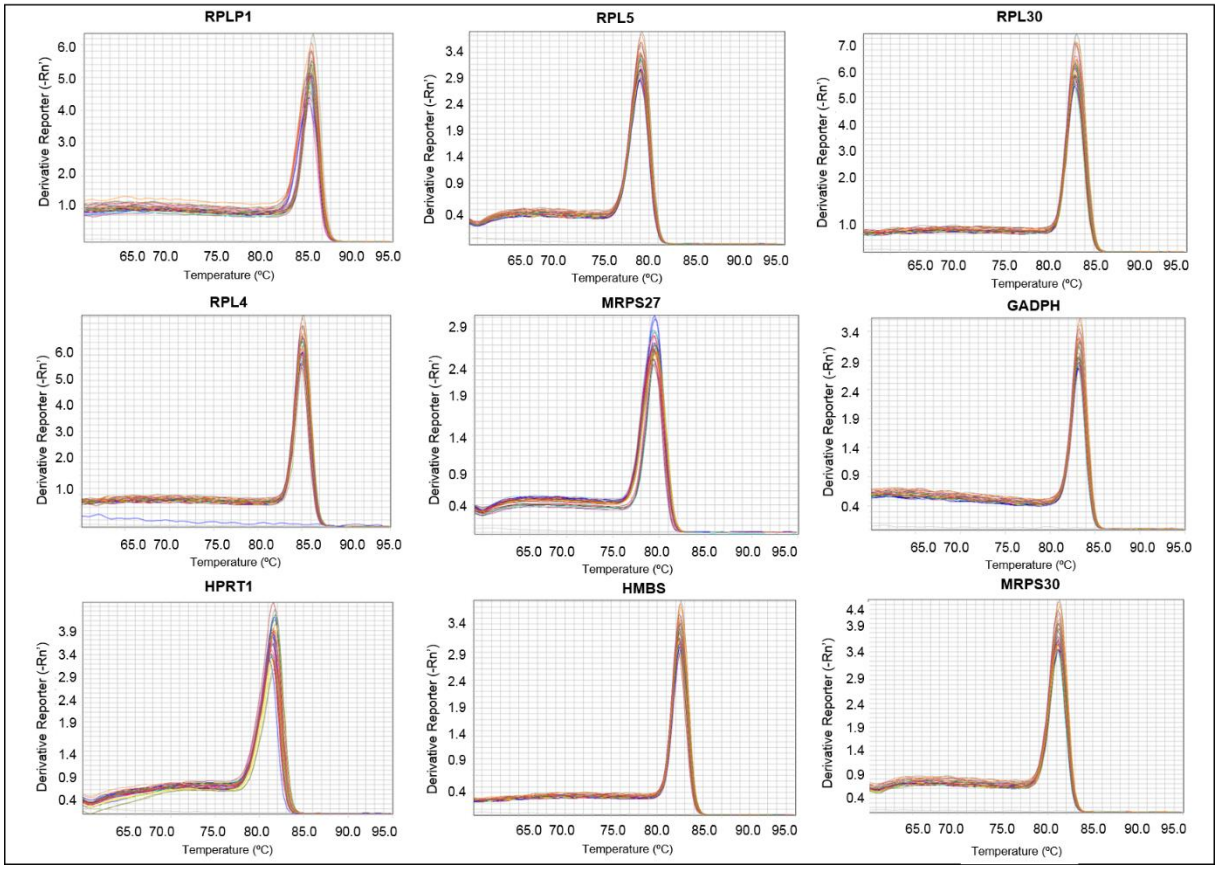

Figure 2. The melting curve for the 9 candidate reference genes evaluated in the femur articular cartilage in broilers.

A similar expression profile among the *RPLP1, MRPS30* and *HPRT1* genes was obtained with the evaluated software (Fig 3, Fig 4, Table 3). The genes *RPL5* and *RPLP1* were classified as the two most stable with the software BestKeeper (Table 3) and GeNorm (Fig 3, Table 3), and *RPLP1* and *RPL4* with NormFinder (Table 3). The gene *RPL5* was classified in the sixth position with the software NormFinder (Table 3) differing from the Bestkeeper and GeNorm results. The least stable gene was *HPRT1* according to all the software evaluated (Fig 3, Fig 4, Table 3). The gene *HMBS* had a divergent classification among BestKeeper, GeNorm and NormFinder, ranking this gene, respectively, in the third, sixth and eighth positions (Table 3).

Since there were some variations in the classification of the reference genes in the results of each software separately, the RankAggreg was used to generate an overall classification, where *RPLP1* and *RPL5* genes were identified as the most stable ones (Fig 4, Table 3). RankAggreg results were similar to those obtained with the evaluation of geNorm, BestKeeper and NormFinder regarding the *MRPS30* and *HPRT1* genes, which were classified as the least stable genes from those evaluated in our study (Fig 3, Fig 4, Table 3).

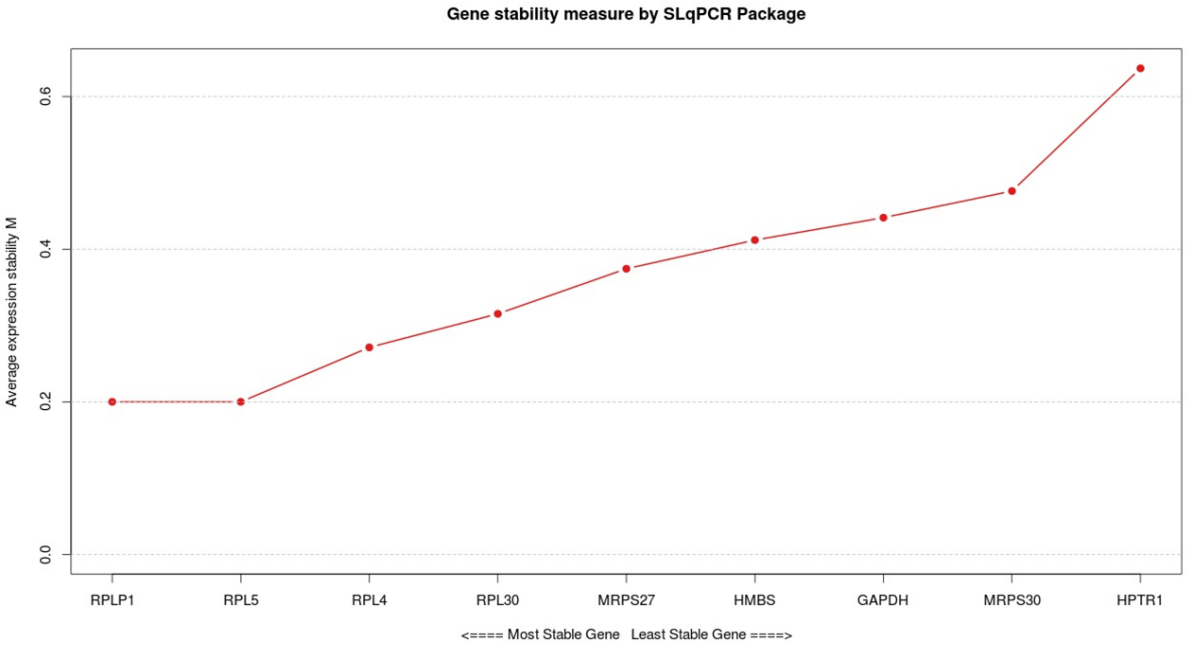

Figure 3. Ranking of candidate reference genes based on the average expression stability using the geNorm software.

Table 3. Gene classification values and ranking (in parenthesis) according to the three algorithms analyzed and the general rank generated with the RankAggreg.

Gene	Bestkeeper (SD of [±Ct])	NormFinder (S-value)	GeNorm (M-value)	RankAggreg (Ranking)
RPLP1	0.266 (2)	0.15 (1)	0.200 (1)	1
RPL5	0.239 (1)	0.22 (6)	0.200 (2)	2
RPL4	0.325 (4)	0.15 (2)	0.271 (3)	3
RPL30	0.367 (5)	0.16 (3)	0.315 (4)	4
MRPS27	0.433 (6)	0.17 (4)	0.374 (5)	5
HMBS	0.311 (3)	0.42 (8)	0.412 (6)	6
GAPDH	0.472 (7)	0.22 (5)	0.441 (7)	7
MRPS30	0.559 (8)	0.39 (7)	0.476 (8)	8
HPRT1	0.929 (9)	0.48 (9)	0.637 (9)	9

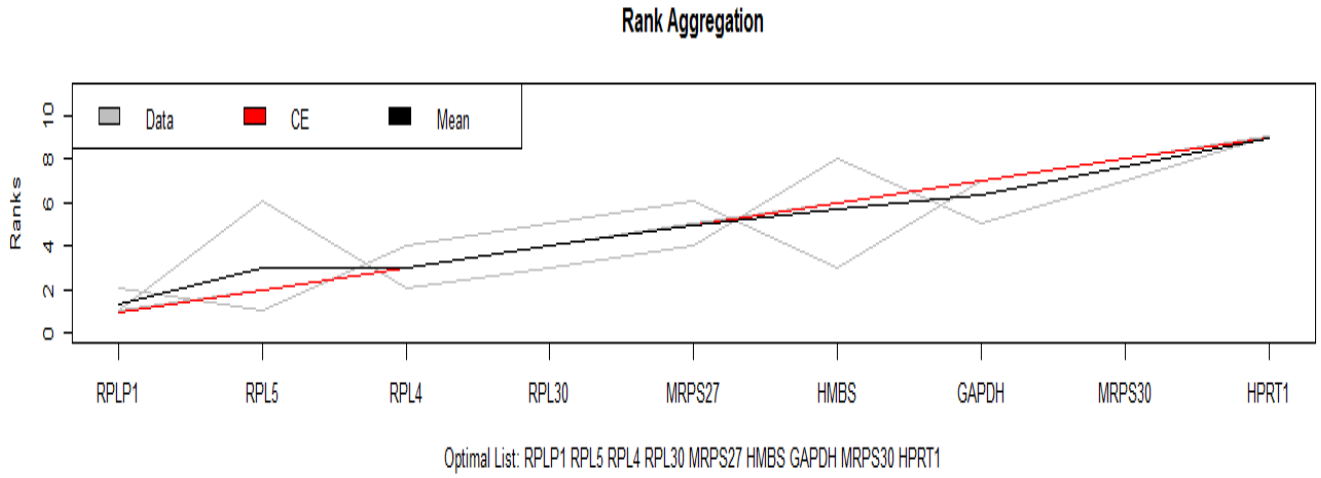

Figure 4. Ranking of candidate reference genes based on the average expression stability using the geNorm, BestKeeper and NormFinder software using the Rankaggreg package analysis.

Discussion

The evaluation of the stability of endogenous genes is an essential step in the relative quantification analysis of gene expression and needs to be performed previously to its use to avoid biased results. Due to the widespread use of qPCR technique, several protocols, reagents, and methods of analysis have emerged, which can lead to contradictory results (8,38). In order to obtain high quality results, the samples must have sufficient quantity and integrity of the RNA, facilitating the results with high precision, sensitivity, and reproducibility of the posterior analysis (29,30,39). Another important factor is the evaluation of a set of candidate reference genes, which is fundamental for obtaining reliable results from qPCR studies (30,39). It should be noted that different conditions and experiments require different genes to be used as normalizers, needing a specific search for genes with stable expression patterns (8,39).

Here, total RNA with good quality was used for the further analysis, according to the MIQE recommendations (40). In the present study, 10 candidate reference genes were evaluated, considering stability parameters and consistency of expression with four different specific software: geNorm, NormFinder, BestKeeper and RankAggreg (Table 3), which are widely used in similar studies (37,41). Although the search for the best reference genes is needed, there is no standard methodology established for this purpose, and a wide variety of approaches is available. Another issue is that there is no standardization of the values used by the algorithms, with some having a clear stability threshold value, while others do not, and this value is relevant for checking the stability of the gene. Thus, the use of several software could lead to a better selection of the most reliable reference genes.

In chickens, studies evaluating the gene stability in many tissues, such as muscle, embryo, ovary, uterus, lung and heart fibroblasts have been reported (35,37,42–45). However, since there are no studies regarding the stability of endogenous genes in poultry cartilage tissue, the results from our study are important to characterize this type of sample, especially because this tissue is involved in several bone disorders or locomotor problems in commercial broilers.

It was possible to observe some variations regarding the stability classification of the genes when evaluating each software separately. For example, *HMBS* was ranked as 3rd in BestKeeper, 8th in NormFinder and 6th in geNorm (Fig 3, Table 3). Nascimento et al. (2015) (35) also found variation in the results obtained by the software used to evaluate reference genes in *pectoralis major* muscle of broilers. The variations found can be explained by the fact that the software are different from each other, and have been developed for addressing different types of experiments. The BestKeeper software allows comparing the expression level of up to ten endogenous genes, also allowing to observe the raw and transformed data, generating an index combining the evaluated genes, which has a high sensitivity to genes with very different expression levels (30). The NormFinder automatically calculates the stability value (S) of all endogenous genes tested on a set of samples, regardless of the number of samples and groups. Their approach is based on a model generating the S value, which has a high sensitivity to the co-regulation of genes (29). On the other hand, geNorm does not consider the co-expression of candidate reference genes and classifies the two most stable reference genes among those tested (28).

