UNIVERSIDADE ESTADUAL PAULISTA - UNESP

CAMPUS DE JABOTICABAL

DETERMINAÇÃO DO TURNOVER PROTEICO E SUPLEMENTAÇÃO COM AMINOÁCIDOS DE CADEIA RAMIFICADA, ARGININA E ÁCIDO DOCOSAHEXAENÓICO PARA CONTROLE DA SARCOPENIA EM CÃES IDOSOS

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Zootecnista

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Orientador: Prof. Dr. Aulus Cavalieri Carciofi

Tese apresentada à Faculdade de Ciências Agrárias e Veterinárias – Unesp, Câmpus de Jaboticabal, como parte das exigências para a obtenção do título de Doutora em Zootecnia.

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TÍTULO DA TESE: DETERMINAÇÃO DO TURNOVER PROTEICO E SUPLEMENTAÇÃO COM AMINOÁCIDOS DE CADEIA RAMIFICADA, ARGININA E ÁCIDO DOCOSAHEXAENÓICO PARA CONTROLE DA SARCOPENIA EM CÃES IDOSOS

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Jaboticabal, 05 de abril de 2022

DADOS CURRICULARES DA AUTORA

Letícia Graziele Pacheco nasceu em Jaboticabal - SP no ano de 1989. Iniciou os estudos na E.E. “José Luiz de Siqueira”, concluindo o ensino médio no ano de 2006. Em 2007 iniciou curso de Letras – Português/Inglês, na Faculdade de Educação São Luis em Jaboticabal – SP, concluindo o curso no ano de 2009. No ano de 2011 ingressou no curso de Graduação em Zootecnia na Universidade Estadual Paulista “Júlio de Mesquita Filho”, Unesp, Jaboticabal, SP. Durante a graduação foi aluna de iniciação científica pela Fapesp sob a orientação das professoras Lizandra Amoroso e Nilva Kazue Sakomura. Concluiu a graduação em fevereiro de 2016. Se tornou Mestra pelo Programa de Pós-Graduação em Zootecnia com ênfase em Nutrição de Monogástricos. Atualmente é doutoranda pelo Programa de Pós – Graduação em Zootecnia, com ênfase em Nutrição de Cães e Gatos, sob a orientação do Professor Doutor Aulus Cavalieri Carciofi.

“Se, a princípio, a ideia não é absurda, então não há esperança pra ela.”

Albert Einstein

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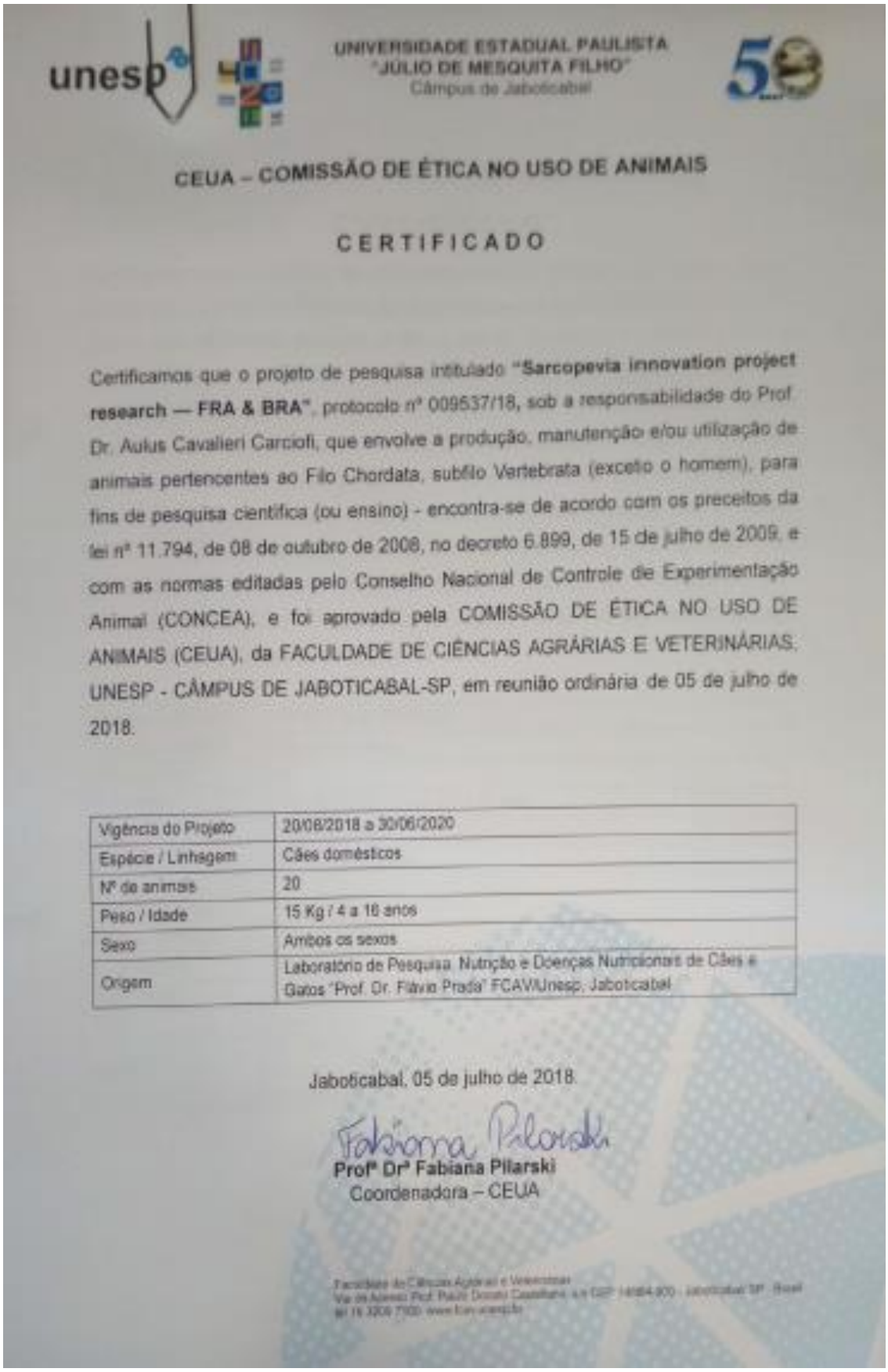

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CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o projeto de pesquisa intitulado **"Sarcopevia innovation project research — FRA & BRA"**, protocolo nº 009537/18, sob a responsabilidade do Prof. Dr. Aulus Cavalieri Carciofi, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 05 de julho de 2018.

Vigência do Projeto	20/06/2018 a 30/06/2020
Espécie / Linhagem	Cães domésticos
Nº de animais	20
Peso / Idade	15 Kg / 4 a 16 anos
Sexo	Ambos os sexos
Origem	Laboratório de Pesquisa Nutrição e Doenças Nutricionais de Cães e Gatos "Prof. Dr. Flávio Prada" FCAV/Unesp, Jaboticabal

Jaboticabal, 05 de julho de 2018.

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RESUMO

A fim de se aprofundar estudos acerca do metabolismo proteico, foram comparados quatro métodos para se determinar o fluxo de proteína em cães adultos e idosos, bem como avaliar os efeitos do fornecimento de uma dieta enriquecida com ácido docosaexaenoico (DHA), arginina e aminoácidos de cadeia ramificada (ACR) sobre o metabolismo de proteína. O primeiro experimento teve por objetivo estudar e padronizar os métodos de utilização dos compostos marcados. O segundo experimento comparou os métodos de determinação do turnover proteico com ^{13}C-Leucina, ^{13}C-Fenilalanina e ^{15}N-Glicina em cães adultos e idosos. Para isto foram avaliadas a digestibilidade, balanço de nitrogênio e fluxo proteico em arranjo fatorial 4x2 (4 métodos e 2 faixas etárias). No terceiro experimento foram avaliadas em cães adultos e idosos duas dietas, uma controle e outra suplementada com DHA, arginina e ACR. Foi adotado delineamento cross over e arranjo fatorial 2x2, com duas dietas (Controle x Teste) e duas faixas etárias (adultos x idosos). Na dieta teste a quantidade analisada de DHA, leucina, isoleucina, valina e arginina foram, respectivamente, 100%, 72%, 22%, 18%, e 35% maiores do que na dieta controle. No primeiro estudo os métodos não diferiram em si, obtendo-se resultados semelhantes de síntese e degradação proteica ($P>0,05$). Após esta experiência, os procedimentos experimentais de aplicação dos métodos foram melhorados para os estudos seguintes. No segundo experimento foi observado efeito de idade no metabolismo de proteína ($P<0,05$) sem efeito de métodos ($P>0,05$). Pelos quatro métodos de estudo, em média as taxas de síntese e degradação de proteína foram, respectivamente, 19% e 21% menores nos idosos em comparação aos adultos ($P<0,05$). Não houve efeito, no entanto, em digestibilidade e balanço de nitrogênio ($P>0,05$). Estes resultados suportam hipótese que alterações metabólicas sejam uma das causas implicadas no desenvolvimento de sarcopenia em cães. No terceiro experimento foi observado efeito de dieta, sendo que a suplementação nutricional proposta elevou o fluxo de proteína corporal dos cães ($P<0,05$), tanto em adultos como idosos. Apesar de estatisticamente semelhante, esta elevação foi 42% maior em adultos em comparação com idosos. Adicionalmente, ao final do período de recebimento das dietas experimentais, tendência à maior massa magra corporal (kg) foi observada para idosos alimentados com a dieta teste em comparação à controle ($P=0,06$). Como conclusão, os quatro métodos avaliados foram equivalentes na determinação de síntese e degradação de proteína. A suplementação alimentar com DHA, ACR e arginina elevou o fluxo proteico, podendo favorecer a desaceleração da perda de massa muscular e controle da sarcopenia, merecendo estudos de longo prazo.

Palavras-chave: geriatria, leucina, massa corporal magra, sarcopenia, isótopos estáveis.

ABSTRACT

To develop better study protocols to access protein metabolism, four methods to estimate protein flux was compared in adult and old dogs. The dietary supply of docosahexaenoic acid (DHA), branched-chain amino acids (BCA), and arginine was also evaluated. The first study aimed to investigate and standardize methods of study protein metabolism with stable isotopes. The second experiment compared the methods of ^{13}C-Leucine, ^{13}C-Phenylalanine, ^{15}N-Glycine in adult and old dogs. Nutrient digestibility, nitrogen balance, and protein flow were evaluated in a 4 (methods) x 2 (ages) factorial arrangement. The third study compared two diets in adult and old dogs, a control and a test diet supplemented with DHA, arginine, and BCA. A cross over design and a 2 (diets) x 2 (ages) factorial arrangement was adopted. The test diet presented analyzed values of DHA, arginine, leucine, isoleucine and valine 100%, 35%, 72%, 22% and 18% higher, respectively, than the control treatment. In the first study, similar results of protein synthesis and degradation was observed for all evaluated methods ($P>0.05$). After this experience, the protocol of the stable isotopes' application was improved for the other studies. On study two, an age effect was observed for protein flow ($P>0.05$), without effect of method ($P<0.05$). Considering the four methods studied, the mean rates of protein synthesis and breakdown was, respectively, 19% and 21% lower in old than adult dogs ($P<0.05$). No age effect was observed for nutrient digestibility and nitrogen balance ($P>0.05$). These results support the hypothesis that metabolic alterations are involved on sarcopenia development in old dogs. On the third study a diet effect was observed: both in adult and old dogs fed the supplemented food higher protein flow was observed than for animals fed the control diet ($P>0.05$). Although statistically similar, this increase in protein flow was 42% higher for adult than for old dogs. In addition, at the end of the period of diet intake a tendency for higher lean body mass was observed for old dogs fed the supplemented food in comparison to control ($P=0.06$). As conclusion, the four methods evaluated was adequate with similar results of protein synthesis and degradation. A diet supplementation with DHA, arginine and BCA increased the protein flow rate, possibly reducing sarcopenia development, deserving future studies.

Keywords: geriatrics, leucine, lean body mass, sarcopenia, stable isotopes.

CAPÍTULO 1 – Considerações gerais

1.1 Introdução

O aumento dos cuidados e melhora da nutrição de cães tem levado ao aumento da expectativa de vida destes animais (Freeman, 2012; Larsen e Farcas, 2014). Com o envelhecimento, cães passam a demonstrar com mais frequência alterações fisiológicas de senectude, muitas das quais requerem estudos sobre possíveis alterações alimentares para se assegurar saúde e qualidade de vida a estes animais. Dentre as alterações frequentes em cães idosos, destacam-se a sarcopenia (Freeman, 2012; Saker, 2021).

O uso de traçadores metabólicos permite extrair informações valiosas acerca da cinética, turnover e metabolismo de um determinado nutriente de interesse, tornando-se uma ferramenta de alto potencial para elucidar questões fisiológicas, independentemente da idade do animal (Kim et al., 2016). O traçador ideal deve ser identificado com precisão suficiente quando fornecido em pequenas doses, devendo ainda representar e não afetar o metabolismo do elemento a ser traçado (Wolfe e Chinkes, 2005)

Os métodos mais conhecidos para se determinar turnover são o do precursor, dos produtos finais e da oxidação aminoacídica (Wagenmakers, 1999). Os três podem ser utilizados para dois propósitos: determinação das taxas de turnover, síntese e degradação proteica geral, ou serem aplicados para estudos de aminoácidos isolados quando em concentrações deficientes ou excessivas.

Dentro do princípio de obtenção, padronização e utilização de métodos minimamente invasivos, objetivou-se com este projeto estudar e comparar o metabolismo proteico de cães adultos jovens e idosos, utilizando-se isótopos estáveis como traçadores metabólicos, com vistas à melhor caracterização e compreensão da sarcopenia. Além disso, foi objetivo comparar e validar três traçadores para quantificação do turnover proteico corporal de cães e também testar uma dieta enriquecida com ácido docosaexaenoico e leucina a fim de verificar seu efeito sobre o metabolismo de proteína.