Although there is a great variation in the classification of genes by different software, there is no recommendation for the best method of gene selection, nor a pattern that indicates good or poor stability (46). After the analysis with BestKeeper, geNorm and NormFinder, the geNorm seemed to be the most suitable software to choose the most reliable genes, because its results were more similar to those found with RankAggreg. Through a general classification obtained with the RankAggreg function, *RPLP1* and *RPL5* were the most stable genes found in our study (Fig 4, Table 3). Regarding the stability of the genes, those best classified in the general ranking also had a good stability in each software separately, since they had values within the parameters suggested by each software: S <0.5, M <1.5 and [± Ct] SD <1, for the NormFinder, GeNorm and Bestkeeper, respectively (Fig 3, Table 3). The use of various tools to choose the reference genes allowed a broad check of the variation of the expression of these genes and although RankAggreg provides a general classification of genes, it does not mean that all genes are stable or vice-versa (39). The evaluation of the output from each software is needed to confirm whether the genes are indeed stable or not (8,39), as shown above.

Several genes have been reported as reference genes, and ribosome proteins are suggested as good endogenous genes, because of their role in the production of ribosomes (47–49), since they are important components of the basic physiological processes in all cells (49). Here, several known endogenous genes, such as *GAPDH, HMBS* and *HPRT1,* as well as many candidate ribosomal proteins (*RPLs* and *MRPLs*) were evaluated for their stability. The *HPRT1* was the least stable, while *RPLP1* was one of the most stable gene. These two genes were unstable at different stages of cardiac development in rats and were not indicated to be used as reference genes in cardiac tissue (50). However, Nakayama et al. (2018) (51) and Nascimento et al. (2015) (35) found similar results from our study, where the *RPLP1* was suitable for normalization of gene expression in nasal tissue in humans and muscle tissue in broilers, respectively. Furthermore, some studies that evaluated the stability of the *HMBS* gene found this gene to be one of the most stable when considering several tissues (34,35,49,52–54). Zhang et al. (2013) (52), for example, evaluating the stability of eight reference genes in 10 types of Boer goat tissues found that the *HMBS* gene was the third most stable and therefore recommended this gene for calibrating gene expression analyses of goat tissues from this breed by real-time qPCR. In broiler chickens *pectoralis* major muscle, the *HMBS* gene was also found to

be the most stable by Nascimento et al. (2015) (35). According to these authors, the *HMBS* and *HPRT1* genes were the most stable and could be used to normalize expression data in the *pectoralis major* muscle of chickens submitted to heat stress (51). Zhang et al. (2017) (53) showed that *HMBS* was the most suitable gene for chickens gut, while Paludo et al. (2016) (34) used *HMBS* as an endogenous gene to study bones from broilers with 45 days of age affected with femoral head necrosis. This gene has been used as endogenous in many species, in different tissues and ages (34,49,54). Here, the *HMBS* gene had a good classification in the Bestkeeper tool, but not in NormFinder and GeNorm (Table 3). These results reinforce that gene stability could be influenced by different factors, such as age, tissue and conditions (35,49). Furthermore, the *HPRT1* gene was classified as the least stable gene by the three software, although this gene has already been considered a good reference gene for different swine tissues (54) and mice (55) and is widely used in rats as a reference gene in qPCR studies.

The *MRPS27* and *MRPS30* are mitochondrial ribosomal proteins encoded by nuclear genes with high activity in muscle tissues and the synthesis of proteins within the mitochondria (56). In quail, the most stable genes evaluated in several tissues were *MRPS30*, *EEF1* and *HMBS* in the thigh muscle, *B2M*, *UBC* and *GAPDH* in the brain, *MRPS30*, *TFRC* and *HMBS* in the heart, and *EEF1*, *LDHA* and *HMBS* in the spleen (57). Furthermore, these authors also recommended testing the expression of endogenous genes that could vary between male and female quails (57). When evaluating the muscle tissue of chickens under stress, the genes *MRPS27, RPL5* and *MRPS30* were considered stable according to the general classification of RankAggreg and can be used as normalizers in qPCR analysis of target genes in this condition (49).

Ribosomal proteins are crucial to the development and tissue homeostasis (58). Wang et al. (2011) (59) suggest ribosomal proteins as good candidates to substitute the traditional reference genes as internal controls in real-time PCR assays. Previously, ribosomal proteins were recommended only for less sensitive detection methods like Northern blot (60). However, recent studies evaluating the stability of reference genes have reported outstanding stabilities of ribosomal proteins in different cell lines and tissues of mammals (61–63), fish (64), shellfish (65) and plants (66). The gene *RPLP1* was studied as a reference gene in several animal species, such as *Homo sapiens* (58), *Mus musculus* (67), *Rattus norvegicus* (68,69), *Gallus gallus* (43,70), *Danio rerio*, *Bos Taurus* (71), *Anopheles gambiae* (72), *Drosophila melanogaster* (73), *Bombyx mori* (74) and *Ailuropoda melanoleuca* (75). This gene plays a role in the elongation step of protein synthesis (75) and its overexpression was associated with tumorigenesis and is involved with human cancer (58). The *RPLP1* had a divergent stability ranking between neonatal and cardiosphere-derived adult cells, indicating that its gene expression was age-dependent (50). *RPLP1* expression seems to be tissue-dependent, because, according to Marciano et al. (2020) (37), when studying breast muscle of chickens, *RPLP1* was the least stable gene and in our results with cartilage it was one of the most stable genes. Benak et al. (2019) (76), when selecting optimal reference genes for gene expression studies in chronically hypoxic rat heart, found that the gene *RPLP1* was one of the most stable genes in the left and right ventricle, similarly to this study. This gene has a variable expression according to the tissue, age and species used at the experiment, reinforcing the importance of checking reference genes even when similar issues have already been studied.

The *RPL5,* a gene that encodes a small protein that is a component of the 60S subunit and is responsible for transporting the *5S rRNA* to the nucleolus, was the 2nd most stable gene in the general ranking in the current study. Our results corroborate with Oliveira et al. (2015) (49), which evaluated the *RPL5* gene in chicken muscle tissue of males and females, and was indicated as suitable for normalization of gene expression. The *RPL5* is a constitutive protein in the large ribosomal subunit that catalyzes mRNA-directed protein synthesis (60). When evaluating assays of the cornea in various murine disease models, Ren et al. (2010) (55) recommended *RPL5* as a reference gene, showing stability in that study. *RPL5* was also a stable gene when studying heart failure of the right ventricles in humans (77) and tissues of red abalone *Haliotis rufescens* (Mollusca, Vetigastropoda) (59,78). Kim et al. (2019) (79) used *RPL5* as a reference gene to calibrate the reverse-transcribed cDNA templates for the samples of cerebral ganglion, pleuro-pedal ganglion, ovary, gills, intestine and adductor muscle of Pacific abalone (*Haliotis discus hannai*). Marciano et al. (2020) (37), when studying breast muscle of chicken also found *RPL5* as one of the most stable genes. These results corroborate with those found in our study, showing that this gene is suitable as normalizer for several species, including chickens.

In the current study, we showed that *RPLP1* and *RPL5* were ranked as the most stable genes when femur head's articular cartilage of broilers were evaluated, contributing to the understanding of gene expression profiles of candidate endogenous genes in chickens. These results can also help to clarify the etiology of bone-related problems in avian and other species. Despite the indication of using two or more reference genes in gene expression studies, it is common to find the use of only one gene, and based merely in the literature, not following the MIQE recommendations (39,40). Therefore, in order to obtain the best reference genes, it is necessary to evaluate a broad panel of genes and software, considering the complexity of the experimental designs and tissues (39). Thus, the choise of the most reliable reference genes for relative quantification analysis reduces the selection of false endogenous genes, improving the accuracy of the results (80).

Conclusions

The *RPL5* and *RPLP1* were the most reliable endogenous genes for qPCR analyses in the femur cartilage tissue of normal and epiphysiolysis-affected broilers. This is the first study evaluating reference genes in the chicken articular cartilage. Our results can be useful for investigating articular disorders in chickens and other species by the analysis of cartilage gene expression.

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Author Contributions
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Performed the experiment: LMH, AMGI, IRS, DEPM, MT.
Data analysis and curation: LMH, AMGI, MRS, IRS, DEPM, MT.
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Writing – Review and Editing: LMH, AMGI, JOP, MRS, IRS, DEPM, MT, MCL.
Funding Acquisition: JOP, MCL.
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Competing Interests
The authors declare that they have no competing interests.

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4. CAPÍTULO II

Artigo a ser submetido na Revista Frontiers in Physiology

Differentially expressed genes in the femur cartilage transcriptome clarifies the understanding of epiphysiolysis in chickens

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Abstract

Locomotor problems are among the main concerns in the current poultry industry, causing major economic losses and affecting animal welfare. The most common bone anomalies in the femur are dyschondroplasia, femoral head epiphysiolysis or femoral head separation (FHS), and Bacterial Chondronecrosis with Osteomyelitis (BCO), also known as Femoral Head Necrosis (FHN). The present study aimed to identify differentially expressed (DE) genes in the articular cartilage of normal and FHS-affected broilers by RNA-Seq analysis. After RNA extraction of the articular cartilage (AC), cDNA libraries were constructed for mRNA sequencing on Illumina platform, following a paired-end protocol. The sequences were mapped against the chicken reference genome (*Gallus gallus*, assembly GRCg6a) using the STAR software. The DE genes between the normal and affected groups were determined using the edgeR package from R, with ae False Discovery Rate (FDR) ≤0.05. In the transcriptome analysis, 12,169 genes were expressed in the femur AC. Of those, 107 genes were DE between normal and affected chickens, in which 9 were downregulated and 98 were upregulated in the affected broilers. For validation, the relative expression of 10 DE genes in the RNA-Seq study was obtained using quantitative PCR (qPCR). From those 10 genes, six were validated: avian beta defensin 1 (*AvBD1*), avian beta defensin 2 (*AvBD2*), ankyrin 1 (*ANK 1*), eosinophil peroxidase (*EPX*), adenosine deaminase (*ADA*), and Rh associated glycoprotein (*RHAG*). In the functional analysis using the DE genes, 79 biological processes (BP) were identified using the DAVID database, being grouped into 12 macrobioprocesses with the REViGO tool. The main PB found were involved in the response to biotic stimulus, gas transport, cellular activation, carbohydrate-derived catabolism, multi-organism regulation, immune system, muscle contraction, multi-organism process, cytolysis, leukocytes and cell adhesion. In this study, the first transcriptome analysis of the broilers femur articular cartilage was performed, and a set of candidate genes that could trigger changes in the broiler´s femoral growth plate was identified.

Keywords: Extracellular Matrix, Femoral Degeneration, Poultry, RNA-Seq, locomotor problems.

INTRODUCTION

In the last decades, an intense selection has been performed for greater feed efficiency and faster growth of broiler chickens (ZUIDHOF et al., 2014). However, some negative impacts in the skeleton structure have appeared, such as locomotor problems that increased significantly, causing a negative impact on welfare, efficiency, performance and other characteristics (COOK, 2000; HAVENSTEIN; FERKET; QURESHI, 2003). Cook (2000) estimated economic losses due to these problems in $ 120 million dollars annually, which represents a loss equivalent to $ 0.16 per animal. These losses are due to the increase in mortality, reduction in feed conversion and weight gain caused directly or indirectly by these pathologies (SULLIVAN, 1994). Currently, bone disorders are still considered one of the main concerns for the poultry industry (LI et al., 2015). Among these conditions, bacterial chondronecrosis with osteomyelitis (BCO) is the most common cause of claudication affecting approximately 1.5% of chickens slaughtered at 42 days of age in the United States and it is an important cause of mortality in broilers, (WIDEMAN; PRISBY, 2013). This condition, also known as femoral head necrosis (WIDEMAN et al., 2012), is one of the most important disturbances in the locomotor system in commercial chickens worldwide (MCNAMEE; SMYTH, 2000). Besides its importance in poultry production, there are few studies on this pathology, especially related to its genetics and molecular mechanisms (ALMEIDA PAZ et al., 2009; OLKOWSKI et al., 2011; WIDEMAN et al., 2012; PALUDO et al., 2017; PEIXOTO et al., 2019).

The proximal femoral epiphysiolysis or femur head separation (FHS) is the separation of the articular cartilage (AC) from the growth plate, being a risk factor for infection and may cause BCO in broilers (RIDDELL, 1983; PACKIALAKSHMI et al., 2015). This can occur because the BCO pathogenesis seems to be initiated by damage the poorly mineralized chondrocyte (cartilage cells) columns in the epiphyseal and physeal growth plates of the leg bones, followed by colonization of the osteochondral clefts by opportunistic bacteria (WIDEMAN et al., 2012; WIDEMAN; PRISBY, 2013).

The genetics play a considerable role in the skeleton development, where the genetic selection and gene mutations influence the development of the skeletal system (COOK, 2000). DNA mutations in different genes are involved with different skeletal

clinical phenotypes (GEISTER; CAMPER, 2015). There is a controversy in the literature regarding BCO and FHN, where some authors consider BCO and FHN as the same pathology (THORP et al., 1993; WILSON et al., 2019) and others consider it different pathologies ALMEIDA PAZ et al., 2009; (SANTILI et al., 2004; MESTRINER et al., 2012;). Some studies evaluating broiler bones found that the genes *RUNX2*, *SPARC* (PALUDO et al., 2017), ADIPOQ, PRRX1, ANGPTL5, ANGPTL7, GFRA2, SFRP5, COL14A1, ABI3BP, COL8A1 and SLC30A10 (PETRY et al., 2018) are associated with BCO and *bFGF* (LI et al., 2015), *LEPR* (MARCHESI et al., 2014), *LRP1B*, *COL28A1*, *PTHrP*, *PERP1*, *FAM180A* e *CHST1* (PEIXOTO et al., 2019) are associated with the FHN. Furthermore, according to the chicken QTL database (https://www.animalgenome.org/cgi-bin/QTLdb/GG/summary), several quantitative trait loci (QTL) for morphometric traits, mineral composition, and tibia and femur resistance were mapped to several chicken chromosomes, indicating important regions related to bone development (JOHNSSON et al., 2015). However, there are no studies investigating the femur articular cartilage in animals affected with epiphysiolysis and the molecular and genetic pathways involved in this condition are still unknown in chickens (JOHNSSON et al., 2015; FAVERI et al., 2019). The functional analysis of genes are interesting to elucidate their contribution to the development of locomotor problems in chickens, and provides a better understanding of human bone disorders. Therefore, this study aimed to identify differentially expressed genes in the femur head cartilage between healthy broilers and those affected by proximal femoral epiphysiolysis using RNA-Seq analysis.

MATERIAL AND METHODS

Animal and sample collection

A total of 29 commercial male broiler chickens from a poultry farm, located in Concórdia/SC, Brazil, was used in this study. Broilers were housed according to standard practices from this line, raised with free access to both feed and water. To reduce environmental effects, broilers used in this study were sampled from the same flock, in a Darkhouse system managed by a high standard producer. At the farm, those animals were selected based on the absence or presence of lameness, being split into two groups: 14 normal and 15 chickens showing lameness as described by Peixoto et al. (2019). This study was approved by the Embrapa Swine and Poultry National

Research Center Ethical Committee of Animal Use (CEUA) under protocol number 012/2012. At the necropsy, the animals were evaluated for the presence or absence of epiphysiolysis and for BCO-levels according to WIDEMAN et al. (2012) and PALUDO et al. (2016). The femur was considered with epiphysiolysis based on the growth plate (GP) separation from the articular cartilage, where animals with separation between GP and AC were classified in the FHS-affected group and the animals with good adhesion of the AC and GP were considered the normal group. Samples of AC were collected in liquid nitrogen and stored at -80ºC.

Total RNA extraction, library preparation and sequencing

For total RNA extraction, eight samples of femoral articular cartilage (4 from each group) were homogenized in liquid nitrogen and 100 mg of tissue was added in 1 mL of Trizol. Then, 200 µL of chloroform were added, the tubes were homogenized for 15 seconds and incubated at room temperature for five minutes, centrifuged at 16,000 xg at 4 ºC for 15 minutes. The aqueous phase was separated into a new microtube, mixed with 70% ethanol. This solution was added to a Qiagen Rneasy silica column (Qiagen, Hilden, NRW, Germany), and the RNA extraction followed the standard protocol of Qiagen RNeasy kit (Qiagen, Germany), according to the manufacturer´s instructions. Total RNA was quantified using the Biodrop spectrophotometer (Thermo Fisher Scientific Inc., Santa Clara, CA, USA) and samples with OD260:OD280 ratio greater than 1.9 were considered pure. The RNA integrity was confirmed in a 1.0% agarose gel electrophoresed for 90 min and in Agilent 2100 Bioanalyzer (Agilent Technologies; Santa Clara, CA, USA). Samples with RNA integrity number (RIN) greater than 8.0 were used for RNA libraries preparation.

Approximately 2 µg of total RNA was submitted to library preparation using the TruSeq Stranded mRNA Library Prep Kits Kit (Illumina, Inc., San Diego CA, USA), according to the manufacturer's recommendations. The size of the libraries was confirmed in Agilent 2100 Bioanalyzer (Agilent Technologies; Santa Clara, CA, USA). Libraries were quantified with qPCR using primers with Illumina adapters and then sequenced in an Illumina HiSeq2500 (Illumina, Inc., San Diego CA, USA) at the Center for Functional Genomics at ESALQ, University of São Paulo, Piracicaba - SP, Brazil, following a paired-end (2 x 100 bp) protocol. All samples were sequenced in the same lane.

Quality control, mapping and differential expression analyses

The reads quality control (QC) was performed in the Seqyclean tool (ZHBANNIKOV et al., 2017) to remove short reads (<70pb), low-quality reads (QPhred <24), PCR artifacts and adapter sequences. Mapping was performed in the STAR 2.7 aligner (DOBIN et al., 2013) using the chicken reference genome (*Gallus gallus*, assembly GRCg6a) available at the Ensembl 95 database (www.ensembl.org). The reads were counted in exon regions using HTSeq count from the STAR (ANDERS; PYL; HUBER, 2015) and the EdgeR package (ROBINSON; MCCARTHY; SMYTH, 2010) from the R environment (R Core Team, 2013) was used to identify the differentially expressed (DE) genes between the normal and affected groups.Genes with false discovery rate (FDR) <0.05 were considered DE, after correcting for the Benjamini-Hochberg (BH) multiple-tests (BENJAMINI; HOCHBERG, 1995). The genes were considered upregulated and downregulated according to the positives and negatives log2 fold-change (LogFC), respectively, in the affected compared to normal broilers. The multidimensional scaling (MDS) plot was create with the LogFC values from each gene using R. Based on DE genes, a heatmap was generated to check the consistence between samples using R (R Core Team, 2013). A smear plot was also created using R, showing the differential expression test results. The FASTQ files obtained in this study were deposited in the SRA database, with BioProject number PRJNA350521.

qPCR validation

To confirm the results obtained in the RNA-Seq analysis, a quantitative PCR analysis (qPCR) was performed using the same eight AC samples from the normal and FHS-affected animals. Total RNA was extracted as described previously in the total RNA extraction, library preparation and sequencing session. The cDNA synthesis was performed according to the recommendations of the SuperScript III, First-Strand Synthesis Supermix protocol (Invitrogen, Carlsbad, CA, USA). For validation, 10 DE genes were chosen based on FDR, LogFC and their functions: avian beta-defensin 1 (*AVBD1*), avian beta-defensin 2 (*AVBD2*), fibrillin 2 (*FBN2*), ankyrin 1 (*ANK-1*), phosphoserine phosphatase (*PSPH*), eosinophil peroxidase (*EPX*), adenosine deaminase (*ADA)*, collagen type XIII alpha 1 chain (*COL13A1*), Rh associated glycoprotein (*RHAG*) and S100 calcium-binding protein A9 (*S100A9*). The primers were designed using the NCBI Primer-BLAST tool (YE et al., 2012) (Table 1) and the quality was evaluated and confirmed by the Netprimer program

(http://www.premierbiosoft.com/NetPrimer). For the relative quantification analyses, reactions were prepared using 1X GoTaq qPCR Master Mix (Promega, Madison, WI, USA), with BRYT Green Dye and CRX as reference dye, 0,13 µM of forward and reverse primers, 2 µL cDNA at 1:10 dilution and ultra-pure water (Nuclease Free Water, Qiagen) to complete a 15 µL reaction. The reactions were distributed in 96-Well plates (0.1 µL Microamp Fast 96-Well Reaction Plate), in duplicate and submitted to the QuantStudio 6 Real-Time PCR equipment (Applied Biosystems, Foster City, CA, USA) with cycling of 95°C for 3 minutes, 40 cycles of 95ºC for 15 seconds and 60ºC for one minute, with melting curve of 95ºC for 15 seconds, 60ºC for 1 minute and 95ºC for 15 seconds. To detect contamination, a negative control sample was included.

The Ct means of each sample were obtained to perform the $2^{-\Delta\Delta Ct}$ analysis (LIVAK; SCHMITTGEN, 2001). For the data normalization, the geometric mean of the Ct values from the reference genes *RPL5* (*Ribosomal Protein L5*) and *RPLP1* (*Ribosomal Protein Lateral Stalk Subunit P1*) were used. These genes were chosen to be used based on their stability evaluation (HUL et al., 2020 - submitted). The Relative Expression Software Tool (REST©) was used to perform the relative quantification and the statistical test, using the non-parametric Pair Wise Fixed Reallocation Randomization Test (PFALL; HORGAN; DEMPLE, 2002). Genes with p-values < 0.05 were considered DE.

Table 1. Primers for the qPCR analysis of the target candidate genes for epiphysiolysis in the femur articular cartilage of broilers.

Gene	Ensembl ID	Primer Squences (5'-3')
AVBD1	ENSGALG00000022815	F: CAGGATCCTCCCAGGCTCTA
avian beta-defensin 1		R: GATGAGAGTGAGGGAAGGGC
AVBD2	ENSGALG00000016669	F: TTCTCCAGGGTTGTCTTCGC
avian beta-defensin 2		R: TGCATTCCAAGGCCATTTGC
FBN2	ENSGALG00000014686	F: TGCATCGATAGCCTGAAGGG
fibrillin 2		R: CTAATTCACACCGCTCACATGG
ANK1	ENSG00000029534	F: CCACCATCCCACCATTCAGT
Ankyrin 1		R: ACGGTCACAAACTCCAGCAT
PSPH	ENSGALG00000002397	F:CAGGAATACGGGAGCTGGTG
phosphoserine phosphatase		R: CCCAGAGACCAGGAAGACCT
EPX	ENSGALG00000043254	F:AAAGGAGGTGGCATTGACCC
eosinophil peroxidase		R: GCCACGCTGCATGTTAAGAG
ADA	ENSGALG00000004170	F:TTCGGCAAGAAAAGAGGGGT
adenosine deaminase		R: GTGTTTGGTAGCTGACGTGC
COL13A1	ENSGALG00000004286	F:CCAAGCAAGGACTAGACACTCA

collagen type XIII alpha 1 chain		R: ACCCTTCATGCCATGTCTTCC
RHAG Rh associated glycoprotein	ENSGALG00000016684	F:TCTGGAGATCACGGCCTTTG R:GCTCCAATATCTGTGGCCTGA
S100A9 S100 calcium binding protein A9	ENSGALG00000024272	F:GGGGACAAAGACACCCTGAC R:TTCACGTGCTTCAGGTAGTTGG

Functional annotation

To investigate the role of DE genes in known metabolic pathways, the list of DE genes was analyzed with the Functional Annotation Clustering (FAC) of the Database for Annotation, Visualization and Integrated Discovery- DAVID (http: //david.abcc.ncifcrf.gov/). Subsequently, the biological processes were clustered in the REVIGO (Reduce and Visualize Gene Ontology) (SUPEK et al., 2011). An enrichment analysis was also performed using NetworkAnalyst (ZHOU et al., 2019) where new biological functions were obtained with the protein-protein interactions (PPI) tool. Furthermore, a gene-disease association network was created with the DE genes using a human curated gene-disease association information of DisGeNET database available in the NetworkAnalyst (ZHOU et al., 2019).

RESULTS

RNA Sequencing and Differential Expression Analysis

The sequencing of the femoral head cartilage samples generated around 190 million (2x100 bp) reads. An average of 26.8 million paired-end reads was obtained per sample, remaining about 23.7 million after the QC. Approximately 87% of the reads were mapped against the chicken reference genome (GRCg6a) available at Ensembl 95 using the STAR software. The percentage of reads mapped per sample ranged from 84.96% to 89.19%, being equally distributed between the normal and FHS-affected group.

The MDS and the heatmap plots showed the separation of samples from each group (normal and affected) (Supplementary figure 1, Supplementary figure 2). In the gene expression analysis, 12,169 genes were expressed in the femoral articular cartilage, where 107 genes were differentially expressed (FDR ≤0.05) between the two groups (normal and FHS-affected) (Supplementary figure 2, Supplementary figure 3).

The log fold change for each gene was plotted against the average abundance and significantly DE genes at a FDR of 5% were highlighted in red (Supplementary figure 3). From the 107 DE genes identified (Supplementary table 1), 91 were annotated and 16 were uncharacterized (Table 2). Out of the total DE genes, 98 (91.6%) were upregulated and 9 (8.4%) were downregulated in the femoral head cartilage of broilers affected by FHS (Table 2, Supplementary table 2).

Table 2. Characterization of the articular cartilage transcriptome showing the differentially expressed and the total number of expressed genes.

Gene type	Expressed genes	DE
lncRNA	409	5
miRNA	18	0
IG_V_gene	3	0
Pseudogenes	154	2
snRNA	10	0
rRNA	2	1
Misc_RNA	1	0
Mt_rRNA	2	0
Protein-coding genes	10,496	83
Uncharacterized-protein genes	1,074	16
Total	**12,169**	**107**

Considering the top 9 DE genes, genes related to collagen (*COL13A1*), myosin (*MYH15*), phosphatases and transferases were downregulated, while genes involved with response to microorganisms and immune systems were upregulated the FHS-affected groups (Table 3).

Table 3: Top 9 downregulated and upregulated differentially expressed genes between normal and epiphysiolysis-affected broilers.

Ensembl Gene ID	Gene symbol	Gene description	logFC
ENSGALG00000039489	*RF00002*	5.8S ribosomal RNA	-2,237
ENSGALG00000010490	*DPYSL4*	dihydropyrimidinase like 4	-2,008
ENSGALG00000015358	*MYH15*	Gallus gallus myosin, heavy chain 15	-1,924
ENSGALG00000006565	*GGT1*	gamma-glutamyltransferase 1	-1,501
ENSGALG00000038225	*SEMA3E*	semaphorin 3E	-1,442
ENSGALG00000002397	*PSPH*	phosphoserine phosphatase	-1,426
ENSGALG00000004286	*COL13A1*	collagen type XIII alpha 1 chain	-1,296
ENSGALG00000043671			-1,237
ENSGALG00000014686	*FBN2*	fibrillin 2	-1,081

ENSGALG00000016669	*AvBD2*	Gallus gallus avian beta-defensin 2	3,668
ENSGALG00000019696	*CATHL2*	Gallus gallus cathelicidin antimicrobial peptide	3,691
ENSGALG00000028273	*HBE1*	Gallus gallus hemoglobin subunit epsilon 1	3,737
ENSGALG00000024272	*S100A9*	Gallus gallus S100 calcium binding protein A9	4,041
ENSGALG00000006572	*TNNT3*	troponin T3	4,045
ENSGALG00000023953	*C4BPA*	Gallus gallus complement component 4 binding protein	4,050
ENSGALG00000002907	*MYL1*	myosin, light chain 1	4,174
ENSGALG00000043254	*EPX*	eosinophil peroxidase	4,265
ENSGALG00000014463	*ACTN2*	Gallus gallus actinin alpha 2	4,339

qPCR validation

According to the qPCR analysis, six out of the 10 analyzed genes were DE between the FHS-affected compared to the normal group, confirming the RNA-Seq results and the involvement of these genes with epiphysiolysis in the femur head (Table 4). It was possible to observe that the expression pattern between the RNA-Seq and the quantification by qPCR were similar (Figure 1).