1.2 Revisão de Literatura

Sarcopenia e suas implicações à saúde de cães

O aumento dos cuidados e melhora da nutrição de cães tem levado ao aumento da expectativa de vida destes animais (Freeman, 2012; Larsen e Farcas, 2014). Com o envelhecimento, cães passam a demonstrar com mais frequência alterações fisiológicas de senectude, muitas das quais requerem estudos sobre possíveis alterações alimentares para se assegurar saúde e qualidade de vida a estes animais. Dentre as alterações frequentes em cães idosos, destacam-se a sarcopenia (Freeman, 2012; Saker, 2021).

O termo sarcopenia é oriundo do grego sarx (carne) e penia (perda) e, embora seja clinicamente aplicado para denotar perda de massa muscular, é frequentemente utilizado para descrever conjunto de processos celulares (denervação, disfunção mitocondrial, inflamatórias e hormonais) e suas consequências como diminuição da força muscular, da mobilidade e da função muscular, além do aumento da fadiga, do risco de distúrbios e aumento do risco de quedas e fraturas esqueléticas (Lang et al., 2010).

Sarcopenia, no entanto, é processo natural caracterizado pela redução do tecido muscular esquelético como resultado do processo de senescência dos organismos. São vários os mecanismos que podem explicar a mudança na massa muscular, entre eles a falta de atividade física regular, mudanças naturais no metabolismo de proteínas quando do déficit entre síntese e degradação, diminuição na concentração de hormônios (crescimento, testosterona e IGF-1), aumento da produção de cortisol e citocinas, perda de funções neuromusculares (contrações musculares), expressão gênica alterada e apoptose (Rudman, 1985; Herbst et al., 2007).

Como processo gradual, a sarcopenia pode vir a não ser notada devido ao aumento de gordura corporal com o envelhecimento. Assim, Rice et al. (1985) encontraram em seres humanos perda considerável de massa magra, embora houvesse manutenção do peso corporal. Estudos neste sentido, infelizmente, não são suficientes para cães de modo a permitir uma caracterização adequada da patogenia e consequências na espécie.

Estudos com humanos em diversos países observaram que de 10 a 25% de idosos com até 70 anos são considerados sarcopênicos, acometendo a

condição entre 40 e 60% dos idosos acima de 80 anos. A prevalência da sarcopenia é mais observada em mulheres do que em homens e cerca de 76% da população não percebe que está desenvolvendo essa síndrome, pois não há uma idade determinada para que a perda de massa muscular comece a afetar as atividades diárias (Baumgartner et al., 1998; Marzetti et al.,2010; Mendes et al., 2016; Gianoudis et al., 2016).

Os riscos da sarcopenia estão associados à mudança da composição corporal do indivíduo devido à perda de proteína corporal. Comumente observa-se maior susceptibilidade à infecções e fraturas ocasionadas pelo enfraquecimento de contrações musculares e outras alterações no tecido muscular esquelético, que resultam em redução da qualidade óssea. Redução na extensão muscular também pode acarretar fraturas por compressão das vértebras, devido à falta de suporte muscular na coluna vertebral. A sarcopenia também pode levar à diminuição de até 50% da capacidade de trabalho e aumento da mortalidade (Dutta, 1997; Tipton, 2001; Short et al., 2004; Rizzoli et al., 2013).

Em cães, estudos que levantam a ocorrência e caracterizaram a sarcopenia são escassos. Alguns autores sugerem que a perda de massa magra é um processo natural com o avançar da idade na espécie com características sintomáticas e fisiológicas similares ao observado nos humanos (Meyer e Stadtfeld, 1980; Lawler et al., 2009). Bentubo et al. (2007), em um estudo sobre a expectativa de vida e causa da morte em cães na cidade de São Paulo, encontraram que cerca de 5,54% dos cães morrem por senilidade com expectativa de vida em torno de 144 meses. Infelizmente, estes estudos não apresentam dados de composição corporal ou caracterizaram sarcopenia na população de estudo.

A nutrição ao longo da vida se reflete diretamente na condição corporal do animal na senescência. O plano alimentar deve auxiliar a manutenção da síntese proteica para evitar o enfraquecimento e redução da massa muscular, que podem resultar em fraturas e perdas hormonais características da sarcopenia (Larsen e Farcas, 2014). Acredita-se que se a ingestão proteica for insuficiente, a perda de massa corporal magra associada à idade pode se agravar e contribuir para redução da qualidade de vida e mortalidade precoce (Laflamme, 2008). É possível que estas mudanças na deposição de tecido

muscular e massa corporal magra nos idosos sejam reflexo de alterações no metabolismo proteico, com menor deposição relativa e maior catabolismo tecidual (William et al., 2001, Wannemacher e McCoy, 1966). Dentre os poucos estudos com cães, maior exigência de proteína para balanço nitrogenado foi verificado em cães idosos (Wannemacher e McCoy, 1966), com aumento das exigências de proteína em cerca de 50% quando comparado aos adultos. Estes resultados foram obtidos ao se utilizar marcadores de turnover proteico e de perda de massa magra, ainda que o balanço de nitrogênio tenha se mantido semelhante entre as idades. Isto é reforçado pelo fato de a senescência não induzir nos cães alterações em digestibilidade proteica, que permanece inalterada com o avançar da idade (Maria et al., 2017), parecendo a utilização pós-absorção ser aspecto relevante na sarcopenia.

Um ponto, no entanto, que pode também somar-se à esta questão é o menor gasto e necessidade energética de cães idosos em comparação à adultos (Harper, 1998; Laflamme, 2000; Harper, 1998). A redução no gasto energético acompanha o envelhecimento de cães, o que resulta em menor ingestão de alimento e nutrientes nos idosos (Maria et al., 2017). Este menor consumo deve então ser compensado na formulação, elevando-se a concentração proteica e de aminoácidos dos alimentos para idosos de modo a se assegurar ingestão suficiente de proteína e aminoácidos. Isto, no entanto, nem sempre é observado na formulação de alimentos comerciais.

Necessidade e metabolismo de proteína

Estudos sobre necessidade de proteína e aminoácidos em cães adultos são poucos e têm se baseado no método de balanço nitrogenado. Para idosos, ainda, estes dados são praticamente inexistentes (NRC, 2006). Pelo método, a necessidade mínima corresponde à ingestão de aminoácidos suficiente para promover equilíbrio no balanço de nitrogênio, em um estado de baixo consumo proteico. No entanto, é sabido que a preservação do turnover proteico e massa corporal magra requer cerca de três vezes mais proteína do que o indicado pelo método de balanço de nitrogênio, que não apresenta, portanto, sensibilidade suficiente para estudo (Wannemacher e McCoy, 1966; Laflamme, 2008).

O turnover proteico é determinado pelo balanço das reações de síntese e degradação, correspondendo às necessidades do animal em determinado status

fisiológico (Waterlow, 2006). O turnover proteico corporal é função complexa pois envolve tanto o turnover de proteínas e aminoácidos individuais, quanto alterações no fluxo de um ou mais aminoácidos específicos. Esse fluxo pode afetar profundamente a regulação proteica (Matthews et al., 1980). As taxas de turnover corporal são geralmente altas apenas na fase de crescimento, porém há diversos fatores decorrentes de processos fisiológicos normais ou patológicos que podem interferir nesta taxa, como por exemplo a realização de exercícios de resistência (hipertrofia), perda de massa muscular devido ao processo de envelhecimento (sarcopenia) ou doenças (câncer) (Wolfe, 2006; Wolfe e Chinkes, 2005).

No processo metabólico, em períodos curtos de deficiência de proteínas, proteínas endógenas do fígado e do trato gastrointestinal são primeiramente utilizadas para suporte ao turnover proteico. Apesar de músculos e pele representarem o maior contingente de reserva proteica, são mobilizados apenas quanto a deficiência proteica persiste por tempos mais prolongados. A mobilização destes tecidos ocorre ainda que o balanço de nitrogênio e o peso corporal continuem inalterados, o que é conseguido pela redução na taxa de turnover proteico corporal e pela utilização das reservas de massa corporal magra para atendimento dos processos metabólicos indispensáveis (Allison e Wannemacher, 1965; Wannemacher e McCoy, 1966; Wolfe, 2006).

Assim, quando se prolonga a deficiência proteica alimentar no idoso, somam-se redução do turnover proteico devido à baixa ingestão de aminoácidos com os efeitos fisiológicos do envelhecimento no metabolismo destes tecidos, exacerbando-se a sarcopenia, acelerando a deposição de gordura corporal e redução de massa magra, com aumento ou estabilidade do peso corporal (Kealy, 1999; Wakshlag et al.,2003). Este quadro de redução das reservas de massa magra e rotatividade reduzida de proteínas corporais, consequente à ingestão deficiente de aminoácidos, pode levar ao aumento de quadros de infecções e piora da saúde (Saker, 2021; Laflamme, 2005).

Métodos para se determinar o turnover proteico

O uso de traçadores metabólicos permite extrair informações valiosas acerca da cinética, turnover e metabolismo de um determinado nutriente de interesse, tornando-os ferramentas de alto potencial para elucidar questões

fisiológicas, independentemente da idade do animal (Kim et al., 2017). O traçador ideal deve ser identificado com precisão suficiente quando fornecido em pequenas doses, devendo ainda representar e não afetar o metabolismo do elemento a ser traçado (Wolfe e Chinkes, 2005).

Isótopos estáveis são átomos que apesar de representarem um mesmo elemento químico, variam quanto ao número de nêutrons. Essa diferença na conformação física dos isótopos não afeta suas propriedades químicas e, desta forma, são considerados excelentes traçadores biológicos (Wolfe e Chinkes, 2005; Kim et al., 2017). A utilização de compostos marcados é vantajosa sobre outros métodos pois, além de permitirem melhor detalhamento do metabolismo e suas vias, possibilita o uso de dietas práticas fornecidas controladamente ou ad libitum (Cerrate et al., 2016).

Os métodos mais conhecidos para se determinar o turnover corporal de proteína são o do precursor, dos produtos finais e da oxidação aminoacídica. Os três podem ser utilizados para dois propósitos: determinação das taxas de turnover, síntese e degradação proteica em geral; serem aplicados para estudos de aminoácidos isolados, quando em concentrações deficientes ou excessivas. Assim, estes facilitam estudos de metabolismo proteico e necessidades de aminoácidos *in vivo*, sem apresentar riscos de saúde ou contaminação ambiental (Wolfe e Chinkes, 2005).

Método do precursor

O método do precursor é representado pelo uso de ^{13}C-leucina. Diferente de outros aminoácidos essenciais que são metabolizados no fígado, a leucina é catabolizada primariamente no músculo, principal reserva proteica corporal. Além disso, a leucina possui efeito regulatório sobre os outros dois aminoácidos de cadeia ramificada (isoleucina e valina), participa da síntese e degradação proteica muscular e do metabolismo oxidativo para produção de energia (Matthews et al., 1980). Segundo o modelo clássico de estudo com ^{13}C-leucina, assume-se um único pool na cinética corporal, a partir do qual este aminoácido é utilizado para se quantificar a síntese e a degradação proteicas, sendo o fluxo calculado a partir da diluição isotópica de leucina marcada no plasma (Goudoever et al., 1995).

De acordo com Tannus (2004), esse marcador, ao ser infundido, se integra ao pool de precursores de proteína corporal e, ao mesmo tempo, participa de reações dentro da célula, podendo ainda recircular no pool de substâncias livres. Esse contexto é exemplificado na figura 1, considerando-se a recirculação da leucina como o ácido a-cetoisocaproico (KIC; análogo de leucina resultante da transaminação intracelular da leucina ao alfa-cetoglutarato). Entre 70 e 80% do enriquecimento do KIC é proveniente da leucina plasmática e a diferença é atribuída à diluição isotópica (Matthews et al., 1982).

O primeiro estudo com cães por este método (Horber et al., 1989) utilizou o radioisótopo ^{14}C-Leucina e demonstrou que a atividade de KIC plasmática pode representar a síntese de proteína na maior parte dos tecidos.

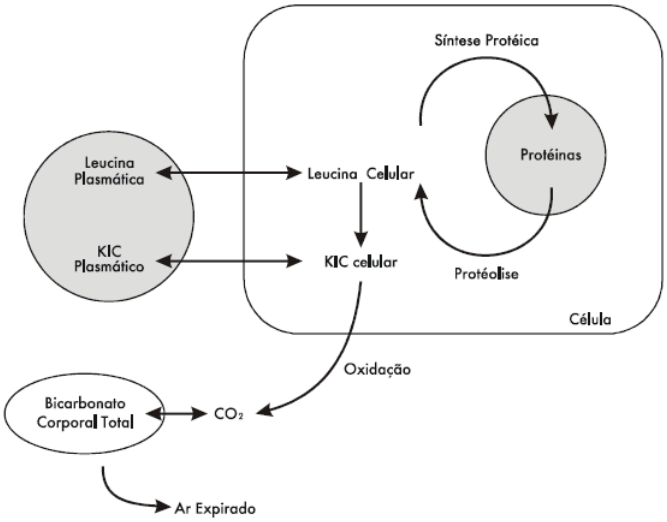

Figura 1. Estudo do metabolismo proteico com uso de leucina marcada (Tannus, 2004). KIC: ácido a-cetoisocaproico

Um problema inerente a esse método é que, devido a essa divisão entre leucina plasmática e KIC faz-se necessário um fator de correção para compensar a recuperação incompleta do ^{13}C no pool oxidativo da leucina. Assim, em situações específicas, como ventilação mecânica, os resultados tornam-se questionáveis. Em conjunto ao fator de correção, o uso de ^{13}C-Bicarbonato auxilia, também, na estabilização do enriquecimento, tornando o método mais rápido e eficiente (Tissot et al., 1993; Matthews et al., 1982).

A administração de uma dose inicial (*priming dose*) apropriada de ^{13}C-Leucina e ^{13}C-Bicarbonato torna possível se reduzir o tempo necessário para se

atingir o estado estacionário do traçador no plasma e no CO_2 expirado. Este se estabiliza em cerca de 2 horas, enquanto sem o ^{13}C-Bicarbonato o processo demoraria entre 8 e 10 horas (Golden e Waterlow, 1977; O'Keefe, Sender e James, 1974).

Método dos produtos finais

O método dos produtos finais envolve o uso de ^{15}N-glicina, o qual foi o primeiro traçador isotópico utilizado para determinar o turnover proteico corporal in vivo. O método foi bastante popular nos anos 1980 devido à característica não invasiva e de fácil execução. Algumas limitações inerentes a este método devem ser ressaltadas: a necessidade de coleta total de urina; o 15N proveniente do traçador é incorporado de maneira diferente entre os aminoácidos; pode-se encontrar diferentes taxas de turnover quando se utiliza outros aminoácidos marcados, mesmo se for outro aminoácido com marcação no ^{15}N (Wagenmakers, 1999; Garlick e Fern, 1985; Fern et al. 1981, 1985).

Vários modelos com 15N foram descritos ao longo do tempo e, exceto por um deles proposto por Halliday e McKeran (1975), todos dependem das medidas isotópicas em produto final de excreção (urina) (Sprinson e Rittenberg, 1949; Waterlow, 1969). Esses métodos podem ser divididos em dois grupos, o primeiro consiste na taxa de renovação calculada a partir do enriquecimento isotópico do produto final em diferentes momentos. Uma única dose do composto marcado é aplicada e a análise compartimental descrita por Long et al. (1977) com o uso de ^{15}N-Alanina. São analisadas a ureia e amônia na urina, extrapolando-se essas medidas para o pool metabólico de proteínas do corpo todo.

O segundo grupo de métodos compreende o enriquecimento contínuo e a medida de taxas calculadas a partir da excreção cumulativa do isótopo no estado estacionário, sendo chamados métodos estocásticos (Shipley e Clark, 1972). Apesar das comparações indicarem que o resultado é semelhante, o grande número de medidas necessárias no primeiro grupo fez com que boa parte dos estudos fossem feitos de maneira estocástica e com medida única (Stein et al., 1976; Nicholson, 1970; Picou e Taylor-Roberts, 1969).

Seu procedimento experimental, porém, é bastante variado e permite diversas adaptações, como por exemplo, enriquecimento a cada 2 a 4 horas, dose única, coletas seguidas ou coletas espaçadas. No entanto, o balanço

positivo de nitrogênio pode ter relação aos erros cumulativos de análise, visto que, comparado com outros métodos, os passos experimentais são bem mais complexos. Após a coleta de urina é necessário realizar a separação de amônia e ureia por resina de troca iônica, para análise posterior separada do ^{15}N em cada um destes dois compostos químicos (Preston e McMillan, 1988; William et al., 2001). Problemas de decaimento isotópico incompleto também são relatados. Diversos fatores podem contribuir para o decaimento inconstante nos casos de aplicação em dose única, como renovação incompleta do pool de aminoácidos, diferença de rotatividade entre proteína e ureia no cólon, entre outros. Porém, sua importância prática é minimizada quando as condições experimentais são adequadamente padronizadas (Waterlow, Golden e Garlick, 1978).

Método indireto de oxidação aminoacídica

O primeiro estudo com o método indireto de oxidação aminoacídica (IAAO) foi desenvolvido por Kim et al. (1983) para determinar as necessidades de aminoácidos durante o desmame de leitões. Os autores utilizaram o traçador radioativo ^{14}C-Fenilalanina, com base no princípio de que o aminoácido a ser avaliado é oxidado em taxas elevadas devido à baixa síntese proteica quando os teores fornecidos via dieta são inadequados e, conforme o fornecimento do aminoácido aumenta, a síntese proteica é maximizada e o sinal do indicador é minimizado (Chamruspollert, Pesti e Bakalli, 2002).

A escolha da fenilalanina como traçador entre os aminoácidos marcados fornecidos oralmente está diretamente ligada à sua via preferencial em oxidação, ou seja, uma grande proporção da primeira passagem dos aminoácidos absorvidos é oxidada, estimando-se para fenilalanina de 29 a 58%, leucina de 20 a 40% e lisina ao redor de 30% (Hoer et al., 1993; Matthews et al., 1993; Biolo et al., 1992; Hoer et al., 1991; Krempf et al., 1990). Essa diferença faz com que ocorra um enriquecimento isotópico menor e fluxo maior no plasma em comparação ao enriquecimento feito via infusão intravenosa devido à oxidação que ocorre nesse tecido e o restante é transportado via circulação sistêmica (Wykes et al., 1992).

Neale e Waterlow (1974) compararam a oxidação e absorção tecidual de fenilalanina, lisina, leucina e valina marcadas em ratos recebendo dietas normal

e livre de proteínas, foi observado que a absorção total pelo fígado e a incorporação do traçador na proteína hepática foi maior quando a fenilalanina foi usada, bem como foi a mais responsiva ao aumento do nível proteico da dieta. A proporção oxidada a CO_2 ou incorporada à proteína também varia de um aminoácido para outro, tendo sido demonstrado que a fenilalanina representou melhor o pool de aminoácidos corporal que os outros aminoácidos avaliados (Aguilar, Harper e Benevenga, 1972).

O método indireto de oxidação aminoacídica (IAAO) foi aplicado, posteriormente, com traçador estável (^{13}C) com o objetivo de se obter modelo minimamente invasivo que permitisse o estudo da cinética de aminoácidos em qualquer grupo de interesse, inclusive grupos vulneráveis que englobam gestantes, lactantes, idosos e filhotes. Basicamente, consiste na união dos métodos do precursor e do produto final, ou seja, ao invés de plasma, coleta-se urina para a quantificação da oxidação (Bross, Ball e Pencharz, 1998).

O método baseia-se no emprego do traçador L-[1-^{13}C] Fenilalanina. Elango, Ball e Pencharz (2008) compararam a infusão intravenosa e oral no fornecimento da L-[1-^{13}C] Fenilalanina e determinaram que, embora a via de administração do isótopo tenha afetado a cinética do traçador, os resultados foram coerentes em ambas as situações. Embora este método tenha sido desenvolvido especificamente para a determinação da necessidade de aminoácidos, pretende-se no presente estudo obter valores que correspondam ao fluxo geral de proteína a partir de uma dieta balanceada, estudando assim seu emprego para a determinação do turnover proteico.

Para estimar as necessidades de aminoácidos em cães adultos, esse método foi considerado mais sensível do que o balanço de nitrogênio e desempenho (Pencharz e Ball, 2003; Levesque et al., 2010), diretamente relacionados ao aumento de massa muscular corporal e, portanto não são adequados para animais adultos em manutenção, já foram estimadas necessidades de fenilalanina, triptofano, treonina e lisina para cães através dessa metodologia (Mansilla et al., 2020; Sutherland et al., 2020; Templeman et al., 2019; Mansilla et al., 2018).

Aminoácidos de cadeia ramificada

Os aminoácidos de cadeia ramificada (ACR) incluem a leucina, isoleucina e valina. Após o início de sua metabolização pela ação da aminotransferase, seus cetoácidos α-cetoisocaproato (KIC), α-ceto-β-metilvalerato (KMV) e α-cetoisovalerato (KIV) são capazes de atuar como análogos dos aminoácidos, bem como terem livre passagem pelas mitocôndrias com capacidade alta de conversão para energia (Lehningher, 2006). São importantes na manutenção e crescimento tecidual e essenciais para a nutrição animal (Ichihara e Koyama, 1966). A valina é um aminoácido glicogênico, o que significa que ela é catabolizada a piruvato ou em intermediários do ciclo do ácido cítrico. A leucina é cetogênica, gerando produção de corpos cetônicos no musculo. Isoleucina é tanto glicogênica quanto cetogênica, apresentando maior flexibilidade metabólica (Lehningher, 2006).

A síntese de proteína é uma das principais funções desses aminoácidos, porém, também estão presentes na regulação metabólica e atuam em determinadas reações como no metabolismo nitrogenado extra-hepático, retardam a fadiga durante o exercício, participam da manutenção da massa muscular e da proliferação de linfócitos e células dendríticas (Harper et al., 1984; Adibi, 1976). Participam de diversas funções bioquímicas cerebrais, como a síntese de proteína, produção de energia, compartimentalização do glutamato e afetam a síntese de catecolaminas e serotonina (Fernstrom, 2005).

Enquanto a maioria dos aminoácidos é degradada no fígado, os ACR são oxidados principalmente pelos tecidos muscular, adiposo, renal e cerebral, pois esses tecidos contêm aminotransferase, ausente no fígado (Lehningher, 2006). Devido ao seu metabolismo extra-hepático, são fundamentais para a massa magra por regularem a síntese e catabolismo proteico no músculo esquelético (Dodd e Tee, 2012). No cérebro, competem pelo mesmo transportador com os aminoácidos aromáticos na barreira hemato-encefálica, especialmente o triptofano, precursor da serotonina. Este, embora pareça ter efeito negativo devido aos feitos da serotonina na regulação do sono, bem-estar e redução da agressividade, parece ocasionar redução da fadiga central durante exercícios, podendo melhorar a performance, pois a serotonina também tem papel importante ao estabelecer a fadiga central no exercício (Monirujjaman e Perdouse, 2014).

Dentre os ACR a leucina é a mais estudada, principalmente visando melhorar a capacidade regenerativa dos músculos, por regular a renovação de proteínas no músculo esquelético, o que pode promover ganho de massa magra ou diminuir sua perda (Dodd e Tee, 2012; Li et al., 2011; Baptista et al., 2010). Um metabólito da leucina, o β-hidroxi-β-metilbutirato (HMB) parece atenuar a perda de massa muscular em animais idosos por possível diminuição de apoptose nuclear (Hao et al., 2011) e aumento da proliferação de células satélites (Alway et al., 2014). Outros efeitos sugeridos da leucina são melhora da recuperação do tamanho da miofibra e da função contrátil de músculo esquelético de camundongos (Pereira et al., 2014).

Todos estes aspectos nutricionais envolvem balanço entre ACR e aminoácidos de cadeia aromática, bem como a relação entre os três ACR. Estas relações são importantes devido ao antagonismo que pode se estabelecer entre estes aminoácidos. A título de exemplo, foi testado para cães relação Leu:Iso:Val de 40:35:25, com suplementação com 7g de ACR para cada 30 kg de peso corporal. Nas condições do estudo foi detectada melhora na função cognitiva de cães idosos (Fretwell et al., 2006), porém não foram encontrados estudos de turnover proteico relacionados a esse conjunto de aminoácidos, bem como estudos dose-resposta para se estabelecer melhores quantidades a serem suplementadas.

No entanto, não se conhecem os efeitos a longo prazo de uma suplementação elevada de ACR (Chen et al., 2009). Especula-se que em excesso este possa interferir na longevidade dos animais (Chen et al., 2009; Yilmaz et al., 2012). Isto é especulado pois fornecimento elevado de nutrientes (não necessariamente apenas ACR) e energia pode ativar a Ser/Thr-cinase mTORC1, que auxilia no equilíbrio da proliferação celular e aumento da célula em resposta aos fatores de crescimento e disponibilidade de nutrientes e energia. O jejum, por outro lado, leva à inativação de mTORC1, resultando em maior degradação de proteína e glicogênio no fígado e no músculo, com ativação de proteínas que promovem autofagia, inibição de proteínas que promovem o crescimento celular e mobilização de triglicerídios do tecido adiposo (Lehningher, 2006). A inibição moderada do complexo mTOR pode ter efeitos prolongadores na longevidade (Blagosklonny, 2006) baseando-se na hipótese de que a autofagia provocada pelo processo facilitaria a reciclagem de materiais

defeituosos e autorrenovação celular (Chen et al., 2009; Yilmaz et al., 2012). Isto tudo, no entanto, não está completamente elucidado e não se distingue, até o momento, os feitos da ingestão excessiva de energia e nutrientes em geral de um possível fornecimento excessivo de ACR.

Arginina

A arginina é precursora de, ao menos, 8 compostos (Figura 2). Está ligada diretamente à síntese de proteínas e óxido nítrico, estando seu metabolismo ligado a diversas vias de interesse nutricional. A arginina é um aminoácido de interesse por dois motivos principais: desempenha importante papel no ciclo da ornitina de formação da ureia e é um substrato indispensável para a produção de óxido nítrico que, por sua vez, atua como relaxante no músculo liso, neurotransmissor e produto para reações imunes (Moncada, 1999).

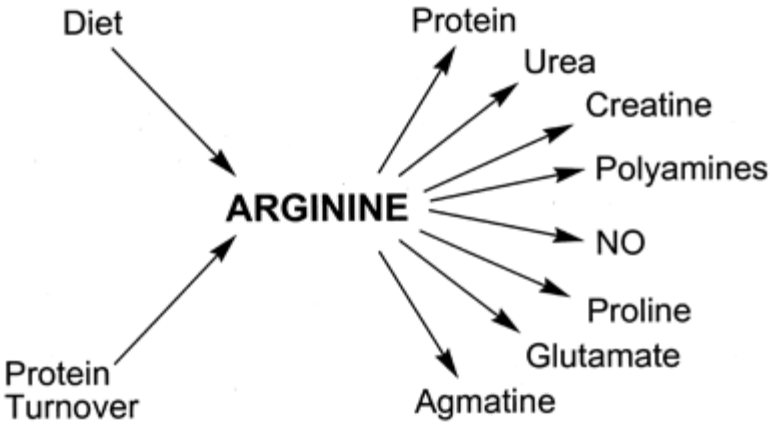

Figura 2. Fontes e caminhos da Arginina. Putrescina, espermina e espermidina são as poliaminas referidas para essa figura. NO, óxido nítrico (adaptado de Morris (2006) com a diferença que a síntese endógena foi removida por se tratar de cães).