Table 4 Relative expression, cycle threshold mean, standard error and p-values for the evaluated candidate genes in the femoral articular cartilage.

Gene	Log2FC	Standard Error	P-value
Avbd1	3,21	2,26	0.005
Avbd2	3,65	1,63	0.006
FBN2	0,52	0,66	0.227
ANK1	2,45	1,28	0.014
RHAG	3,04	1,69	0.006
EPX	3,48	1,24	0.007
ADA	2,14	1,43	0.003
COL13A1	-0,22	0,38	0.562
S100A9	2,51	0,4	0.093
PSPH	-1,13	0,22	0.218

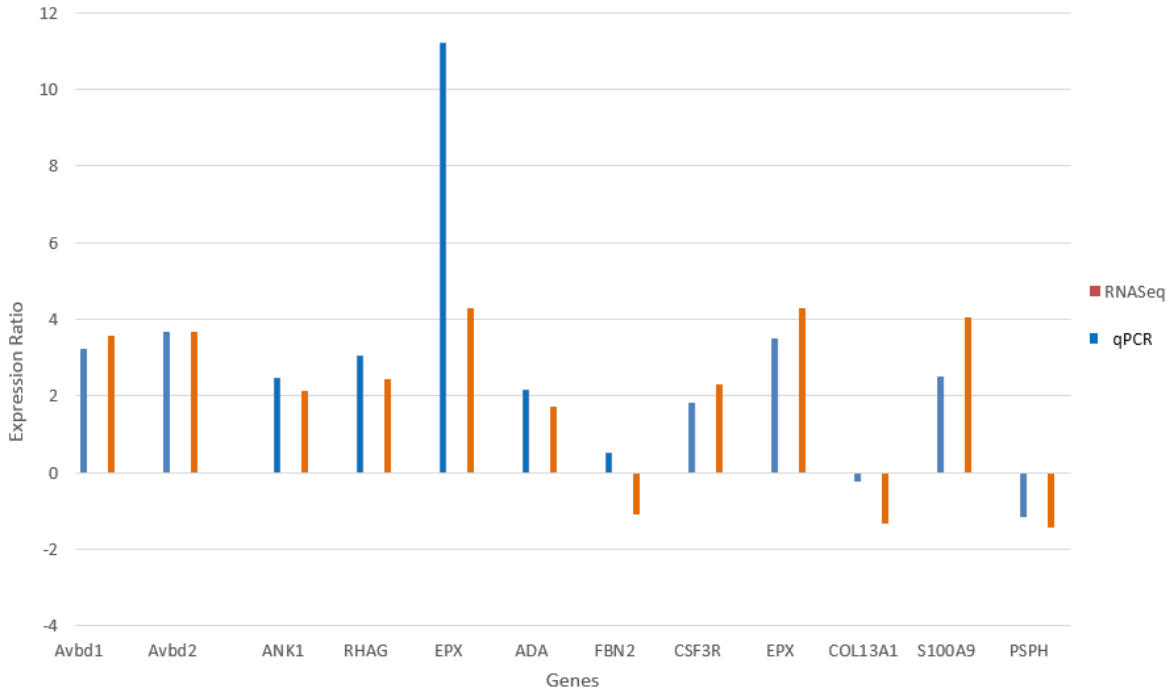

Figure 1. Comparison of the expressed values (Log2FC) between the RNA-Seq and qPCR methodology.

Gene ontology and Gene Network Analyses

In the gene ontology (GO) analysis using DAVID database, the genes with defined biological function were grouped into 79 functional groups according to their most relevant biological processes (BP) identified through the GO enrichment analysis of the REVIGO program. The main biological functions identified were related to immune system processes, response to external stimulus, defense response and leukocyte migration (Figure 2, Supplementary Table 2).

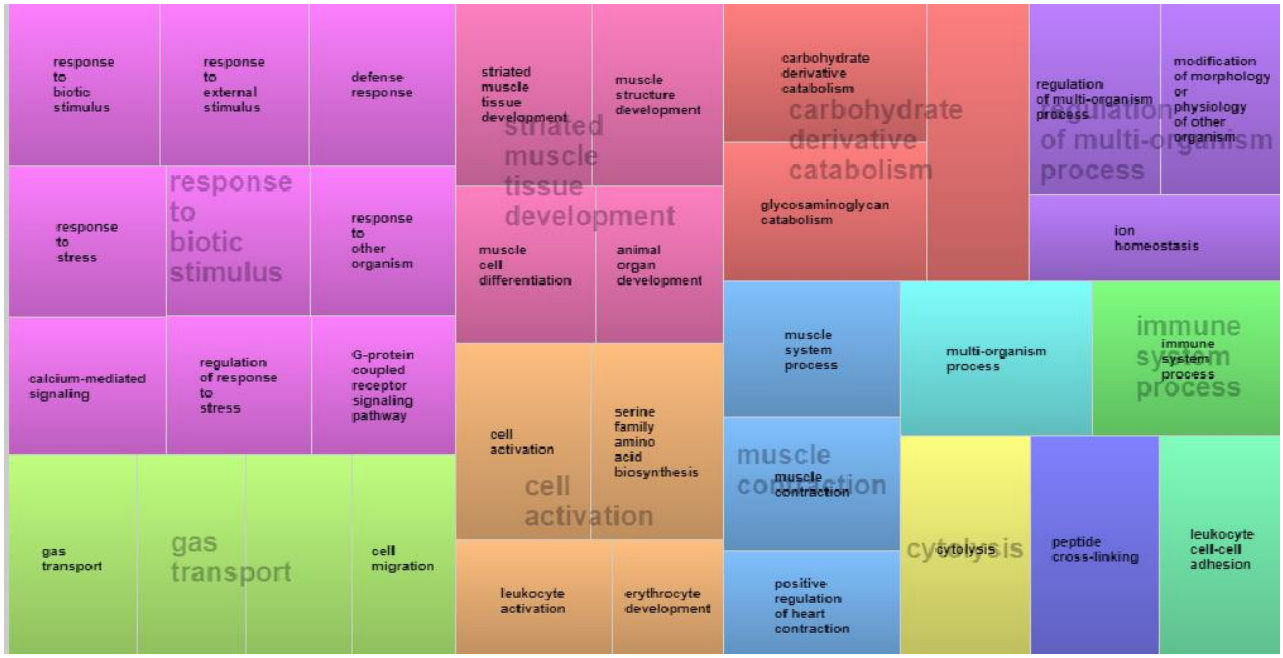

Figure 2. Superclusters of biological processes enriched for up- and downregulated genes in the articular cartilage related to femur epiphysiolysis. Different colors show different superclusters and the size of each box is determined by the uniqueness of the categories.

To verify the known interactions among the DE genes aiming to improve the knowledge about them, an analysis at the NetworkAnalyst platform was performed. An enriched network was generated, where genes were linked to biological processes, such as Wnt signaling pathway (*PLCB2, CCND3, JUN, RAC2*), GnRH signaling pathway (*PLCB2, MAPK12, JUN),* adrenergic signaling in cardiomyocytes (*PLCB2, MYH15, MAPK12)* VEGF, signaling pathway (*MAPK12, RAC2),* Bacterial infection (*MAPK12, JUN), f*ocal adhesion (*CCND3, JUN, RAC2*), gap junction (*TUBB1, PLCB2*) and toll-like receptor signaling pathway (*MAPK12, JUN)* (Figure 3). Furthermore, some DE genes were associated with bone-related disease problems when compared to a human database. The *JUN* and *PGM5* genes were related to osteosarcoma, *ACTA1* with waddling gait and difficulty to walk, *ADA* with abnormality of pelvic girdle bone morphology, *TNNT3* with joint stiffness, arthrogryposis, distal, type 1, ulnar deviation of the wrist, ulnar deviation of the fingers, abnormality of the hip bone, metatarsus varus, and *ANK1* with developmental bone (Figure 4).

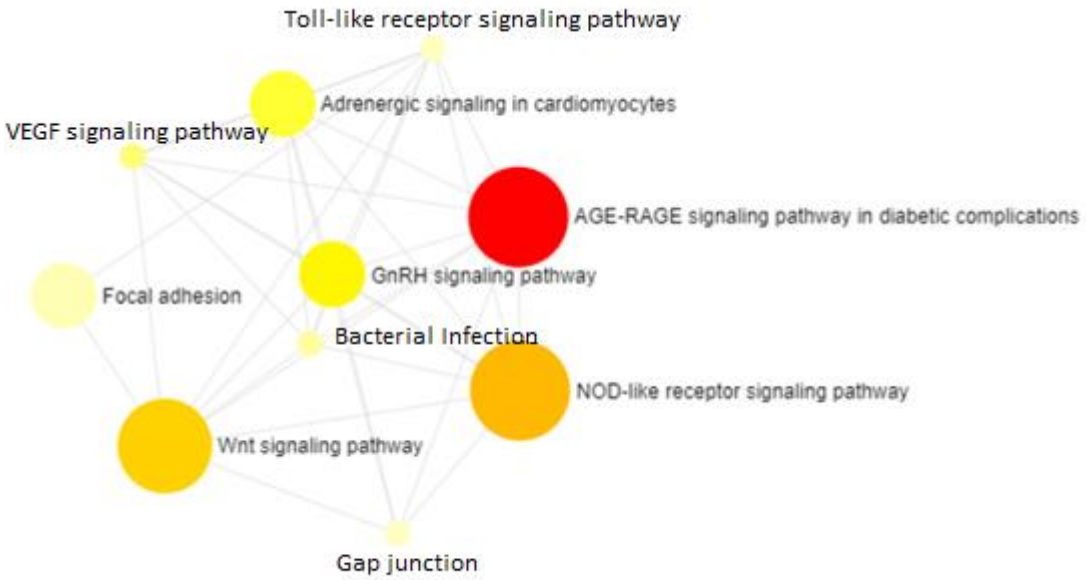

Figure 3. Gene network of differentially expressed genes and metabolic pathways. Circles represent biological process and connecting lines represent interactions between them, according to the active NetworkAnalyst prediction method.

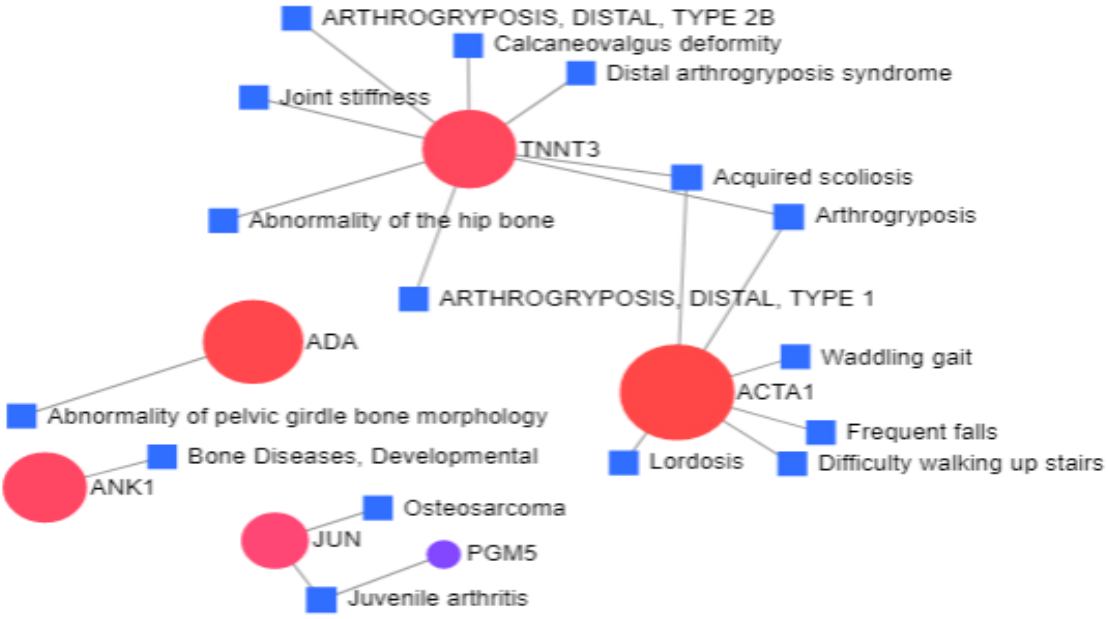

Figure 4. Gene network of differentially expressed genes related to locomotor diseases. Circles represent genes, the squares represent the diseases and connecting lines represent interactions between the genes and diseases, according to the active prediction method of NetworkAnalyst.