As fontes de arginina incluem a proteína proveniente da dieta e renovação das proteínas corporais (*turnover* proteico). Para o cão e o gato não há síntese endógena, diferentemente do que ocorre com o ser humano e roedores. A arginina é considerada um aminoácido essencial para cães e gatos. Dietas livres de arginina foram testadas em cães e causaram salivação excessiva, vômito, tremores e hiperglicemia logo no segundo dia de teste, houve também relato de

redução no consumo e no peso desses animais, tanto para animais em crescimento quanto adultos (Burns, Milner e Corbin, 1981; Hai, Milner e Corbin, 1978), tornando necessário seu fornecimento via dieta.

Estudos metabólicos sobre a utilização de nutrientes, como vitamina D, niacina e taurina, sugerem que cães têm mais características metabólicas e nutricionais carnívoras do que ratos, mas menos do que gatos, portanto, em uma escala de sensibilidade à deficiência de Arginina, encontra-se, respectivamente, gatos, cães e ratos (Morris e Rogers, 1978; Burns et al., 1981; Czarnecki e Baker, 1984; Milner et al., 1975; Rogers, 1994).

Em relação ao atendimento das necessidades nutricionais de arginina, é importante que haja o suficiente desse aminoácido proveniente da dieta para a promoção do crescimento muscular e de outros tecidos dependentes de proteína (NRC, 2006), Burns et al. (1981) relataram também que a suplementação de arginina via dieta ajuda na prevenção de heperamonemia e acidúria orótica no cão adulto.

Ácido docosahexaenoico

O ácido docosahexaenoico (DHA) é um ácido graxo poliinsaturado de cadeia longa da família ômega-3 (n-3). Metabolicamente está relacionado a outros ácidos graxos também derivados do ácido α-linolênico (ALA), embora essa conversão não seja considerada muito eficiente para cães (Lenox e Bauer, 2013). O DHA é encontrado em algas marinhas e peixes marinhos, sendo habitualmente incorporados às dietas ou fornecido como suplementos (Calder, 2016). O baixo consumo de DHA pode afetar o desenvolvimento cognitivo, a fisiologia e a função celular e tecidual por diversos mecanismos, incluindo alteração na estrutura e função das proteínas da membrana, sinalização celular e produção de mediadores lipídicos (Miles e Calder, 1998).

Os ácidos graxos poliinsaturados, incluindo ácido linoleico, ácido dihomo-γ-linolênico (20:3n-6), ácido araquidônico, ácido eicosapentaenoico (EPA) e DHA originam diversos mediadores lipídicos bioativos, provenientes das vias das ciclooxigenase e lipoxigenases (Calder, 2015). Os mediadores produzidos a partir do EPA e DHA induzem menor resposta de inflamação e promovem funções imunológicas, facilitando a defesa e diminuindo o efeito patológico da

inflamação orgânica (Bannenberg e Serhan, 2010; Serhan, Yacoubian e Yang, 2008).

Vários estudos para cães verificaram na espécie resultados semelhantes aos encontrados para seres humanos e camundongos. A suplementação de EPA e DHA induziu modulação da resposta inflamatória, neurodesenvolvimento, melhora da capacidade cognitiva e do sistema imunológico (Heinemann et al., 2005; Kelley et al., 2009). Em estudo com suplementação a curto prazo e alta dosagem de DHA e EPA com testes de resolução de problemas encontrou resultados positivos nas habilidades de cães idosos (Casini et al., 2011). Hadley, Bauer e Milgram (2017) estabeleceu o uso de alga *Schizochytrium sp*. como fonte de ácidos graxos poli-insaturados de cadeia longa ômega-3, incluindo DHA, por 25 semanas para avaliações cognitivas de discriminação de objetos visuais, aprendizado e consolidação de memória, observando que o aumento de DHA na dieta melhorou o aprendizado inicial dos protocolos e memória de curto prazo, mas não para memória de longo prazo e tarefas de discriminação concorrente.

No entanto, como a conversão de ácido alfa-linolênico em EPA e DHA é ineficiente em cães (Bauer, 2007), torna-se necessário o fornecimento destes ácidos graxos poliinsaturados derivados via alimentação, não sendo efetivo o fornecimento apenas de ácido alfa-linolênico. No mercado pet food atual, as principais fontes de DHA são o óleo de peixe marinho e de água fria (Kus e Mancini-Filho, 2010) e a microalga do gênero *Schizochytrium*, que possui concentração de DHA próxima à 20%.

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^{13}C-Leucine, ^{13}C-Phenylalanine, and ^{15}N-Glycine comparison for the evaluation of protein turnover in old dogs

Abstract

Stable isotopes use to study protein metabolism are a reality in human nutrition, however, they are still poorly explored for dog nutrition. The present study compared the methods of precursor, end-products, and amino acid oxidation to determine the protein turnover rate of old dogs (10.5±1.98 years). Six dogs were fed a kibble diet for maintenance, and enriched with ^{13}C-Leucine, ^{13}C-Phenylalanine, or ^{15}N-Glycine. Depending on the isotope the traces elimination was measured on breath test (collected with a mask), blood plasma or urine by isotope ratio mass spectrometry. The study considered four methods (^{13}C-Leucine, ^{13}C-Phenylalanine measured on breath test and urine, and ^{15}N-Glycine), each dog was oneas considered the experimental unit. Data was submitted to variance analysis, Tukey test ($P<0.05$), Pearson correlations and Bland & Altman statistics. Enrichment plateau was achieved in all methods. Results of protein synthesis (3.39 ± 0.33 $g.kg^{-0,75}.d^{-1}$) and breakdown (3.26 ± 0.18 $g.kg^{-0.75}.d^{-1}$) was similar among methods ($P>0.05$). This indicated that the adopted procedures to collect samples were adequate, as the results calculated indicate equally efficient rates of isotopes recovery. However, no Pearson correlation was found ($P>0.05$) and the bias was high between methods, what should be considered during their selection.

Keywords: geriatric, protein synthesis, sarcopenia, stable isotopes

Introduction

Stable isotopes are powerful tools allowing studies on protein metabolism without presenting health or environmental contamination risks (Waterlow, 1984; Bross, Ball and Pencharz, 1998, Wagenmakers, 1999). They are a reality in human nutrition (Liu et al., 2002, Wagenmakers, 1999; Nair et al., 1992), however although not new, they are still poorly explored in studies about dog nutrition (Williams et al., 2001).

Many nutritional, physiological or pathological processing's can interfere on protein turnover in adult animals, such as diet composition (Gaine et al., 2006), resistance exercise (hypertrophy) (Rodriguez et al., 2007; Phillips et al., 1999),

diseases or aging (Laflamme, 2008; Rennie, 1985). This process can induce changes on muscle mass with consequences to health, physical activity and resistance to diseases.

In the first published study about protein metabolism in old dogs, Wannemacher and McCoy (1966) found an increase of approximately 50% in protein requirements of old dogs compared to adults. These results were obtained using markers of protein turnover and lean body mass changes, although the nitrogen balance results remained similar between ages. If metabolic tracers are incorporated on such type of studies, in a minimally invasive, precise, and “friendly” way they may allow extracting valuable information about the kinetics, turnover and metabolism of a particular nutrient of interest, becoming a tool to better elucidate physiological issues such as the age effect on protein metabolism (Kim et al., 2017).

The methods of the precursor, end-products and of the amino acid oxidation are well stablished in animal models to determine rates of turnover, synthesis, and overall protein breakdown (Claydon et al., 2012; Duggleby & Waterlow, 2005; Millward & Garlick, 1972). They also can be used in studies about individual amino acids requirements (Mansilla et al., 2020; Templeman et al., 2019; Mansilla et al., 2018). The precursor method is represented by the use of the stable isotope L-[^{13}C] leucine. Despite other essential amino acids that are metabolized in the liver, leucine is primarily catabolized in muscle, the main body protein reserve, in addition to participating in muscle protein synthesis and breakdown and oxidative metabolism (Matthews et al., 1980). The final products method involves the use of [^{15}N] glycine, which was the first isotope tracer used to determine body protein turnover in vivo (Picou & Taylor-Roberts, 1969). Some potential limitations to apply this method to dogs are the need of total urine collection,^{15}N from the tracer is incorporated differently among amino acids, and different turnover rates are observed when using other labeled amino acids, including different results for other ^{15}N-labelled amino acid (Fern et al. 1981, 1985; Garlick & Fern, 1985; Wagenmakers, 1999).

Finally, the indirect method of amino acid oxidation with L-[1-13C]Phenylalanine as a tracer was developed with the aim to obtain a minimally invasive model to study of amino acid kinetics in any group of interest, including vulnerable groups that include pregnant, nursing mothers, elderly or newborns

(Bross, Ball & Pencharz, 1998). The approach consists of the association of the precursor and the final product methods, and instead of plasma, urine is collected to quantify oxidation (Bross, Ball and Pencharz, 1998). The isotope deliver can be intravenous or oral, as although the route of administration affects the tracer kinetics, the results were consistent in both situations (Elango, Ball, and Pencharz, 2008). The method was originally developed to study amino acid requirements, but there are interest in their use to obtain the general values of protein flow, thus applying it to determine protein turnover. The rates of L-[1-13C]Phenylalanine incorporation into body reserves or oxidation in the postprandial period can be accessed using urine or breath test (Darling et al., 1999), and both approaches might have advantages or disadvantages that should be evaluated.

Considering that only studies with the methods of final products, indirect amino acid oxidation and a radioactive isotope alternative for the precursor method were localized in dogs (Shoveller et al., 2017; Williams et al., 2001; Horber et al., 1989), and that a direct comparison of results between then is not available, the objective of the present study was to compare the methods of precursor, end-products, and amino acid oxidation to determine the protein turnover rate of old dogs fed a kibble diet for maintenance.

Material and Methods

The study was conducted at the Research Laboratory of Nutrition and Nutritional Diseases of Dogs and Cats “Prof. Dr. Flávio Prada”, Universidade Estadual Paulista (UNESP), Jaboticabal, Brazil. All procedures in this research were previously approved by the Ethics Committee on the Use of Animals (CEUA) of the same institution (protocol number 009537/18).

Animals, housing and experimental design

The health of all dogs was previously verified by a veterinarian by physical examination, hemogram and serum biochemical analysis, and all were considered health. Six beagle dogs (12.7 ± 2.6 years, 13.6 ± 0.6 kg) were fed with an extruded diet in an adjusted amount to maintain a constant body weight (Natural Senior, Guabi Nutrição e Saúde Animal LTDA, Campinas, Brazil. Analyzed chemical composition: 26% crude protein, 13% crude fat; 4,5% crude

fiber; 1.92% leucine; 0.99% phenylalanine; 1.83% glycine (DM basis); 3.85 kcal.g^{-1} of metabolizable energy). Dogs received the food for 35 days: from day 1 to 10 for adaptation; from days 11 to 15 the Indirect amino acid oxidation method was conducted; from days 16 to 32 the End-products method was evaluated; at day 31 the precursor method was performed. On day 35 the volume of CO_2 produced by each dog was obtained using a respirometry system.

	Minutes after isotope administration																	
Item	-60	-40	-20	0	20	40	60	80	100	120	140	160	180	200	480	720	1440	2160
11° Day – Amino acid oxidation method																		
Food intake	■	■	■	■														
^{13}C-phe				■	■	■	■	■										
NaH$^{13}CO_3$				■														
Breath				■				■		■		■		■				
Urine				■											■	■	■	■
21° Day – Final products method																		
^{15}N-gly				■														
Urine				■											■	■	■	■
31º Day – Precursor method																		
^{13}C-leu				■	■	■	■	■										
NaH$^{13}CO_3$				■														
Breath				■			■	■	■	■	■	■	■					
Blood				■			■	■	■	■	■	■	■					

^{13}C-Phenylalanine

For this test dogs were housed individually in metabolic cages equipped with apparatus to separate feces and urine for collection, measuring 0.9 m × 0.9 m × 0.9 m. After 24h fasting animals were fed with their usual amount of food divided in four equal meals, and fed each amount at every 20 min until complete the daily food intake. Only dogs that ate all offered food was tested. Dogs that did not complete the meal was tested on the other day.

Immediately after completed the meal, dogs received a prime dose of 0.7 mg/kg of L-[1-^{13}C]Phenylalanine and 0.2 mg.kg^{-1} of NaH$^{13}CO_3$ (Cambridge Isotope Laboratories, Tewksbury, US) supplied orally in gelatin capsules. After, dogs were orally dosed every 20 minutes four times with 1.3 mg/kg of L-[1-^{13}C]Phenylalanine in gelatin capsules.

Samples of breath test were collected prior to enrichment, and 80, 120, 160, 200 min of the priming dose administration. At each sampling time breath test was collected using a specific designed mask for at least 2 minutes, directly into a glass vial coupled to the mask and stored at room temperature for latter analysis.

Urine samples were collected prior to enrichment, and then after 12h, 24h, and 36h of the meal and stored frozen (-20 ºC) until analysis. Urine samples were quantitatively collected in plastic containers placed under the collecting funnel of the metabolic cages, added with 4 mL of 6N HCl as a preservative (Synth, LABSYNTH, Diadema, Brazil). Before analysis urine samples were thawed and lyophilized (Thermo VLP200, ThermoFisher, Massachusetts, EUA).

^{15}N-Glycine

Dogs were individually housed in metabolic cages with apparatus to separate feces and urine for collection, measuring 0.9 m × 0.9 m × 0.9 m. After 24h fasting, animals were fed with their usual amount of food. Immediately after completed their meal, animals were enriched with a single dose of 20mg/kg of [15N] Glycine (Cambridge Isotope Laboratories, Tewksbury, US). The labeled compound was orally dosed in gelatin capsules. Only dogs that ate all offered food was tested, and animals that did not complete the meal was evaluated on the other day.

Urine samples were collected prior to enrichment, and then after 8h, 12h, 24h, and 36h of the meal and stored frozen (-20 ºC) until analysis. Urine samples were quantitatively collected in plastic containers placed under the collecting funnel of the metabolic cages, added with 4 mL of 6N HCl as a preservative (Synth, LABSYNTH, Diadema, Brazil).

Prior to analysis, urine samples were thawed at room temperature. In the procedure, the incorporation of labeled N is quantified on both the urinary ammonia and urea. To this, the separation of ammonia and urea on urine samples was conducted using the cation exchange method in resin (AG50W-X8, Bio-Rad Laboratories, California, EUA), as described by Read (1961) and Preston and McMillam (1988). On the assay the resin binds ammonia in a neutral solution. Urea stays in the supernatant, that is after converted into ammonia with urease (Sigma Aldrich, Missouri, EUA), and incorporated in the resin. Further, ammonia is extracted from the resin and subjected to N isotopic analysis.

^{13}C-Leucine

The cephalic vein was catheterized (Descarpack®, São Paulo, Brasil) and with dogs after 24h fasting a priming dose of a combination of 2 mg/kg of L-[1-^{13}C] Leucine and 0.1 mg/kg of [13C] Na$H^{13}CO_3$ (Cambridge Isotope Laboratories, Tewksbury, US) was infused. After five minutes of this prime dose, an infusion of 0.7 mg/kg of L-[1-^{13}C] Leucine was repeated every 20 minutes for a total period of 80 minutes.

Samples of breath test and blood plasma were collected prior to enrichment, and 60, 80, 100, 120, 140, and 180 min after the prime dose enrichment. At each sampling time breath test was collected using a specific designed mask for at least 2 minutes, directly into a glass vial coupled to the mask and stored at room temperature for latter analysis.

The animals were trained to use the masks prior to the collections, they were encouraged to put their muzzles on the mask voluntarily (with wet food and positive reinforcement). When they lost the fear of the mask, adaptation was made in relation to the time of wearing the mask. In addition, all collections were performed with positive reinforcement (affection) while wearing the mask.

Blood samples were collected using the catheter and a syringe, deposited in heparinized tubes, centrifuged for plasma separation and the plasma kept frozen (-20 ºC). Immediately before analysis, plasma samples were lyophilized (Thermo VLP200, ThermoFisher, Massachusetts, EUA).

Determination of CO_2 volume

A respirometry system (Animal respirometry system, Sable System International, Las Vegas, USA) was used to determine the volume produced of CO_2 (VCO_2) of each dog. Dogs were housed in hermetically sealed chambers with internal control of temperature and moisture, with the dimensions of 1m x 1m x 1m (ALB 1000 CG Respirometry Chamber, Inbras Equipamentos para Saúde, Ribeirão Preto, Brazil). The chamber temperature was adjusted to 26±1 °C and the relative humidity of the air in the interval of 55% to 60%. Dogs were previously adapted to stay and be fed inside the chamber, avoiding stress that could interfere on results. Dogs were fasted for 24h, placed on the cages and fed with their usual amount of food. Water was provided ad libitum. Each run latest 8 hours. According to previous data of pilot studies, 2 hours was waited to equilibrate the gases inside the chambers.

Ambient air flows were introduced into the chambers by a negative pressure system, with an average flow of 10 L min^{-1}, with control and adjustments provided by a mass flow pump with a maximum flow capacity of 100.0 L min^{-1} (Sable Systems International, Las Vegas, Nevada, USA). The air flow volumes were established to maintain CO_2 saturations below 1.0% (average CO_2: 0.3±0.1% and average O_2: 20.4±0.2%) inside the chambers. Channel air sampling was performed by independent pressure gauges (RM-8 Gas Flow Multiplexer, Sable Systems International, Las Vegas, Nevada, USA). The air samples were routed by a subsampler (SS-4 Gas Analyzer Subsampler, Sable Systems International, Las Vegas, Nevada, USA) to a humidity meter (RH-300 Relative Humidity & Dew Point Analyzer, Sable Systems International, Las Vegas, Nevada, USA). The oxygen concentrations were measured by a paramagnetic analyzer (PA-10, Sable System International, Las Vegas, Nevada, USA), and the CO_2 concentrations were measured with a carbon dioxide analyzer (CA-10, Sable System International, Las Vegas, USA). Before measuring the gas concentrations, moisture was removed from the air by using anhydrous magnesium perchlorate (Exodus Científica, Sumaré, São Paulo, Brazil). The control and gauging of the gas metres were carried out using a standard air mixture (CO_2: 1.0031% and O_2: 21.00%; White Martins, Vinhedo, São Paulo, Brazil). The data obtained by the mass flow metre and gas analyzers were collected by an ExpeData system (Sable System International, Las Vegas, Nevada, USA).

The VCO_2 produced by each dog was determined by the following equation (Lighton, 2008):

$$VCO_{2(mL \cdot min^{-1})} = FR_e \frac{[(F_eCO_2 - F_iCO_2) + F_iCO_2(F_iO_2 - F_eO_2)]}{(1 + F_iCO_2)}$$

Where FR_e is the excurrent flow rate, F_e is the fractional concentration of a gas in the excurrent airstream, and F_i in the incurrent airstream.

Indirect calorimetry validation

Before and at the end of the evaluations, the chambers and the respirometry system were validated for their gas recovery efficiency by burning Ethanol (Ethyl alcohol, Signma-Aldrich, Missouri, USA) for 430 minutes, assuming the combustion values proposed by Elia (1992). The volume of ethanol

burned (initial weighing - final weighing), the inflow and the outflow of air was considered and the volumes of O_2 and CO_2 that should have been detected by the device were calculated. Based on these values, the recovery percentage was established. To validate the system a CO_2 recovery greater than 95% was established.

Isotopic analysis

The isotopic analyzes were performed in the Stable Isotopes Center, Universidade Estadual Paulista (Unesp), Botucatu, Brazil. All samples were analyzed in a continuous-flow isotope ratio mass spectrometry system (CF-IRMS). The CO_2 samples were analyzed in an automated breath ^{13}C analyzer isotope ratio mass spectrometer (ABCA2, SerCon, Cheshire, UK). The system extracts the sample from the tube by washing with He, utilizing a two-way needle. The He flow carries the sample to the IRMS, were the isotopic ratio $R(^{13}C/^{12}C)$ is expressed as an atom fraction $x(^{13}C)$ (%) and relative difference between the carbon isotopes $\delta^{13}C$ (‰), calculated with the equations described by Coplen (2011).

$$x\left(^{13}C\right) = \frac{R\left(^{13}C/^{12}C\right)_{sample}}{1 + R\left(^{13}C/^{12}C\right)_{sample}}$$

$$\delta^{13}C = \frac{R\left(^{13}C/^{12}C\right)_{sample}}{R\left(^{13}C/^{12}C\right)_{VPDB}} - 1$$

To calculate the $\delta^{13}C$ was used the value of $R(^{13}C/^{12}C)_{VPDB}$, and results were previously normalized by two-point anchorage (Paul et al., 2007) using the NBS-22 and USGS87 standards. The ABCA-IRMS uncertainty was estimated at ±0.16‰, according Carlos et al. (2019). To calculate the excess atom fraction $x^E(^{13}C)$ by difference between $x(^{13}C)_{final}$ and $x(^{13}C)_{initial}$.

Urine and plasma samples were weighed in 5 x 8 mm tin capsules (PN 24006400, Thermo Scientific, Germany), and analyzed on an Elementary Analyzer (EA) type CF-IRMS. To this an IRMS (Delta V, Thermo Scientific, Germany) coupled to an EA (Flash EA, Thermo Scientific, Germany) and a gas interface (ConFlo IV, Thermo Scientific, Germany) was used. The capsules were inserted with an autosampler into the EA oxidation/reduction reactor at 1020°C,

were the carbon and nitrogen of the sample were converted into CO_2 and N_2 through combustion and carried to the IRMS, where $R(^{13}C/^{12}C)$ and $R(^{15}N/^{14}N)$ were determinates. The $\delta^{13}C$ and $x^E(^{13}C)$ was calculated as previously shown and $x(^{15}N)$ and $\delta^{15}N$, calculated with the equations described by Coplen (2011).

$$x\left(^{15}N\right) = \frac{R\left(^{15}N/^{14}N\right)_{sample}}{1 + R\left(^{15}N/^{14}N\right)_{sample}}$$

$$\delta^{13}C = \frac{R\left(^{15}N/^{14}N\right)_{sample}}{R\left(^{15}N/^{14}N\right)_{VAIR}} - 1$$

To calculate $\delta^{15}N$ the default value $R(^{15}N/^{14}N)_{VAIR}$ were used. The EA-IRMS uncertainty was estimated at ±0.15‰ and ±0.20‰ for $\delta^{13}C$ and $\delta^{15}N$, respectively. The results were normalized by two-point anchorage (Paul et al., 2007) using the NBS-22 and USGS87 standards for $\delta^{13}C$, and IAEA-N-1 and USGS90 for $\delta^{15}N$. To calculate the excess atom fraction $x^E(^{15}C)$ by difference between $x(^{13}C)_{final}$ and $x(^{13}C)_{initial}$.

Whole body protein turnover

The flux of labeled compounds and rates of protein synthesis and breakdown were calculated according to the model proposed by Picou and Taylor-Roberts (1969):

$$\mathbf{Q = O + S = I + B}$$

Where Q is tracer flux ($g.kg^{-1}.d^{-1}$); O is the oxidation of the amino acid measured through the breath test (not considered in the other tracers); S is the protein synthesis rate ($g.kg^{-1}.d^{-1}$); I is the N intake from the diet ($g.kg^{-1}.d^{-1}$); and B is the protein breakdown rate ($g.kg^{-1}.d^{-1}$).

$$Q_{Leucine(g\cdot kg^{-1}\cdot d^{-1})} = D\left(\frac{x^E\left(^{13}C\right)_{Leucine}}{1.33 \cdot x^E\left(^{13}C\right)_{Plasma}}\right) MW \cdot 24 \times 10^6$$

$$Q_{Glycine(g\cdot kg^{-1}\cdot d^{-1})} = D\left(\frac{x^E\left(^{15}N\right)_{Glycine}}{x^E\left(^{15}N\right)_{Urine}}\right) MW \cdot 24 \times 10^6$$

$$Q_{Phenylalaline(g\cdot kg^{-1}\cdot d^{-1})} = D\left(\frac{x^E\left(^{13}C\right)_{Phenylalanine}}{x^E\left(^{13}C\right)_{Gas/Urine}}\right) MW \cdot 24 \times 10^6$$

Where Q is the tracer flux (g.kg^{-1}.d^{-1}), D is the tracer infusion rate (µmol.kg-1.h-1); $x^{E}_{(tracer)}$ is the absolute enrichment of the tracer; $x^{E}_{(sample)}$ is the atom percent in excess and represents the enrichment in the steady state; MW is the molar weight of the tracer, which together the multiplication by 10^6 is used to transform µmol to g; 24 is to transform hour in day (Adapted from Mathews et al., 1980).

S (g.kg^{-1}.d^{-1}) = Q - O

Protein synthesis (S) for ^{13}C leucine or ^{13}C-phenylalanine was calculated from the flux (Q) and oxidation (O) data. In the case of urine for the ^{15}N glycine and ^{13}C-phenylalanine tracers it equals the flux (Q), provided the other parameters are calculated in the enrichment plateau (Mathews et al., 1980).

B (g.kg^{-1}.d^{-1}) = Q - I

Protein breakdown (B) was calculated from the difference between the flux (Q) of ^{13}C-leucine or ^{15}N glycine or ^{13}C phenylalanine and the intake of the amino acids (I) in the case of glycine or phenylalanine (when the animals were fed) or equals the isotope dose for ^{13}C leucine (when the animals were fasted) (Mathews et al., 1980).

$$O_{(g\cdot kg^{-1}\cdot d^{-1})} = F^{13}CO_2 \left(\frac{1}{x^E\left(^{13}C\right)_{tracer}} - \frac{1}{x^E\left(^{13}C\right)_{sample}} \right) \times 100$$

O (g.kg^{-1}.d^{-1}) = $\boldsymbol{F^{13}CO_2 \left(\frac{1}{X^E_{(13C)tracer}} - \frac{1}{X^E_{(13C)sample}} \right) * 100}$

The oxidation (O) of ^{13}C phenylalanine and ^{13}C leucine was calculated with this equation, where: $X^{E}_{(tracer)}$ is the absolute enrichment of the tracer; $X^{E}_{(sample)}$ is the atom percent in excess and represents the enrichment in the steady state; $F^{13}CO_2$ represents the rate of $^{13}CO_2$ released by the tracer oxidation (g $^{13}CO_2$.kg^{-1}.d^{-1}), calculated by the following equation:

$$F^{13}CO_{2(g\cdot kg^{-1}\cdot d^{-1})} = \left(\frac{VCO_2 \cdot ECO_2}{BW} \right) \cdot \left(\frac{60 \times 41.6}{100} \right) MW \cdot 24$$

Where VCO_2 is the CO_2 production rate of the animal determined in respirometry chambers (mL.min^{-1}), ECO_2 is the $^{13}CO_2$ enrichment plateau in the breath test (APE); BW is the weight of the animal (kg). The constants 41.6 µmol.ml^{-1} and 60 min.h^{-1} convert VCO_2 to mol.h^{-1} and the factor 100 changes the plateau to a fraction. The factor 1.0 accounts for the $^{13}CO_2$ retained in the body because of

bicarbonate fixation (Hoerr et al., 1989). MW is the molar weight of the tracer, which together the multiplication by 10^4 is used to transform µmol to g. The 24 is to transform hour in day (Adapted from Mathews et al., 1980).