DISCUSSION

Studies related to epiphysiolysis and other bone integrity problems, such as BCO, are scarces in chickens (WIDEMAN et al., 2012; LI et al., 2015; WIDEMAN et al., 2015b; PALUDO et al., 2017; PETRY et al., 2018; PEIXOTO et al., 2019), and most of the bone tissue studies are still mainly conducted with humans and rodents (JOHNSSON et al., 2015; SIMPSON; MURRAY, 2015; ABUBAKAR et al., 2016; GABRIELE SOMMER et al., 2019). Furthermore, there are no studies evaluating the femoral cartilage gene expression of chickens and its impacts in bone-related problems. Therefore, a global expression of the femoral head articular cartilage of normal and FHS-affected chickens at 35 days of age was used to clarify genes and molecular pathways involved in the separation of the cartilage and femoral growth plate.

In the cartilage transcriptome, approximately 91.5 % of the genes were upregulated while 8.5% were downregulated in the FHS-affected compared to the normal group (Supplementary table 2). The expression profile of the AC tissue differed from the femoral growth plate, in which approximately 49% of the genes were upregulated and 51% downregulated in the FHS-affected group (PEIXOTO et al., 2019). In the femoral growth plate, several biological processes were related to angiogenesis, blood coagulation, cell adhesion, bone development and lipid metabolism, with several genes downregulated in FHS group (PEIXOTO et al., 2019). Here, in AC tissue, 79 BP were found and after Revigo clusterization (Supplementary table 2), the genes that draw the most attention due to their functionality were related to response to biotic stimulus, immune response, cytolysis, striated muscle development, carbohydrate derivative catabolism and cell activation processes. Several BP were similar between growth plate (PEIXOTO et al., 2019) and AC tissues, however, in the AC transcriptome, a highly proportion of the genes had an increased level of expression in the affected animals. This BP set could help to understand the etiology of this disturb and clarify genes related to this condition in fast-growing broilers as well as the interaction between the two tissues. In our study, 107 genes were identified as DE in the AC and 28 genes were the same as those identified by PEIXOTO et al. (2019) in the bone . Furthermore, some genes previously described as potentially involved with FHS, such as interferon alfa-6 inducible protein *(IFI6),* adenosine deaminase *(ADA),* cathelicidin-3 *(CATH3),* avian beta defensin 1 *(AVBD1),* avian beta

defesin 2 *(AvBD2),* ankirine 1 *(ANK 1),* leukocyte cell-derived chemotaxin 2 *(LECT2),* collagen type XII alpha chain 1 *(COL13A1)*(LI et al., 2015; PACKIALAKSHMI et al., 2015; PALUDO et al., 2017; PETRY et al., 2018; PEIXOTO et al., 2019) were also DE in the current study. Among the DE genes, some of them (*Avbd1, Avbd2, ANK1, RHAG, ADA* and *EPX)* were confirmed by qPCR, which validate the results found with the RNA-Seq approach (Table 4).

Here, only 9 genes were downregulated in the cartilage of broilers with epiphysiolysis, in which the collagen type XIII alpha 1 chain *(COL13A1*) can be highlighted. This gene encodes a transmembrane collagen protein, which has a small cytosolic domain and a large ectodomain, rich in collagenous sequences (HÄGG et al., 1998). *COL13A1* major sites of expression are brain, cornea, kidney, lung, skin, and tendon (KOCH et al., 2006). This gene shows a wide tissue distribution and occurs at the cellular junctions and cell-matrix interaction sites in epithelial, mesenchymal, and neural tissues. It is a component of focal adhesion in cultured fibroblasts (HÄGG et al., 2001) interacting with the collagen-binding integrin α1β1, as well as with the matrix macromolecules fibronectin, nidogen-2, perlecan and heparin, suggesting involvement in multiple cell-matrix interactions (NYKVIST et al., 2000; TU et al., 2002). Therefore, the downregulation of *COL13A1* and possibly other collagen genes could reduce the synthesis of the extracellular matrix, facilitating the separation of the cartilage and bone tissues, favoring the occurrence of proximal femoral epiphysiolysis. Considering the downregulated DE genes, the BP involved were animal organ development, muscle cell development, muscle cell differentiation, muscle structure development, muscle tissue development, serine family amino acid biosynthetic process, striated muscle cell development, striated muscle cell differentiation and striated muscle tissue development (Supplementary Table 2). Most of them were involved in muscle development processes and could be related to the cytoskeleton since the lower expression of these genes can cause malformation in the femur cartilage structure, which could contribute to the separation of the articular cartilage from the growth plate.

Through the evaluation of the upregulated genes, most of the BP was involved with the recruitment of immune cells to enhance the adaptive immune, blood circulation, angiogenesis, circulatory system development and cellular adhesion. The *CCND3, CDK6, JUN, ADD2, ANK1, RHAG, EPB42, SLC4A1, STOM, CAMP, SERPINB10, LYG2, CATHL3, AvBD2, CATHL3, S100A9, CSF3R, RAC2, FGL2, PTPRC, LYVE1,* and *ITGAB2* genes can be highlighted due to their involvement in

direct antimicrobial activities and immunomodulatory properties response (VAN DIJK; VELDHUIZEN; HAAGSMAN, 2008; WANG, 2013) (Supplememtary Table 1, Figure 2). The main BP identified in the AC from DE genes between FHS-affected and normal broilers are discussed below.

Genes related to immune response

Immune biological processes were very representative in DAVID and Revigo (Supplementary table 2), with 18 genes grouped. The gene expression profile observed in this study showed a global activation of the immune system process. The main BP found in DAVID were regulation of immune system process, immune response, immune system development, positive regulation of immune system process, response to bacterium, defense response to bacterium, leukocyte migration, regulation of leukocyte migration, myeloid leukocyte migration, positive regulation of leukocyte migration, leukocyte chemotaxis and leukocyte activation. Among the enriched genes, *AvBD1, ACTN2, ADA, C7, CATH2, CCND3, CSF3R, EDN2, JUN, TF, RHAG, S100A9, SERPINB10, SSTR2, AvBD2, EPB42, LECT2, LYG2, PTPRC*, and *STOM* were upregulated in the FHS-affected group. The *AvBD1, AvBD2, AvBD7, CATHL2, CATHB1, LECT2, SERPINB10, and S100A9* genes were enriched in the host immune response BP. Mainly *AVBD1, AvBD2* and *CATHL2* are key components of the innate immune system (HARWIG et al., 1994; CHENG et al., 2015). *LECT2 (Leukocyte cell-derived chemotaxin 2)* encodes a multifunctional protein characteristically similar to cytokines that improve protective immunity in bacterial sepsis (LU et al., 2013).

The BP of defense response to other organism was enriched with *RSFR, LYG2, AvBD1, STOM, CATH2, AvBD2* and *SERPINB10* genes and defense response to bacterium enriched with *RSFR, LYG2, AvBD1, CATH2, AvBD2* genes. The identification of these BP indicates a probable presence of microorganisms in the AC tissue. The initiation of BCO can be caused by a mechanical micro fracturing of the growth plate, followed by colonization of osteochondrotic clefts by different opportunistic bacteria hematogenous spread (WIDEMAN et al., 2015a). One of the causes of BCO is bacterial translocation from the intestinal tract and proliferation in bone fissures (WIDEMAN; PRISBY, 2013). Among them, *Staphylococcus aureus* was found to be the most frequent bacteria associated to osteomyelitis (BERENDT; BYREN, 2004; WRIGHT; NAIR, 2010).

The host-defense peptides (HDPs) are a group of small peptides that are cationic and amphipathic with direct antimicrobial activities and immunomodulatory properties that are responsible for the recruitment of immune cells to enhance the adaptive immune response (VAN DIJK; VELDHUIZEN; HAAGSMAN, 2008; WANG, 2013). HDPs are one of the innate immunity components. Its antimicrobial activity aims to combat bacteria, enveloped viruses, fungi and protozoa, while the immunomodulatory properties help in boosting adaptive immunity through chemotaxis of lymphocytes. HDPs kill microbes through the binding to the cell and then producing pores that lead to cell leakage and lysis (YANG et al., 2000). In avian species, three classes of HDP are described: avian beta-defensins (*AvBDs*), cathelicidins (*CATHs*), and liver-expressed antimicrobial peptide 2 (*LEAP-2)* (CUPERUS et al., 2013; ZHANG; SUNKARA, 2014).

The AvBD's group comprises 14 genes that are different in their chemical structure, mainly amino acid sequence and composition (KLÜVER; ADERMANN; SCHULZ, 2006). In this study, the beta-defensins *AvBD1* and *AvBD2* were upregulated in the affected broilers, comparing to the normal animals, confirming the possible influence of these genes in epiphysiolysis and probable FHS progression to BCO manifestation. These genes participate in the defense response and response to biotic stimulus, modification of morphology or physiology of other organism and animal organs development BP, indicating that alterations in their expressions can contribute to tissue necrosis and the appearance of epiphysiolysis in broilers (LEHRER; GANZ, 2002; SCHUTTE; MCCRAY, 2002; THOMMA; CAMMUE; THEVISSEN, 2002; FROY; GUREVITZ, 2003; HONG et al., 2012). The *AVBD1, AVBD2, ANK-1, EPX, ADA* and *RHAG* were 12.56, 9.26, 5.45, 11.19, 4.41 and 8.24 times upregulated in the affected group, respectively, compared to the control group, possibly being involved with bacterial chondronecrosis with osteomyelitis. The genes *AvBD1* and *AvBD2* belong to the avian β-defenses family genes, which form a set of 14 genes that have antibacterial action and play an important role in immune defense, being part of the innate immune system of birds (CHENG et al., 2015). Primarily, the *AvBDs* were called gallicins, when first isolated from chicken heterophils, but were renamed as *AvBDs* (EVANS et al., 1994; LYNN et al., 2007). The defensins make up a group of small cysteine-rich cationic peptides that have already been identified in several species, including insects, plants, humans and animals (LEHRER; GANZ, 2002; SCHUTTE; MCCRAY,

2002; THOMMA; CAMMUE; THEVISSEN, 2002; FROY; GUREVITZ, 2003). The *AvBD1* gene upregulation in the intestine, spleen and liver of broilers infected with *E. maxima* indicated the action of this gene against bacterial infections (HONG et al., 2012). In the current study, the upregulation of the *AvBD1* and *AvBD2* genes can be related to a consequence of epiphysolysis, since they bind the cell and produce pores that lead to cell leakage and lysis and their immunomodulatory properties help in boosting adaptive immunity through chemotaxis of lymphocytes, developing an antimicrobial activity (YANG et al., 2000). Therefore, these genes probably got upregulated in order to eliminate the progression of the epiphysiolysis .

The *ANK1* gene encodes a protein related to the binding of the structural constituent of the cytoskeleton, proteins that aid in the attachment of other proteins in the membrane to the actin-spectrin cytoskeleton. The spectrin is another cytoskeletal protein that interacts directly with the actin present in the cytoskeleton, performing processes such as cell motility, activation and proliferation, membrane contact and maintenance (RUBTSOV; LOPINA, 2000; BAGNATO et al., 2003). Recently, two sites of the *ANK1* gene were identified as hypermethylated and associated with the neuropathology of Alzheimer's disease (CHI et al., 2016). According to HALL et al. (2016), ANK1 has an adaptive function as a membrane adapter protein, making connections between the cell membrane proteins and the spectrin-actin cytoskeleton, resulting in cell migration. Ankirin-1 has a role in supporting cell movement after damage. As the ankirin-1 can affect the structure of the actin filament and the cellular motility, it is possible that the increase of the levels of ankirin-1 may inhibit the organization of the actin filament by increasing the binding of the spectin-actin or, alternatively, the ankyrin-1 could act modulating the signaling pathways of actin remodeling (HALL et al., 2016). The gene *ANK1* is co-regulated by p53, which is involved in a variety of cellular functions, including cell-cycle arrest, DNA repair, and apoptosis (HALL et al., 2016; VOGELSTEIN; LANE; LEVINE, 2000). The upregulation of *ANK1* can be related to the cause or consequence of the epiphysiolysis and BCO. Its high expression can alter the structure of actin cytoskeleton affecting the structural integrity of the femur articular cartilage, contributing to the occurrence of proximal femoral epiphysiolysis. Also, its expression is related to cellular damage, so it could be a consequence, since after the damage process from the proximal femoral

epiphysiolysis is initiated, the upregulation can act as a sign of trying to combat the progression of this condition.

The inflammation is a vital component of host defense, but on the other hand, excessive inflammation can cause tissue damage (SITKOVSKY et al., 2004). The adenosine deaminase *(*ADA*)* is an enzyme that acts as an endogenous regulator of the adaptive immune, playing an important role on T-lymphocytes proliferation and differentiation (ARAN et al., 1991). Furthermore, adenosine regulates cell metabolism and triggers a variety of physiological effects in cell proliferation (DESROSIERS et al., 2007). The *ADA* gene acts as a sensor and provides information to the immune system about tissue damage, protecting the host cells from excessive tissue injury associated with strong inflammation (KUMAR; SHARMA, 2009). The upregulation of *ADA* can be related to the fact that its elevated levels downregulates the activation of lymphocytes during inflammation, also playing a regulatory role on neutrophils in immune responses (DESROSIERS et al., 2007; MILLS et al., 2012). Extracellular adenosine signaling hasbeen shown to play a role in inflammation during hypoxia and ischemia-reperfusion injuries, usually resulting in vascular leakage, accumulation of inflammatory cells, and elevated serum cytokine levels. Furthermore, just as hypoxia can induce inflammation, inflamed tissues often become severely hypoxic (ELTZSCHIG; CARMELIET, 2011). The *ADA* upregulation can be considered a consequence of the proximal femoral epiphysiolysis, since its high expression is related to immune responses, trying to combat inflammatory process already installed. Therefore, the hypothesis is that through the increased number of bacteria in the tissue, there is an upregulation of the *ADA* gene, aiming to fight and eliminate the bacteria that are causing damage.