Statistical analysis

Results were applied to equations and protein flux, synthesis and breakdown were calculated. After confirming the presupposition normality of residue and variance homoscedasticity, data were submitted to ANOVA in a completely randomized design, with 4 methods (treatments) and 6 experimental units (dogs) per treatment. When differences were verified on F test means compared by Tukey's test. As a complementary comparison, the confidence intervals of agreement between the results of the different methods were determined as the bias (mean difference) ± 1.96 SD, according to Bland and Altman (1986), and the mean values were compared using the Pearson correlation. The Bland and Altman statistics and Pearson correlation was performed on R Software (PBC, Boston, MA, 2020), and the remaining analyses were performed on SAS software 9.1 using the Proc MIXED (SAS Institute, Cary, NC, USA, 2003). Values of $P<0.05$ were considered significant.

Results

The indirect calorimetry systems validation with ethanol obtained a CO_2 recovery higher than 95%, showing accuracy. Due to this, the VCO_2 obtained can be considered reliable, with a mean value of 75±6 ml of $VCO_2.min^{-1}$ for the experimental group of dogs. This VCO_2 and the observed VO_2 resulted in a mean energy expenditure (EE) of 97 ± 3.5 $kcal.kg^{-0.75}. d^{-1}$ for the old dog group under study. Considering the food intake records and the protein content of the diet, the mean protein intake of the dogs along the study was 6.96 ± 0.26 $g.kg^{-0.75}.d^{-1}$.

The isotopes enrichment according to the different methods and biological samples utilized are illustrated on Figure 3. It is possible to see the enrichment curve for all isotopes, from each the tracer flux were calculated.

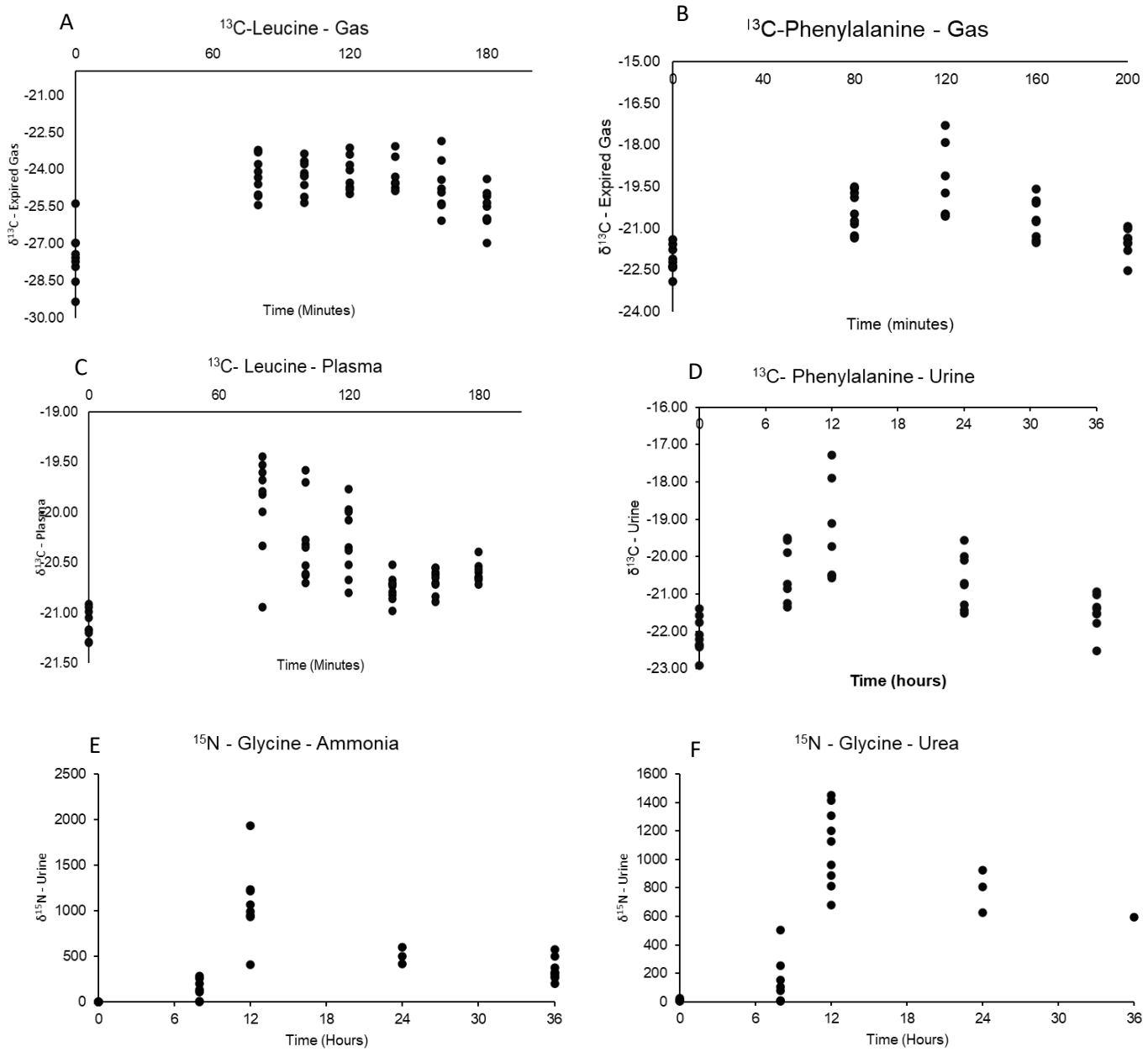

Figure 1. Fractional enrichment curve for each method of analysis: y = $\delta^{13}C$ or $\delta^{15}N$; x=sampling time in hours or minutes. A. ^{13}C-Leucine method, $^{13}CO_2/^{12}CO_2$ evaluated along 180 min of breath test analysis. B. ^{13}C-Phenylalanine method, $^{13}CO_2/^{12}CO_2$ evaluated along 200 min of breath test analysis. C. 13C-Leucine method, $^{13}CO_2/^{12}CO_2$ evaluated along 180 min of blood plasma analysis. D. ^{13}C-Phenylalanine method, $^{13}CO_2/^{12}CO_2$ evaluated along 36 hours of urine analysis. E. ^{15}N-Glycine method, $^{15}N/^{14}N$ evaluated in urinary ammonia along 36 hours analysis. F. 5N-Glycine method, $^{15}N/^{14}N$ evaluated in urinary urea along 36 hours analysis.

Protein synthesis and breakdown results were similar among methods (P>0.05), showing a good precision between them, as shown on Table 1. The adopted procedure to collect samples of breath test, utilizing the masks and storing the air samples on the vials might be considered successful, as the results calculated with plasma (^{13}C-Leucine) and urine (^{15N}-Glycine and ^{13}C-Phenilalanine) was similar with the calculated with gas (P>0.05). This indicates equally efficient rates of isotopes recovery, or at least recoveries precise enough to result in similar estimations of synthesis and breakdown of protein. The estimated protein flux, however, was almost double for ^{15}N-Glycine, which may be a characteristic of the tracer and the calculation procedure adopted.

Table 1. Protein flux, synthesis, and breakdown of old beagle dogs obtained with different traces and biological samples.

Method	**Flux**	**Synthesis**	**Breakdown**
		($g.kg^{-0.75}.d^{-1}$)	
^{13}C-Phenylalanine (gas)	3.48[a]	3.46	3.21
^{13}C-Leucine	3.70 [a]	3.67	3.48
^{15}N-Glycine (ammonia and urea)[1]	6.03 [b]	3.29	3.21
^{13}C-Phenylalanine (urine)	2.91 [a]	2.91	2.90
SEM [2]	0.28	0.16	0.15
P value	0.005	0.418	0.647

[1] The atom percent in excess to calculate enrichment correspond to the mean of urea and ammonia on urine (Waterlow, 2006).

[2] SEM = standard error of the mean (n = 6 dogs per method).

[a,b] – means in a column not sharing a common letter differs (P<0.05).

The rates of synthesis and breakdown obtained with the different methods was compared with the Bland & Altman statistics and Pearson correlation (Table 2).

Table 2. Pearson correlation and Bland & Altman statistics of the results obtained with the different methos studied.

	Pearson Correlation	P value	Bias	Lower limit	Upper limit	Concordance Correlation Coefficient	Bias Correction Factor
Synthesis			$g.kg^{-1}.d^{-1}$				
^{13}C-Phe (gas) x ^{13}C-Phe (urine)	0.59	0.213	0.55	-0.85	1.94	0.45	0.75
^{13}C-Leu x ^{13}C-Phe (gas)	0.33	0.513	0.25	-1.31	1.82	0.18	0.54
^{13}C-Leu x ^{13}C-Phe (urine)	0.01	0.986	0.80	-0.68	2.28	0.00	0.29
^{13}C-Leu x ^{15}N-Gly	-0.79	0.056	0.42	-2.20	3.04	-0.32	0.40
^{13}C-Phe (gas) x ^{15}N-Gly	-0.32	0.535	0.16	-2.96	3.29	-0.30	0.95
^{13}C-Phe (urine) x ^{15}N-Gly	0.18	0.724	-0.38	-2.72	1.95	0.15	0.82
Breakdown							
^{13}C-Phen (gas) x ^{13}C-Phe (urine)	0.58	0.229	0.34	-1.05	1.74	0.50	0.88
^{13}C-Leu x ^{13}C-Phe (gas)	0.49	0.320	0.68	-0.74	2.09	0.18	0.54
^{13}C-Leu x ^{13}C-Phe (urine)	0.24	0.643	1.02	-0.35	2.39	0.05	0.23
^{13}C-Leu x ^{15}N-Gly	-0.73	0.095	0.65	-1.97	3.28	-0.27	0.37
^{13}C-Phe (gas) x ^{15}N-Gly	-0.34	0.514	-0.02	-3.12	3.07	0.96	-0.32
^{13}C-Phe (urine) x ^{15}N-Gly	0.18	0.730	-0.36	-2.70	1.97	0.83	0.15

Probably the small number of replicates (n = 6 dogs) and the variability between animals did not allow to detect correlation between methods (P>0.05). It was also possible to see that the bias was high, and in general de concordance correlation coefficient and the accuracy (bias correction factor) were low.

Discussion

The ^{15}N-Glycine method was considered popular in the 1980s due to its non-invasive and easy-to-apply proposal, as it requires a single dose of the tracer and a urine collection for 9 to 12 hours (Fern et al., 1981, 1985; Garlick and Fern, 1985). However, in conditions outside from a laboratory, the application of the method presents some complications, as urine collection is not as easily controlled and it may be not a sensitive method to be used in different physiological and pathological conditions (Wagenmakers, 1999).

In humans is possible to request the individual to urinate, emptying the bladder before the enrichment and then collecting the produced urine at the required times. In dogs this was not possible, and the animal may have a residual amount of urine in the bladder, before the enrichment that may dilute the first point of the plateau (the first spontaneous voided urine of the animal). Additionally, dogs do not urinate in the necessary times, but when they want, and the sampling time do not correspond exactly with the required on the protocol. This issue was already discussed by cats on the doubly labelled water method with urine as the sampling fluid (Goloni, et al., 2019), but as in the present study, the final values were close and similar, open the opportunity to explore this method to dogs. To increase the precision someone would argue that it would be possible to place an urinary catheter to collect the urine samples of dogs, but this is too invasive and out of the scope of the present study.

The big criticism in the use of 15N as a tracer for studies about protein metabolism in general is the assumption that all amino acids integrate in a single nitrogen compartment and achieve simultaneously the same enrichment. It is known that nitrogen breaks down into ammonia and urea, and duet that in some studies an average between the two was used (Fern et al., 1981, 1985) and, although the path of each amino acid is demonstrably independent and adopts different rates (Garlick and

Fern, 1985), in this study, only the flux differed from the other tracers (P<0.05) while synthesis and breakdown were similar (Table 2, P>0.05), this difference is justified because the tracer is different (15N and 13C).

13C-Leucine is characterized in the literature with continuous intravenous infusion for about two hours with an enrichment plateau defined by arterial leucine (Matthews et al., 1980), it is considered the gold standard for the determination of protein turnover due to its true representation to the incorporation of free amino acids (Matthews et al., 1982). Rates calculated by leucine are converted to the whole body using the average leucine content of the body protein (8g/100g protein, Matthews et al., 1980). Oxidation can be quantified when the bicarbonate pool is prepared and corrected by the indicated values (Van Hall, 1999), such corrections allowed for more accurate protein synthesis and breakdown values in this study, where we found no difference between the methods (Table 2, P> 0.05).

The big difference between 13C-Leucine and the other methods is that the animal is fasting, and although it does not reflect the normal conditions of the animal's daily life, the measurements are fair and adequate for the conditions of collection and most physiological conditions (Wagenmakers, 1999), in addition to favoring routine collections in a veterinary hospital or with domesticated animals that, in most cases, do not feed when under stress or disease.

In a study, Williams et al. (2001) used different concentrations of protein in the feed comparing synthesis and breakdown in young and old dogs with 15N-Glycine, finding no difference between the categories of animals, suggesting that the measures are more influenced by protein intake than by the condition of the animal in this methodology. However, the benefits of using this methodology are that this marker demonstrates fewer deviations because the analysis is performed by ammonia and urea metabolic compartments by harmonic mean, in addition to being a non-invasive method (Garlick, Clugston and Waterlow, 198; and Fern, Garlick and Waterlow 1985, van Waardenburg et al., 2004).

A study with elderly humans comparing protein metabolism used 15N-Gly with the harmonic mean between ammonia and urea and 13C-Leu as metabolic tracers, although there was no statistical difference, there was a slight increase in rates by 13C-Leu (Pannemans et al., 1997), which was also observed in this study (Table 2, P>0.05).

Therefore, caution is suggested when comparing methodologies due to the time of the experiment, route of administration and the choice of tissue/product for the calculation.

Non-invasive methods are incorporated into the most vulnerable study models, such as children and people with illness, where the isotope infusion is oral or intragastric, for an interval of 12 to 60 hours for studies of amino acid and protein metabolism, as well as the urine used as a sample for enrichment of plasma amino acids, demonstrating good correlation of plasma results between L- [1-13C] leucine, L- [1-15N] glycine and L- [1-13C] phenylalanine (Basile-Filho et al. al. 1997, from Benoist et al. 1984, Sanchez et al. 1995, Waterlow et al. 1978, Wykes et al. 1992, Zello et al., 1994).

Bross, Ball and Pencharz (1998) developed a 1-day protocol for protein metabolism studies comparing intravenous and oral infusion of L-[1-13C] phenylalanine with analyzes through urine and blood plasma, where they found no difference between the routes of collection. Corroborating the results found in this study, where the different routes and protocols for supplying the compounds, did not change the final result (Table 2, $P>0.05$).

Conclusions

All methods obtained similar values and can be used for research purposes. The adopted gas and urine sampling procedure can be considered adequate, as the isotopes enrichment allowed to estimate similar values of protein synthesis and breakdown rates. The agreement and concordance between methods, however, were low and should be considered. The selection of the most suitable method will depend on the experimental conditions, as ^{13}C-Leucine evaluates animals in fasted state, ^{13}C-Phenilalanine and ^{15}N-Glycine in fed state, and the feasibility of total urine collection will depend on the research structure and the dog population of study.

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Capítulo 3 - Determination of whole-body protein turnover in old and adult dogs with different metabolic tracers, digestibility, nitrogen balance and corporal composition

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Abstract

Sarcopenia affects part of the elderly dog population, being characterized by the loss of lean body mass, probably resulting from decreased muscle protein synthesis. In order to further investigate protein metabolism in dogs of different ages, we propose the comparison of three methods to determine protein flux in adult and elderly dogs. The study was carried out in a completely randomized design, in a 3x2 factorial arrangement of treatments, with three methods and two age groups. For the study of protein metabolism, the precursor method with L- [1-^{13}C] Leucine, the [^{15}N] Glycine end products method and the indirect oxidation method L- [1-^{13}C] Phenylalanine were compared. The study included 20 dogs, male and female, 10 adults (age between 1 and 3 years) and 10 elderly (age older than 10 years). The animals were fed commercial feed extruded for elderly dogs in sufficient quantity to maintain body weight. Dogs received food for 33 days, distributed as follows: day 1 to 10 for diet adaptation, days 11 to 15 for total collection of faeces urine for the test of digestibility, nitrogen balance and elimination of urine of 24 hours by urine, days 16 to 19 for rest, days 20 to 23 for the test of phenylalanine (expired gas and urine), days 24 and 25 for the glycine (urine) test, days 26 to 32 for rest and elimination of the previous markers, day 33 for the Leucine test (expired gas and plasma). Through the collection of plasma, urine, and expired gas, the parameters of isotopic enrichment were obtained and applied in flux equations, protein synthesis and degradation. Before the comparisons of means will be verified the assumptions of homogeneity of variances and normality of the errors. The data will be submitted to analysis of variance, considering the effects of method, age and their interactions (method x age). When significance is obtained in Test F, the means will be compared by the Tukey test. The methods of evaluation of protein turnover will also be compared by Pearson correlation and Bland and Altman analysis. The value of $P < 0.05$ will be considered significant. With the results, we hope

to obtain a more in-depth view on the effect of age on the protein metabolism of dogs, as well as to study in further the three methods of determination of body protein turnover.

Keywords: [^{15}N] Glycine, L- [1-^{13}C] Leucine, L- [1-^{13}C] Phenylalanine, Protein, Sarcopenia

Introduction

The nutrition throughout the life is linked by the corporal condition in the senescence, and it may help in the maintenance of the protein synthesis to avoid the muscle-weakening , fractures and hormonal losses caused by sarcopenia. If the protein from feed is not enough, the body muscle mass losses linked by the age can be graver and contribute to the early death (Laflamme, 2008).

It is possible that these changes in muscle tissue deposition and lean body mass in the elderly are a reflection of changes in protein metabolism, with less relative deposition and greater tissue catabolism. This is reinforced by the fact that senescence does not induce changes in protein digestibility in dogs, which remains unchanged with advancing age (Maria et al., 2017).

Protein turnover is determined by the balance of synthesis and degradation reactions, corresponding to the animal needs in a given physiological status (Waterlow, 2006). Body protein turnover is a complex function as it involves both the turnover of individual proteins and amino acids and changes in the flow of one or more specific amino acids. This flux can profoundly affect protein regulation (Matthews et al., 1980). Body turnover rates are generally high only in the growth phase, but there are several factors resulting from normal or pathological physiological processes that can interfere with this rate, such as resistance exercises (hypertrophy), loss of muscle mass due to aging process (sarcopenia) or disease (cancer) (Wolfe, 2005; Wolfe e Chinkes, 2005).

In the metabolic process, in short periods of protein deficiency, endogenous proteins from the liver and gastrointestinal tract are primarily used to support protein turnover. Although muscles and skin represent the largest contingent of protein reserves, they are mobilized only when protein deficiency persists for longer periods of time. Mobilization of these tissues occurs even though nitrogen balance and body

weight remain unchanged, which is achieved by reducing the rate of body protein turnover and using lean body mass reserves to meet the essential metabolic processes (Allison and Wannemacher, 1965; Wannemacher and McCoy, 1966; Wolfe, 2006). When protein deficiency is prolonged in the elderly, the reduction in protein turnover is added to the effects of sarcopenia, accelerating the deposition of body fat and reduction of lean mass, with an increase or stability of body weight (Kealy, 1999; Wakshlag et al., 2003). However, this reduced turnover of proteins, resulting from the deficient intake of amino acids, can lead to an increase in infections and worsening of body health, especially in the elderly.

The use of metabolic tracers allows extracting valuable information about the kinetics, turnover and metabolism of a particular nutrient of interest, making them high potential tools to elucidate physiological issues, regardless of the age of the animal (Kim et al., 2016). The ideal tracer must be identified with sufficient precision when given in small doses, and must still represent and not affect the metabolism of the element to be traced (Wolfe e Chinkes, 2005).

The best known methods for determining turnover are precursor, end products and amino acid oxidation. The three can be used for two purposes: to determine the rates of protein turnover, synthesis and degradation in general, or to be applied to studies of isolated amino acids when in deficient or excessive concentrations, facilitating studies of protein metabolism in vivo, without presenting risks of health or contamination.

The precursor method is represented by the use of L-[13C] leucine. Unlike other essential amino acids that are metabolized in the liver, leucine is primarily catabolized in muscle, the body's main protein store. In addition, leucine has a regulatory effect on the other two branched-chain amino acids (isoleucine and valine), in addition to participating in muscle protein synthesis and degradation and oxidative metabolism (Matthews et al., 1980). According to the classical model of study with [13C] leucine, a single pool in body kinetics is assumed from which this amino acid is used to quantify protein synthesis and degradation, with the flux being calculated from the isotopic dilution in the plasma of labeled leucine (Goudoever et al., 1995).

The end products method involves the use of [15N] glycine, which was the first isotope tracer used to determine in vivo body protein turnover. The method was very popular in the 1980s due to its non-invasive and easy-to-perform characteristics.

Some limitations inherent to this method should be highlighted: the need for total urine collection, and that the 15N from the tracer is incorporated differently between amino acids and, usually, different turnover rates are found when using other labeled amino acids. , even if it is another 15N-tagged amino acid (Fern et al. 1981, 1985; Garlick & Fern, 1985; Wagenmakers, 1999).

The indirect method of amino acid oxidation (IAAO) was developed with the objective of obtaining a minimally invasive model that would allow the study of amino acid kinetics in any group of interest, including vulnerable groups that include pregnant women, lactating women, the elderly and puppies. Basically, it consists of combining the methods of the precursor and the final product, that is, instead of plasma, urine is collected for the quantification of oxidation (Bross, Ball and Pencharz, 1998).

Elango, Ball, and Pencharz (2008) compared intravenous and oral infusion to deliver L-[1-13C]phenylalanine and determined that, although the route of isotope administration affected tracer kinetics, the final results were consistent in both. the situations. Although this method was developed specifically for the determination of amino acid requirements, in the present study we intend to obtain values that correspond to the general flow of protein from a balanced diet, thus studying its use to determine protein turnover.

The aim of this study was to compare the three methods for determining protein turnover in young and old adult dogs fed the same diet, as well as comparing digestibility, nitrogen balance, urinary urea and body composition between age groups.

Material and Methods

The study was conducted at the Research Laboratory of Nutrition and Nutritional Diseases of Dogs and Cats “Prof. Dr. Flávio Prada”, Universidade Estadual Paulista (UNESP), Jaboticabal, Brazil. All procedures in this research were previously approved by the Ethics Committee on the Use of Animals (CEUA) of the same institution (protocol number 009537/18).

Animals, housing and experimental design

20 adult dogs, male and female, were used, 10 young adults, aged between 1 and 3 years and 10 elderly, aged over 10 years. These were previously evaluated through physical examination and complementary analyzes including blood count, urinalysis and serum biochemistry (alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, albumin, urea and creatinine) so that no pathological condition interferes with the results. The experiment followed a 3x2 factorial scheme of treatments, consisting of three methods for determining protein turnover and two age groups (young and senile adults), totaling six experimental treatments. This was developed in a completely randomized design, with 10 experimental units for each combination of age and method. For this, all dogs were tested by the three methods of protein turnover assessment.

The animals were fed with feed formulated in accordance with the FEDIAF (2018), with a nutritional profile shown in Table 1. Food samples were collected throughout the study, creating a representative pool of what was supplied. At the end of the study, the food was analyzed for dry matter, mineral matter, crude protein, amino acid composition, ether extract, acid hydrolysis, starch and dietary fiber. The dogs received the food for 33 days, distributed as follows: days 1 to 10 for adaptation to the diet (Kriengsinyos et al., 2002), days 11 to 15 for total collection of feces and urine for the digestibility, nitrogen balance and 24h urine elimination of urea, days 16 to 19 for rest, days 20 to 23 for the phenylalanine assay, days 24 and 25 for the glycine assay, days 26 to 32 for rest and elimination of previous markers, day 33 for the leucine assay.

The feed (Table 1) was formulated to meet all the nutritional needs contained in the FEDIAF (2018) and processed at the feed factory of the Faculty of Agrarian and Veterinary Sciences, FCAV/UNESP, Jaboticabal, SP.

Table 3. Chemical composition of food.

Item	**Use (%)**
Crude protein (%)	19.5000
Crude fat (%)	14.5000
Ac.linolenic (W-3) (%)	0.2487

Linoleic acid (W-6) (%)	3.2060
Crude fiber (%)	2.4357
Moisture (%)	10.6406
Ash (%)	5.9308
Dietary fiber	4.3295
Calcium (%)	1.1231
Total P (%)	0.6812
Ca:P (%)	1.64:1.0
Starch (%)	39.2379
Estimated metabolizable energy (kcal/kg)	3800
Total amino acids (%)	
Lysine	1.2682
Methionine	0.3800
Cystine	0.2860
Met + Cys	0.6657
Alanine	1.1394
Tyrosine	0.6046
Threonine	0.6626
Tryptophan	0.2000
Leucine	1.3500
Isoleucine	0.7400
Valine	0.9240
Glycine	1.4065
Serine	0.8270
Arginine	1.2071
Histidine	0.5677

During the test the dogs were fed to maintain body weight. For this, they were initially fed based on their individual energy needs, established by the food history of each animal in the kennel, and considering the metabolizable energy of the food. The daily amount of food was provided in a single meal. The amount supplied and leftovers were weighed, and consumption was recorded. The animals were then weighed every

5 days, always in the morning before the first meal, and the amount of feed was adjusted so that the dogs had a constant body weight throughout the experimental period.

Digestibility, nitrogen balance, 24-hour urinary urea

The digestibility and nitrogen balance assay was performed using the total collection method of feces and urine, according to FEDIAF recommendations (2018). For this, the dogs were housed in stainless steel metabolic cages, with an apparatus for the separate collection of feces and urine, with dimensions of 100 x 100 x 100 cm. Metabolic cages were washed daily, rinsed with water and dried. Feces and urine were collected at least twice a day. Feces were weighed and frozen (-15°C). Urine was collected in a collection flask containing 4mL of 6N hydrochloric acid solution, the volume was quantified and stored frozen (-15°C) until analysis. At the end of the collection period, feces and urine were thawed and homogenized, composing one sample per animal. The stool samples were dried in a forced ventilation oven (320-SE, FANEM, São Paulo) at 55°C for 72h. The pre-dried samples of feces and feed were ground in a knife mill (MOD 340, ART LAB, São Paulo), with a 1mm sieve. Feed and feces were analyzed according to the procedures described by the AOAC (1995) for dry matter, mineral matter, crude protein and ether extract in acid hydrolysis. The gross energy content of diets and feces was determined by bomb calorimetry (IKA Calorimeter System C 200, IKA-Werke GmbH & Co. KG, Staufen, Germany). Based on these results, the digestibility of dry matter, organic matter, crude protein, ether extract acid hydrolysis and gross energy of the feed were calculated for both young and elderly adult dogs, considering the calculation procedures described by FEDIAF (2018).