The *RHAG* is one of the genes of the RH gene family (*RH, RHAG, RHBG,* and *RHCG*) (KITANO; SATOU; SAITOU, 2010). When studying plasma of laying hens associated with heat stress, the *RHAG* was upregulated (ZHU et al., 2019). Also Hasanpur; Nassiri; Salekdeh (2019) studied two groups of chickens: normal and affected with ascites syndrome and found that the *RHAG* was overexpressed in the affected group. This gene is usually expressed in tissues that produce blood cells, but it is also expressed in heart cells and those related to gas transferring system from the lungs to organelles within the cells (SCHEELE et al., 2005; NAVARRO et al., 2006). The elevated expression of genes related to the gas transfer system of the heart cell can indicate a more pronounced O_2 reduction or CO_2 enhancement in the heart cells.

Therefore, the raise of the heart cells workload and energy metabolism, as compared to other organs, may suffer more from the imbalance between the O_2 supply and CO_2 removal of the gas transferring elements. In such tissue, hypoxia or hypercapnia may damage the heart cells (ENKVETCHAKUL et al., 1993; DANESHYAR; KERMANSHAHI; GOLIAN, 2012). One of the main BP enriched in the current study was gas transport, being related to gas transportation and cell migration (Figure 2). The upregulation of *RHAG* can be related to the proximal femoral epiphysiolysis, since in consequence of the pressure in the bone structure, caused by inflammation, the blood supply is cut, causing hypoxia due to the lack of oxygen. *RHAG* upregulation can be related to a consequence of FHS since the increase of this gene expression aims the oxygenation of the affected tissue.

The *EPX* gene is activated during a immune response, going to the area of injury or inflammation, releasing proteins and other components that have a toxic effect on severely damaged cells or invading organisms. One of these proteins is called eosinophil peroxidase, that are extremely cytotoxic to bacteria (JOHNSTON; BAEHNER, 1971; MICKENBERG; ROOT; WOLFF, 1972), parasites (AURIAULT; CAPRON; CAPRON, 1982; LOCKSLEY; WILSON; KLEBANOFF, 1982), eukaryotic cells (GLEICH; ADOLPHSON, 1986) and neoplastic cells (JONG; KLEBANOFF, 1980; NATHAN; KLEBANOFF, 1982) and also has anti-inflammatory and pro-inflammatory activities, regulating inflammation by fighting microbial invaders. The upregulation of the *EPX* may be related to a consequence of FHS, since a possible infection could pressure the bone structure, cutting the blood supply to this area, developing the necrosis. Furthermore, the *EPX* could drive the inflammatory process regulating genes related to fight the infection. Our results showed several biological processes and genes-related to immune response, indicating that the overexpression of these genes is activating the immune system to eliminate the progression of FHS, evidencing the presence of an inflammatory process, even at the early stages of epiphysiolysis.

Bone-related bioprocesses

The enrichment network at NetworkAnalyst platform indicates associations between DE genes and Wnt signaling pathway, GnRH signaling pathway, Adrenergic signaling in cardiomyocytes, VEGF signaling pathway, Bacterial infection, Focal adhesion, Gap junction, Toll-like receptor signaling pathway, AGE-RAGE signaling pathway in diabetic complications and NOD-like receptor signaling pathway (Figure 3). The Wnt signaling

is an ancient and evolutionarily conserved pathway responsible for the regulation of crucial aspects of cell fate determination, cell migration, cell polarity, neural patterning and organogenesis during embryonic development (HABAS; DAWID, 2005), important for bone development. Most of the genes enriched in this BP also appeared in focal adhesion and related to diabetic and Toll-like receptors. Some of these processes have already been described by PEIXOTO et al. (2019) and could be intrinsically correlated with FHS.

In humans, the metabolic syndrome is characterized by the presence of at least three of the following changes: visceral obesity, associated with insulin resistance, hypertension, high levels of triglycerides, and low HDL-cholesterol. The obesity and the insulin resistance are the major alteration associated to the metabolic syndrome, becoming almost mandatory at the metabolic syndrome cases. These associated factors in humans increase the predisposition of the development of type two diabetes and cardiovascular diseases (DESPRÉS; LEMIEUX, 2006). Durairaj et al. (2009) suggested that FHS could be a metabolic problem, related to fat metabolism disorders, facilitating an unbalanced growth in the articular-epiphyseal complex that leads to its separation under sheer stress. They observed that the blood parameters such as cholesterol, triglycerides, and low-density lipoproteins were slightly increased in FHS chickens. Despite the physiological differences between humans and chickens, the appearance of the GnRH signaling pathway, AGE-RAGE signaling pathway in diabetic complication and adrenergic signaling in cardiomyocytes indicates that chickens may have a similar physiology, needing more studies to better elucidate these pathways. The genes *PLCB2, MYH15, MAPK12* were related to the Adrenergic signaling in cardiomyocytes process. This pathway can be associated to epiphysiolysis as a consequence, due to the raise of the heart cells workload and energy metabolism, suffering from the imbalance between the O_2 supply and CO_2 removal of the gas transferring elements and the damage that hypoxia may cause to the heart cells (ENKVETCHAKUL et al., 1993; DANESHYAR; KERMANSHAHI; GOLIAN, 2012).

The genes *MAPK12, RAC2* were connected to the VEGF signaling pathway. This pathway is crucial at the vascular development stages and processes, like vasculogenesis, angiogenesis and lymphangiogenesis, which are essential for specification, morphogenesis, differentiation, and homeostasis of vessels during development and in the adulthood (ADAMS; ALITALO, 2007). Several studies have

reported bacterial infection as one of the causes of the epiphysiolysis. However, there is a controversy in the literature regarding the bacterial infections in the FHS and whether it is or not related to the cause of epiphysiolysis (THORP et al., 1993; SANTILI et al., 2004; ALMEIDA PAZ et al., 2009; MESTRINER et al., 2012; WILSON et al., 2019). The toll-like receptor signaling pathway was related to the genes *MAPK12* and *JUN.* This pathway plays a key role in the innate immune system and can be correlated as a consequence of the epiphysolisis, through the recognition of structurally conserved molecules derived from microbes that breached physical barriers and being recognized by the tool-like receptors, activating the immune response (MAHLA et al., 2013)

The initiation of FHS can be caused by a mechanical microfracturing of the growth plate, followed by colonization of osteochondrotic clefts by different opportunistic bacteria hematogenously dispread (WIDEMAN et al., 2015). The BCO can also be caused by bacterial translocation of the intestinal tract and proliferation in bone fissures, where the *Staphylococcus aureus* is the most frequent organism associated to osteomyelitis (BERENDT; BYREN, 2004; WRIGHT; NAIR, 2010; WIDEMAN et al., 2013). Furthermore, using the NetworkAnalyst 3.0, the DE genes were investigated for associations with locomotor problems in humans (Figure 4). This analysis showed that the *ADA, ANK1, JUN, ACTA1, TNNT3* and *ACTA1* genes were related to locomotor problems, showing a similar pattern in chickens and humans. However, more studies with chickens are needed to elucidate better its physiology and understand its response in the occurrence of locomotor problems.

Response to biotic stimulus, cell activation, cytolyses and immune system process.

At 35 days of age, the broilers are in an important phase of the femur development, where the length of the femur reaches a plateau (APPLEGATE; LILBURN, 2002). Biomechanical local stress, given through continuous mechanical stress, and impaired blood flow to the epiphyseal-physical cartilage are some of the factors that favor the pathogenesis of osteochondrosis, reported in several animal species (TRUETA; AMATO, 1960; RIDDELL, 1975; BOSS; MISSELEVICH, 2003; YTREHUS et al., 2004b, 2004a; YTREHUS; CARLSON; EKMAN, 2007). The FHS has been associated to the growing phase and a large number of DE genes in this study were associated

to bioprocesses related to immune system process, response to biotic stimulus, and multi organism process, which are relevant to the animal's locomotor system development. The DE genes in these BP associated to the epiphysiolysis are *ADA, AvBD1, AvBD2, C7, CCND3, CSF3R, EDN2, EPB42, GGT1, JUN, LECT2, LYG2, PTPRC, RAC2, RHAG, RSFR, S100A9, SELP, SERPINB10, STOM.*

The apoptosis is probably involved in the FHS in broilers, since it is a physiological mechanism crucial in the development and tissue homeostasis. In our study, these bioprocesses were not enriched in the DAVID database, but some genes associated to apoptosis were DE (*ADA, JUN, IFI6*). The gene *IFI6,* also known *as ISG12*, has an important role in the apoptosis regulation (PETRY et al., 2016; GENECARDS, 2019). In humans, this gene encodes a hydrophobic protein that acts in intracellular signaling (STARK et al., 1998; SATO; TANIGUCHI; TANAKA, 2001), but in birds, it does not have its function fully established. Furthermore, the ISGs family isknown to generate cellular and physiological diversity and it is associated with antiviral, anti-tumor and immunomodulatory activity mechanisms (PARKER; PORTER, 2004). In our results, the gene *IFI6* was DE and co-located with the gene *STEAP4*. These genes are expressed at the same site (cell or tissue), and their functions are related to regulation of cellular metabolism during osteoblast differentiation and regulation of apoptosis (ZHOU et al., 2013). The upregulation of the gene *IFI6 c*an be related to a causal factor, stimulating an excessive apoptosis at the articular cartilage, turning the animal more susceptible to the FHS.

Extracellular Matrix

The carbohydrate derivate catabolism was one of the superclusters observed, which contained the carbohydrate derivate catabolism and glycosaminoglycan catabolic bioprocesses (Figure 2, Supplementary Table 2). The extracellular matrix (ECM) is a structurally stable component that is located under the epithelium and surrounds connective tissue cells (HAY, 1981). Due to its structure, ECM is responsible for providing support and resistance to tissues and organs throughout the body, and acts in the body in biochemical processes that will assist in tissue morphogenesis, differentiation and homeostasis (FRANTZ; STEWART; WEAVER, 2010). In addition, in ECM there are molecules responsible for cell modulation, such as adhesion, migration,

proliferation, differentiation and cell survival of the tissue (DALEY; PETERS; LARSEN, 2008).

ECM is a very organized structure, divided into molecules responsible for the formation of fibers (collagen, elastin, and fibronectins) and the interfibrillar (proteoglycans and glycoproteins) (JÄRVELÄINEN et al., 2009; BRUCE et al., 2014). The glycosaminoglycan are fundamental components in the ECM, fulfilling various biological functions, such as cell adhesion, migration and proliferation, protein secretion and gene expression. They are highly polar and can also contribute to permeability properties, connective tissue structure and as a guide to enzymes and growth factors in both the matrix and cell surface (CECHOWSKA-PASKO, 1996).

The glycosaminoglycan and aminoglycan BP are related to ECM where DE genes were upregulated in FHS- affected broilers (*ADA, SERPINB10, AvBD1, STOM, JUN, RHAG, KEL, TF, EDN2, EPB42*) (Supplementary Table 2). Here, important upregulated genes are *ADA, RHAG,* and *JUN,* which participate in the glycosaminoglycan (GAGs) and aminoglycan metabolic processes involved in the ECM metabolism. The *RHAG, ADA* and *AvBd1* differential expression pattern between health and FHS-affected group were also confirmed by qPCR (Table 4).

The genes grouped in these previous BP were upregulated in the FHS-affected broilers, indicating that the body tries to fix the damage through the remodeling. Altogether, the results indicate that the upregulation of the genes could be a consequence of the damage by the epiphysiolysis, where the upregulation of these genes is an attempt to diminish the injury, since glycosaminoglycans mediate various receptor-ligand interactions on the cell surface and, as a result, play an important role in development as well as in lesion repair.

In this study, response to biotic stimulus, immune response, cell activation processes were BP highly represented. Epiphysiolysis may cause important physiological implications to the broilers development, which leads to more severe disorders.

There are some studies conducted with chicken bone tissues, evaluating locomotor problems, but none has been done with cartilage tissue. Both tissues are important to the development of the disease, so it is very important to have results regarding the relation between bone and cartilage tissues with the disease. The

identification of young broilers with vulnerable femoral joint can help genetic selection to reduce this anomaly. The BCO pathology does not show clinical signs at early stages, only at late stages or after necropsy when the diagnostic is possible (PACKIALAKSHMI et al., 2015). The use of Infrared thermography (IRT) was suggested as a technique to detect lesions attributed to BCO (WEIMER et al., 2019). The IRT consists of a noninvasive technique that measures infrared radiation from an object and can be a useful tool to evaluate clinical health. Although there are options to confirm the diagnosis of this condition, there are still limitations. The functional analyses of the DE genes help to elucidate their contribution to the development of the epiphysiolysis. These results contribute to a better understanding of the bacterial chondronecrosis with osteomyelitis in chickens and possible femur disorders in humans.

CONCLUSION

The first transcriptome of the femoral articular cartilage was generated, and biological processes and genes involved with femur epiphysiolysis in rapid growth chickens were identified. A total of 107 genes were differentially expressed between normal and epiphysiolysis-affected broilers. The *IFI6, CATH3, LECT3* and *COL13A1* genes were previously described as potentially involved with FHS. Several genes such as *AvBD1*, *AvBD2, ANK1, RHAG, ADA* and *EPX* were firstly associated to FHS in broilers, showing that the disruption in the AC could favor this phenotype. These results might help developing strategies to reduce the manifestation of this disorder in poultry, improving welfare and reducing economics loses.

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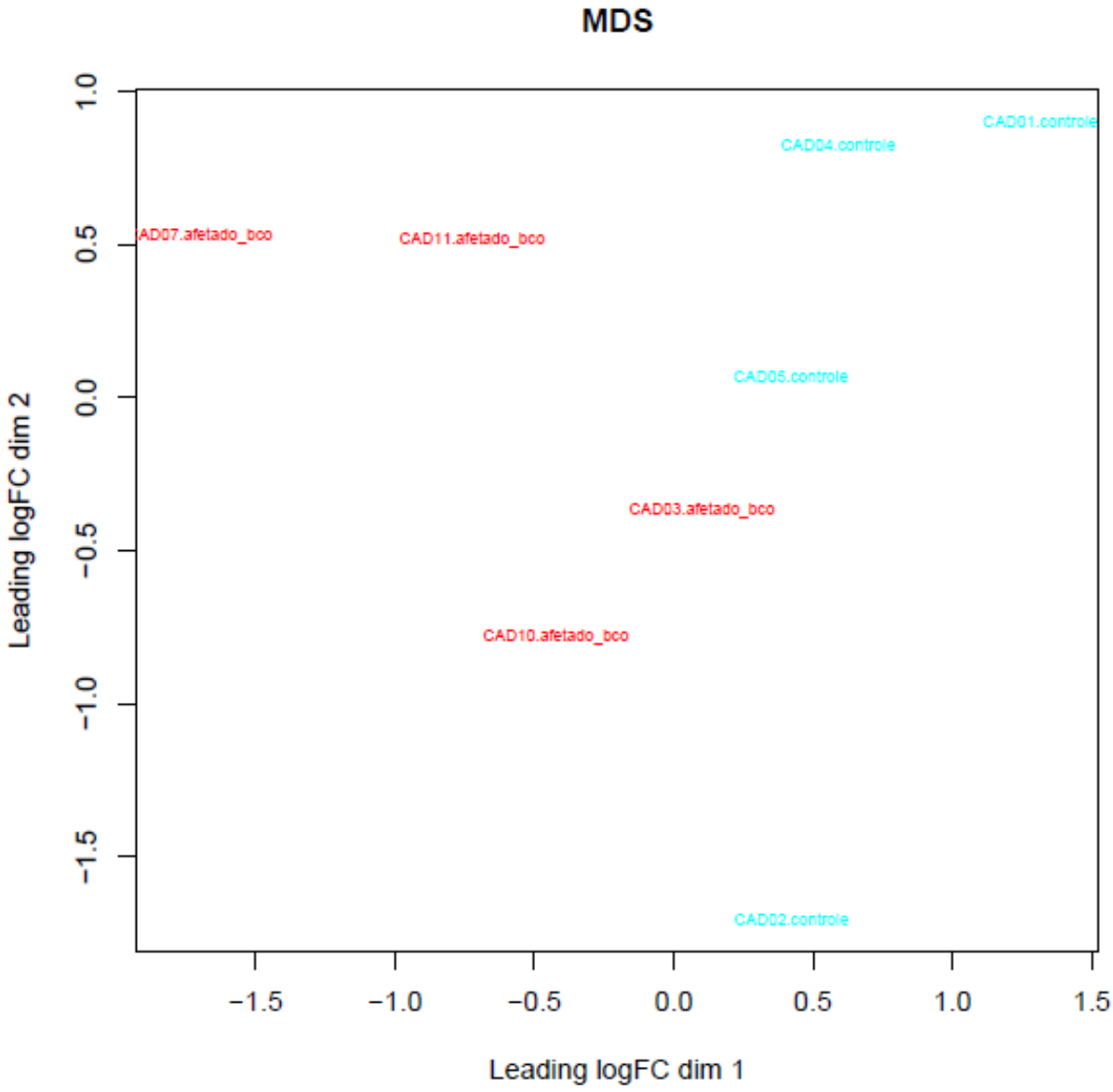
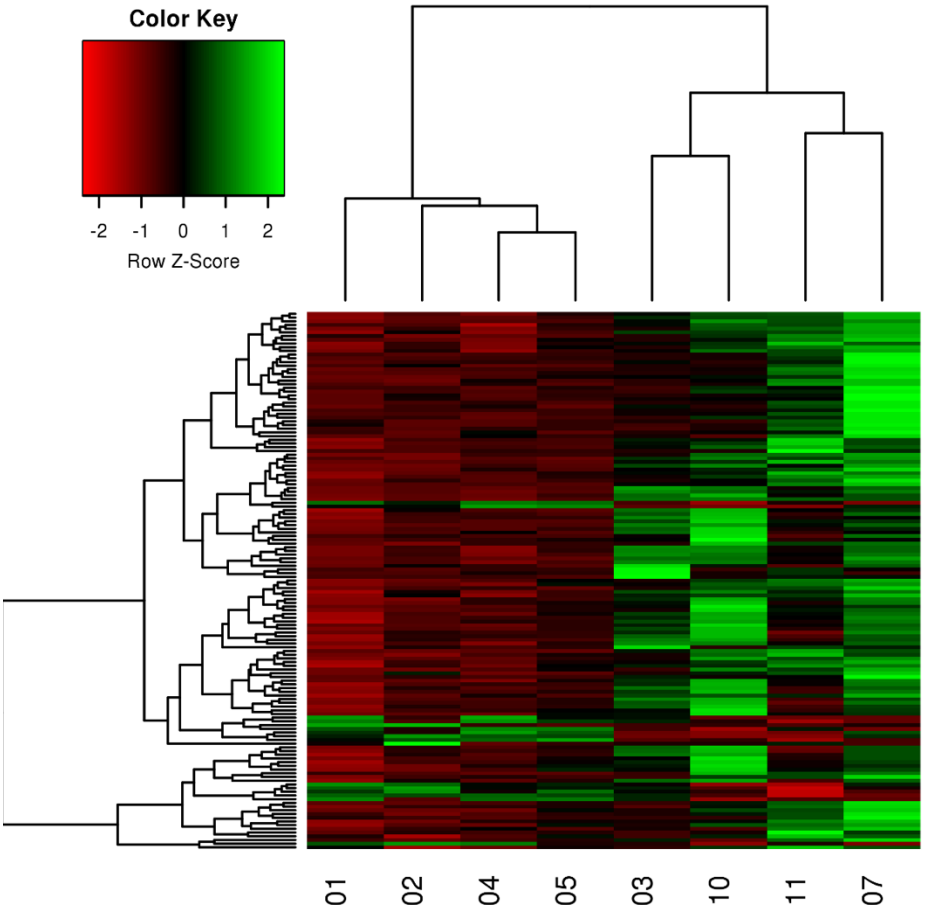
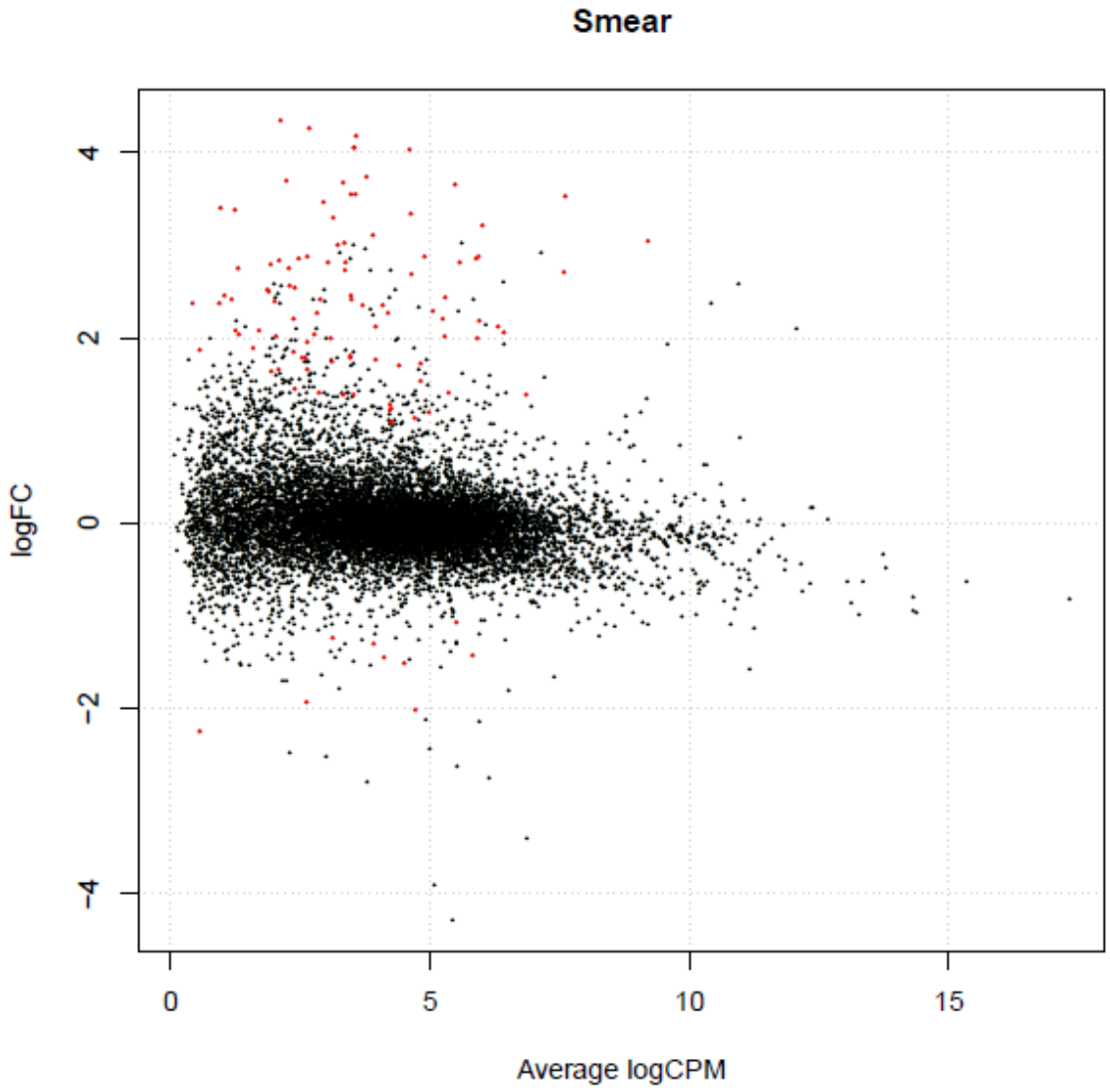
5. ANEXOS



Supplementary Figure 1: Multidimensional scale (MDS) plot showing the separation between samples from normal in blue (CAD 01, CAD 02, CAD 04, CAD 05) and epiphysiolysis-affected broilers in red (CAD 03, CAD 07, CAD 10, and CAD 11).



Supplementary Figure 2: Heatmap with 107 differentially expressed genes between samples of the femur head cartilage tissue from normal (01, 02, 04, 05) and epiphysiolysis-affected animals (03, 07, 10, 11). Each gene expression is shown in the lines and the samples in columns, resulting in a hierarchical group of genes and samples. In red are the downregulated and in green are the upregulated genes in the affected animals.



Supplementary Figure 3 - Representation of 107 differentially expressed genes (FDR = 0.05 in red). X-axis values are based on mean expression values and the Y-axis values are Log2FC.

Supplementary Table 1 - Genes differentially expressed in the femur head cartilage of healthy and epiphysiolysis-affected broiler chickens.

Gene_id	Gene_name	logFC
ENSGALG00000043254	*EPX*	4,265140953
ENSGALG00000022815	*AvBD1*	3,550775499
ENSGALG00000006323	*LECT2*	2,87851666
ENSGALG00000016761	*LYG2*	3,410986532
ENSGALG00000024272	*S100A9*	4,041420369
ENSGALG00000034150	*CATHL3*	3,379953627
ENSGALG00000052026		2,742915118

ENSGALG00000019696	*CATHL2*	3,691216119
ENSGALG00000019553	*SERPINB10B*	2,732183598
ENSGALG00000003212	*TSPO2*	3,664873729
ENSGALG00000047285	*BD7*	3,471856529
ENSGALG00000016669	*AvBD2*	3,668135915
ENSGALG00000040279		2,022187971
ENSGALG00000014463	*ACTN2*	4,339315826
ENSGALG00000032854	*ACP5*	1,954830762
ENSGALG00000023953		4,050020284
ENSGALG00000036925	*CADM3*	2,809547453
ENSGALG00000004804	*TGM3*	2,419571981
ENSGALG00000008306	*FGL2*	3,213223185
ENSGALG00000002112	*CSF3R*	2,279124695
ENSGALG00000016285		2,454983677
ENSGALG00000039978	*SLC4A1*	2,704019357
ENSGALG00000026518	*RUNDC3A*	2,44875269
ENSGALG00000050327		2,375542377
ENSGALG00000023436	*HDC*	2,367744865
ENSGALG00000044661		2,420926354
ENSGALG00000007447	*TUBB1*	1,994595325
ENSGALG00000011190		2,557061826
ENSGALG00000042105	*PLCB2*	1,817209049
ENSGALG00000006572	*TNNT3*	4,045292494
ENSGALG00000027165	*RNASE4*	2,289248426
ENSGALG00000021230	*EPB42*	2,698399492
ENSGALG00000014585		2,362356709
ENSGALG00000004170	*ADA*	1,722427845
ENSGALG00000015358	*MYH15*	-1,92470467
ENSGALG00000016684	*RHAG*	2,441152594
ENSGALG00000002397	*PSPH*	-1,42600672
ENSGALG00000029857		1,79314388
ENSGALG00000013575	*IFI6*	2,869748753
ENSGALG00000011086	*ACTA1*	2,818513187
ENSGALG00000045846	*PDK2*	1,389003693
ENSGALG00000009479		3,102175743
ENSGALG00000027716		3,345872146
ENSGALG00000005418	*FRRS1*	1,19379141
ENSGALG00000031177		1,879249968
ENSGALG00000000378	*SLC25A37*	1,774334938
ENSGALG00000053964		1,658019558
ENSGALG00000013414	*PDLIM3*	2,344183418
ENSGALG00000051174		3,540345331
ENSGALG00000030886		3,534161726
ENSGALG00000053676		2,842935934
ENSGALG00000045478		2,27544674
ENSGALG00000036805		2,194548712

ENSGALG00000003845	*MKNK2*	1,403430128
ENSGALG00000003485	*CCND3*	1,086308954
ENSGALG00000041693		2,80817001
ENSGALG00000026948	*ADD2*	2,125035463
ENSGALG00000027765	*MMR1L1*	2,424712197
ENSGALG00000051068		2,394354286
ENSGALG00000002907	*MYL1*	4,173853292
ENSGALG00000040101		2,196113993
ENSGALG00000004418	*SSTR2*	2,860543857
ENSGALG00000054766	*ITGA2B*	2,021757598
ENSGALG00000014736		2,005043165
ENSGALG00000003594	*ANK1*	2,115633452
ENSGALG00000005643	*LYVE1*	3,021253996
ENSGALG00000041988	*SIK1*	1,27368248
ENSGALG00000043671		-1,23726451
ENSGALG00000038902	*PIM1*	1,140373551
ENSGALG00000007728		1,209186391
ENSGALG00000014835	*C7*	2,851353942
ENSGALG00000028273	*HBE1*	3,737900568
ENSGALG00000039269	*RNF213*	2,058591669
ENSGALG00000019384	*MAPK12*	1,448465085
ENSGALG00000006565		-1,50157899
ENSGALG00000001558		1,699245218
ENSGALG00000039489	*RF00002*	-2,2369755
ENSGALG00000005473	*PNAT3*	2,206103471
ENSGALG00000010870	*JUN*	1,385299355
ENSGALG00000000558	*SLC1A6*	2,887383641
ENSGALG00000051148		2,803168187
ENSGALG00000001434	*STOM*	1,409886505
ENSGALG00000037077	*CCR5*	2,035946818
ENSGALG00000012456	*RAC2*	1,384361626
ENSGALG00000039480		2,760811941
ENSGALG00000002192	*PTPRC*	1,232680188
ENSGALG00000050131		2,995177847
ENSGALG00000002643		1,657345034
ENSGALG00000028357	*MMR1L2*	2,494044185
ENSGALG00000045776	*CPN2*	1,784928007
ENSGALG00000004286	*COL13A1*	-1,29613515
ENSGALG00000014686	*FBN2*	-1,08076797
ENSGALG00000014995	*PGM5*	2,531983418
ENSGALG00000052196		1,64131705
ENSGALG00000006889	*TMC7*	2,076636513
ENSGALG00000009552		1,780010801
ENSGALG00000054746		1,856211784
ENSGALG00000030247	*TMOD4*	2,047927724
ENSGALG00000000667	*EDN2*	2,083372559

ENSGALG00000038225	*SEMA3E*	-1,44204757
ENSGALG00000016325	*GSTA3*	1,734887629
ENSGALG00000044326		1,540641036
ENSGALG00000052853		3,305369159
ENSGALG00000032239	*GCHFR*	2,528047135
ENSGALG00000052537		1,8970309
ENSGALG00000006453	*LTF*	3,047053185
ENSGALG00000010490	*DPYSL4*	-2,00800872

Supplementary Table 2 - Biological processes related to genes differentially expressed in the femur head cartilage of healthy and epiphysiolysis-affected broiler chickens. The downregulated genes are represented in bold.

Term ID	Description	Gene	P-Value
GO:0006026	aminoglycan catabolic process	*LYG2, LYVE1.*	0.072532
GO:0048513	animal organ development	***FBN2***, *RHAG, CSF3R, KKEL, ACTN2, TGM3,* ***MYH15***, *ACTA1, JUN, LECT2, EDN2, EPB42, ADA, SERPINB10.*	0.036044
GO:0019722	calcium-mediated signaling	*SELP, EDN2, ADA.*	0.034973
GO:1901136	carbohydrate derivative catabolic process	*LYG2, LYVE1, ADA.*	0.046475
GO:0055080	cation homeostasis	*RHAG, KEL, TF, EDN2, EPB42.*	0.063932
GO:0001775	cell activation	*CCND3, CD62, JUN, EDN2, ADA, RAC2.*	0.044457
GO:0060326	cell chemotaxis	*CSF3R, CCLI10, EDN2, RAC2.*	0.024848
GO:0016477	cell migration	*CSF3R, SELP, JUN, CCLI10, EDN2, ADA, RAC2.*	0.090366
GO:0030593	neutrophil chemotaxis	*CSF3R, CCLI10, EDN2, RAC2.*	0.095546
GO:0019835	cytolysis	*LYG2, CATH2.*	1.12E-05
GO:0006952	defense response	*S100A9, LYG2, STOM, CATH2, TF, CCLI10, GGT1, RSFR, AvBD1, SELP, AvBD2, JUN, ADA, SERPINB10.*	5.40E-04
GO:0042742	defense response to bacterium	*RSFR, LYG2, AvBD1, CATH2, AvBD2.*	4.55E-04

GO:0098542	defense response to other organism	*RSFR, LYG2, AvBD1, STOM, CATH2, AvBD2, SERPINB10.*	0.072532
GO:0048821	erythrocyte development	*RHAG, SERPINB10.*	0.048946
GO:0015669	gas transport	*HBBA, RHAG.*	0.053709
GO:0006027	glycosaminoglycan catabolic process	*LYG2, LYVE1.*	0.091301
GO:0007186	G-protein coupled receptor signaling pathway	*SSTR2, ACTN2, CCLI10, ADA, RAC2.*	0.001343
GO:0071621	granulocyte chemotaxis	*CSF3R, CCLI10, EDN2, RAC2.*	0.001529
GO:0097530	granulocyte migration	*CSF3R, CCLI10, EDN2, RAC2.*	0.016146
GO:0048534	hematopoietic or lymphoid organ development	*RHAG, CSF3R, JUN, LECT2, ADA, EPB42, SERPINB10.*	0.042442
GO:0030097	hemopoiesis	*RHAG, CSF3R, JUN, LECT2, ADA, SERPINB10.*	0.010333
GO:0006955	immune response	*S100A9, PTPRC, CATH2, C7, TF, CCLI10, ADA, RAC2.*	0.020347
GO:0002520	immune system development	*RHAG, CSF3R, JUN, LECT2, ADA, EPB42, SERPINB10.*	2.74E-06
GO:0002376	immune system process	*S100A9, RHAG, CSF3R, STOM, CATH2, PTPRC, C7, TF, CCLI10, CCND3, SELP, JUN, LECT2, EDN2, EPB42, ADA, RAC2, SERPINB10.*	0.069001
GO:0098771	inorganic ion homeostasis	*RHAG, KEL, TF, EDN2, EPB42.*	0.084708
GO:0050801	ion homeostasis	*RHAG, KEL, TF, EDN2, EPB42.*	0.012406
GO:0055072	iron ion homeostasis	*RHAG, TF, EPB42.*	0.078364
GO:0045321	leukocyte activation	*CCND3, JUN, EDN2, ADA, RAC2.*	0.094013
GO:0007159	leukocyte cell-cell adhesion	*CCND3, SELP, ADA, RAC2.*	0.009850
GO:0030595	leukocyte chemotaxis	*CSF3R, CCLI10, EDN2, RAC2.*	3.89E-04
GO:0050900	leukocyte migration	*CSF3R, SELP, CCLI10, EDN2, ADA, RAC2.*	0.037459
GO:0055065	metal ion homeostasis	*RHAG, KEL, TF, EDN2, EPB42.*	0.016925
GO:0035821	modification of morphology or physiology of other organism	*AvBD1, STOM, JUN.*	0.002542

GO:0051704	multi-organism process	*RSFR, LYG2, AvBD1, STOM, CATH2, AvBD2, TF, JUN, GGT1, ADA, SERPINB10.*	0.011100
GO:0055001	muscle cell development	***MYH15**, ACTA1, KEL, ACTN2.*	0.018540
GO:0042692	muscle cell differentiation	***MYH15**, ACTA1, KEL, MAPK12, ACTN2.*	0.004147
GO:0006936	muscle contraction	*MYL1, TNNT3, ACTN2, EDN2, ADA.*	0.065180
GO:0061061	muscle structure development	***MYH15**, ACTA1, KEL, MAPK12, ACTN2.*	0.007637
GO:0003012	muscle system process	*MYL1, TNNT3, ACTN2, EDN2, ADA.*	0.086250
GO:0060537	muscle tissue development	***MYH15**, ACTA1, KEL, ACTN2.*	0.060656
GO:0030099	myeloid cell differentiation	*RHAG, JUN, LECT2, SERPINB10.*	0.040567
GO:0002274	myeloid leukocyte activation	*JUN, EDN2, RAC2.*	0.005277
GO:0097529	myeloid leukocyte migration	*CSF3R, CCLI10, EDN2, RAC2.*	0.053709
GO:1901380	negative regulation of potassium ion transmembrane transport	*KEL, ACTN2.*	0.063167
GO:0043267	negative regulation of potassium ion transport	*KEL, ACTN2.*	7.45E-04
GO:1990266	neutrophil migration	*CSF3R, CCLI10, EDN2, RAC2.*	9.43E-04
GO:1901565	organonitrogen compound catabolic process	*LYG2, LYVE1, HDC, ADA.*	0.031974
GO:0018149	peptide cross-linking	*TGM3, EPB42.*	0.072532
GO:0030335	positive regulation of cell migration	*SELP, JUN, EDN2, RAC2.*	0.091068
GO:2000147	positive regulation of cell motility	*SELP, JUN, EDN2, RAC2.*	0.095003
GO:0045823	positive regulation of heart contraction	*EDN2, ADA.*	0.063167
GO:0010460	positive regulation of heart rate	*EDN2, ADA.*	0.034513
GO:0002684	positive regulation of immune system process	*PTPRC, SELP, EDN2, ADA, RAC2.*	0.081857
GO:0002687	positive regulation of leukocyte migration	*SELP, EDN2, RAC2.*	0.017737
GO:0045933	positive regulation of muscle contraction	*EDN2, ADA.*	0.090989
GO:0032103	positive regulation of response to external stimulus	*CCLI10, EDN2, RAC2.*	0.096732

GO:0045987	positive regulation of smooth muscle contraction	*EDN2, ADA.*	0.063167
GO:1904062	regulation of cation transmembrane transport	*KEL, STOM, ACTN2.*	0.077201
GO:0031347	regulation of defense response	*S100A9, STOM, CCLI10, GGT1, ADA, SERPINB10.*	0.009528
GO:0002682	regulation of immune system process	*CSF3R, STOM, PTPRC, SELP, EDN2, GGT1, ADA, RAC2, SERPINB10.*	0.004495
GO:0050727	regulation of inflammatory response	*S100A9, CCLI10, GGT1, ADA.*	0.020026
GO:0002685	regulation of leukocyte migration	*SELP, EDN2, ADA, RAC2.*	0.004299
GO:0043900	regulation of multi-organism process	*STOM, JUN, ADA, SERPINB10.*	0.071619
GO:0006937	regulation of muscle contraction	*TNNT3, EDN2, ADA.*	0.040567
GO:0090257	regulation of muscle system process	*TNNT3, EDN2, ADA.*	0.061830
GO:0032101	regulation of response to external stimulus	*S100A9, STOM, SELP, CCLI10, EDN2, GGT1, ADA, RAC2, SERPINB10.*	5.33E-04
GO:0080134	regulation of response to stress	*S100A9, STOM, SELP, CCLI10, GGT1, ADA, SERPINB10.*	0.062854
GO:0009617	response to bacterium	*RSFR, LYG2, AvBD1, CATH2, AvBD2, TF.*	0.002036
GO:0009607	response to biotic stimulus	*LYG2, AvBD1, STOM, CATH2, AvBD2, TF, SERPINB10.*	0.001864
GO:0043207	response to external biotic stimulus	*RSFR, LYG2, AvBD1, STOM, CATH2, AvBD2, TF, SERPINB10.*	0.001363
GO:0009605	response to external stimulus	*S100A9, LYG2, CSF3R, STOM, CATH2, TF, GGT1, CCLI10, RSFR, AvBD1, SELP, AvBD2, JUN, EDN2, ADA, RAC2, SERPINB10.*	6.00E-06
GO:0051707	response to other organism	*RSFR, LYG2, AvBD1, STOM, CATH2, AvBD2, TF, SERPINB10.*	0.001363
GO:0006950	response to stress	*S100A9, LYG2, STOM, CATH2, TF, CCLI10, GGT1, RSFR, AvBD1, SELP, AvBD2, JUN, ADA, SERPINB10.*	0.045040
GO:0019932	second-messenger-mediated signaling	*SELP, EDN2, ADA.*	0.078654

GO:0009070	serine family amino acid biosynthetic process	***PSPH,*** *GGT1.*	0.053709
GO:0055002	striated muscle cell development	***MYH15****, ACTA1, KEL, ACTN2.*	0.007870
GO:0051146	striated muscle cell differentiation	***MYH15****, ACTA1, KEL, ACTN2.*	0.028565
GO:0014706	striated muscle tissue development	***MYH15****, ACTA1, KEL, ACTN2.*	0.069877
GO:0055076	transition metal ion homeostasis	*RHAG, TF, EPB42.*	0.030737