To quantify urine nitrogen, the samples were thawed, homogenized and centrifuged at 3500 rpm for 15 minutes to precipitate possible feces and hair residues, and then analyzed in tin capsules (Tin Capsules 501-059, LECO Corporation, St. Joseph, MI, USA) in LECO equipment (AOAC, 1995; method 990.03; FP528, LECO Corporation, St. Joseph, MI, USA). Based on this procedure, the nitrogen balance was calculated by computing the N ingested – N feces – N urine. Urinary urea determination was performed using a commercial kit (Blood Urea Nitrogen kit no. 535-B, Sigma, St. Louis,

MO) in a spectrophotometer (Labquest, Labtest Diagnóstica S.A, Lagoa Santa, MG, Brazil). Considering that urea is a product of amino acid catabolism (HENDRIKS et al., 1997; RUSSEL et al., 2000), it is expected with this evaluation to verify the influence of age on the renal excretion of this compound.

Determination of CO_2 volume

A respirometry system (Animal respirometry system, Sable System International, Las Vegas, USA) was used to determine the volume produced of CO_2 (VCO_2) of each dog. Dogs were housed in hermetically sealed chambers with internal control of temperature and moisture, with the dimensions of 1m x 1m x 1m (ALB 1000 CG Respirometry Chamber, Inbras Equipamentos para Saúde, Ribeirão Preto, Brazil). The chamber temperature was adjusted to 26±1 °C and the relative humidity of the air in the interval of 55% to 60%. Dogs were previously adapted to stay and be fed inside the chamber, avoiding stress that could interfere on results. Dogs were fasted for 24h, placed on the cages and fed with their usual amount of food. Water was provided ad libitum. Each run latest 8 hours. According to previous data of pilot studies, 2 hours was waited to equilibrate the gasses inside the chambers.

Ambient air flows were introduced into the chambers by a negative pressure system, with an average flow of 10 L min^{-1}, with control and adjustments provided by a mass flow pump with a maximum flow capacity of 100.0 L min^{-1} (Sable Systems International, Las Vegas, Nevada, USA). The air flow volumes were established to maintain CO_2 saturations below 1.0% (average CO_2: 0.3±0.1% and average O_2: 20.4±0.2%) inside the chambers. Channel air sampling was performed by independent pressure gauges (RM-8 Gas Flow Multiplexer, Sable Systems International, Las Vegas, Nevada, USA). The air samples were routed by a subsampler (SS-4 Gas Analyzer Subsampler, Sable Systems International, Las Vegas, Nevada, USA) to a humidity meter (RH-300 Relative Humidity & Dew Point Analyzer, Sable Systems International, Las Vegas, Nevada, USA). The oxygen concentrations were measured by a paramagnetic analyser (PA-10, Sable System International, Las Vegas, Nevada, USA), and the CO_2 concentrations were measured with a carbon dioxide analyser (CA-10, Sable System International, Las Vegas, USA). Before measuring the gas concentrations, moisture was removed from the air by using anhydrous magnesium perchlorate (Exodus

Científica, Sumaré, São Paulo, Brazil). The control and gauging of the gas metres were carried out using a standard air mixture (CO_2: 1.0031% and O_2: 21.00%; White Martins, Vinhedo, São Paulo, Brazil). The data obtained by the mass flow metre and gas analysers were collected by an ExpeData system (Sable System International, Las Vegas, Nevada, USA).

Before and at the end of the evaluations, the chambers and the respirometry system were validated for their gas recovery efficiency by burning Ethanol (Ethyl alcohol, Signma-Aldrich, Missouri, USA) for 430 minutes, assuming the combustion values proposed by Elia (1992). The volume of ethanol burned (initial weighing - final weighing), the inflow and the outflow of air was considered and the volumes of O_2 and CO_2 that should have been detected by the device were calculated. Based on these values, the recovery percentage was established. To validate the system a CO_2 recovery greater than 95% was stablisehd.

The VCO_2 produced by each dog was determined by the following equation (Lighton, 2008):

$$VCO_{2(mL \cdot min^{-1})} = FR_e \frac{[(F_eCO_2 - F_iCO_2) + F_iCO_2(F_iO_2 - F_eO_2)]}{(1 + F_iCO_2)}$$

Where FR_e is the excurrent flow rate, F_e is the fractional concentration of a gas in the excurrent airstream, and F_i in the incurrent airstream.

^{13}C-Leucine

The cephalic vein was catheterized (catheter, Descarpack®, São Paulo, Brasil) and with dogs after 24h fasting a priming dose of a combination of 2 mg/kg of L-[1-13C] Leucine and 0.1 mg/kg of [13C] NaH13CO3 (Cambridge Isotope Laboratories, Tewksbury, US) was infused. After five minutes of this prime dose, an infusion of 0.7 mg/kg of L-[1-13C] Leucine was repeated every 20 minutes for a total period 80 minutes (Figure 1).

Item	Minutes after isotope injection								
	0	20	40	60	80	100	120	140	180
^{13}C-Leu infusion	●	●	●	●	●				
$NaH^{13}CO_3$ infusion	■								
Expired gas collection	♠	♠	♠	♠	♠	♠	♠	♠	♠

Plasma Collection	♦	♦	♦	♦	♦	♦	♦	♦	♦

Figure 2. Sampling protocol of the 13C-Leucine method.

Samples of expired gas and blood plasma were collected prior to enrichment, and 20, 40, 60, 80, 100, 120, 140 and 180 min after the prime dose enrichment. At each sampling time expired gas was collected using a specific designed mask for at least 2 minutes, directly into a glass vial coupled to the mask and stored at room temperature for latter analysis.

Blood samples was collected using the catheter and a syringe, deposited in heparinized tubes, centrifuged for plasma separation and the plasma kept frozen (-20 ^{o}C). Immediately before analysis, plasma samples were lyophilized (Thermo VLP200, ThermoFisher, Massachusetts, EUA).

^{13}C-Phenylalanine

For this test dogs were housed individually in metabolic cages equipped with apparatus to separate feces and urine for collection, measuring 0.9 m × 0.9 m × 0.9 m. After 24h fasting animals were fed with their usual amount of food divided in four equal meals, and fed each amount at every 20 min until complete the daily food intake. Only dogs that ate all offered food was tested. Dogs that did not complete the meal was tested on the other day.

Immediately after completed the meal, dogs received a prime dose of 0.7 mg/kg of L-[1-^{13}C]Phenylalanine and 0.2 mg.kg^{-1} of $NaH^{13}CO_3$ (Cambridge Isotope Laboratories, Tewksbury, US) supplied orally in gelatin capsules. After, dogs were orally dosed every 30 minutes four times with 1.3 mg/kg of L-[1-^{13}C]Phenylalanine in gelatin capsules (Figure 2).

Item	Minutes after isotope administration									
	-60	-30	0	30	60	90	120	150	180	210
Food intake	●	●	●	●	●					
^{13}C-Phe infusion			●	●	●	●	●			
$NaH^{13}CO_3$ infusion			■							
Expired gas collection			♠	♠	♠	♠	♠	♠	♠	♠

Figure 3. Sampling protocol of the ^{13}C-Phenylalanine method. Urine was collected before enrichment and then all urine made after the meal.

Samples of expired gas were collected prior to enrichment, and 30, 60, 90, 120, 150, 180 and 210 min of the priming dose administration. At each sampling time expired gas was collected using a specific designed mask for at least 2 minutes, directly into a glass vial coupled to the mask and stored at room temperature for latter analysis.

Urine samples were collected prior to enrichment, and then all urine made after the meal and stored frozen (-20 ^{o}C) until analysis. Urine samples were quantitatively collected in plastic containers placed under the collecting funnel of the metabolic cages, added with 4 mL of 6N HCl as a preservative (Synth, LABSYNTH, Diadema, Brazil). Before analysis urine samples were thawed and lyophilized (Thermo VLP200, ThermoFisher, Massachusetts, EUA).

^{15}N-Glycine

Dogs were individually housed in metabolic cages with apparatus to separate feces and urine for collection, measuring 0.9 m × 0.9 m × 0.9 m. After 24h fasting, animals were fed with their usual amount of food. Immediately after completed their meal, animals were enriched with a single dose of 20mg/kg of [15N] Glycine (Cambridge Isotope Laboratories, Tewksbury, US). The labeled compound was orally dosed in gelatin capsules. Only dogs that ate all offered food was tested, and animals that did not complete the meal was evaluated on the other day.

Urine samples were collected prior to enrichment, and then then all urine made after the meal and stored frozen (-20 ^{o}C) until analysis. Urine samples were quantitatively collected in plastic containers placed under the collecting funnel of the metabolic cages, added with 4 mL of 6N HCl as a preservative (Synth, LABSYNTH, Diadema, Brazil).

Prior to analysis, urine samples were thawed at room temperature. In the procedure, the incorporation of labeled N is quantified on both the urinary ammonia and urea. To this, the separation of ammonia and urea on urine samples was conducted using the cation exchange method in resin (AG50W-X8, Bio-Rad Laboratories, California, EUA), as described by Read (1961) and Preston and

McMillam (1988). On the assay the resin binds ammonia in a neutral solution. Urea stays in the supernatant, that is after converted into ammonia with urease (Sigma Aldrich, Missouri, EUA), and incorporated in the resin. Further, ammonia is extracted from the resin and subjected to N isotopic analysis.

Isotopic analysis

The isotopic analyzes were performed in the Stable Isotopes Center, Universidade Estadual Paulista (Unesp), Botucatú, Brazil. All samples were analyzed in a continuous-flow isotope ratio mass spectrometry system (CF-IRMS). The CO_2 samples were analyzed in an automated breath ^{13}C analyzer isotope ratio mass spectrometer (ABCA2, SerCon, Cheshire, UK). The system extracts the sample from the tube by washing with He utilizing a two-way needle. The He flow carries the sample to the IRMS, were the isotopic ratio R (iE/jE) is expressed as a relative difference between the carbon isotopes (δiE), calculated with the equation described by Coplen (2011).

To calculate the $\delta^{13}C$ was used the value of $R(^{13}C/^{12}C)_{V\text{-}PDB}$, results were previously normalized considering the delta over baseline. The ABCA-IRMS uncertainty was estimated at ±0.16‰ according to Carlos et al. (2019).

Urine and plasma samples were weighed in 5 x 8 mm tin capsules (PN 24006400, Thermo Scientific, Germany), and analyzed on an Elementary Analyzer (EA) type CF-IRMS. To this an IRMS (Delta V, Thermo Scientific, Germany) coupled to an EA (Flash EA, Thermo Scientific, Germany) and a gas interface (ConFlo IV, Thermo Scientific, Germany) was used. The capsules were inserted with an autosampler into the EA oxidation/reduction reactor at 1020°C, were the carbon and nitrogen of the sample were converted into CO_2 and N_2 through combustion and carried to the IRMS. The R(iE/jE) was calculated as previously shown.

To calculate $\delta^{15}N$ the default value $R(^{15}N/^{14}N)_{V\text{-}AIR}$ were used. The EA-IRMS uncertainty was estimated at ±0.15‰ and ±0.20‰ for $\delta^{13}C$ and $\delta^{15}N$, respectively. The results were normalized by two-point anchorage (Paul et al., 2007) using the NBS-22 and USGS87 standards for $\delta^{13}C$, and IAEA-N-1 and USGS90 for $\delta^{15}N$.

Whole body protein turnover

The flux of labeled compounds and rates of protein synthesis and breakdown were calculated according to the model proposed by Picou and Taylor-Roberts (1969):

$$\mathbf{Q = O + S = I + B}$$

Where Q is tracer flux (g.kg^{-1}.d^{-1}); O is the oxidation of the amino acid measured through the expired gas (not considered in the other tracers); S is the protein synthesis rate (g.kg^{-1}.d^{-1}); I is the N intake from the diet (g.kg^{-1}.d^{-1}); and B is the protein breakdown rate (g.kg^{-1}.d^{-1}).

$$\mathbf{Q\ leucine}\ (\mathbf{g}.\boldsymbol{kg^{-1}}.\boldsymbol{d^{-1}}) = \left(\boldsymbol{D} * \left(\left(\frac{\boldsymbol{X^{E}_{(13C)Leucine}}}{\boldsymbol{X^{E}_{(13C)Plasma}} * \mathbf{1.33}} - \mathbf{1}\right)\right)\right)\boldsymbol{MW} * \mathbf{10^{6}} * \mathbf{24}$$

$$\mathbf{Q\ glycine}\ (\mathbf{g}.\boldsymbol{kg^{-1}}.\boldsymbol{d^{-1}}) = \left(\boldsymbol{D} * \left(\left(\frac{\boldsymbol{X^{E}_{(15N)Glycine}}}{\boldsymbol{X^{E}_{(15N)Urine}}} - \mathbf{1}\right)\right)\right)\boldsymbol{MW} * \mathbf{10^{6}} * \mathbf{24}$$

$$\mathbf{Q\ phenylalanine}\ (\mathbf{g}.\boldsymbol{kg^{-1}}.\boldsymbol{d^{-1}}) = \left(\boldsymbol{D} * \left(\left(\frac{\boldsymbol{X^{E}_{(13C)Phenylananine}}}{\boldsymbol{X^{E}_{(13C)Gas/Urine}}} - \mathbf{1}\right)\right)\right)\boldsymbol{MW} * \mathbf{10^{6}} * \mathbf{24}$$

Where Q is the tracer flux (g.kg^{-1}.d^{-1}), D is the tracer infusion rate (µmol.kg-1.h-1); $X^{E}_{(tracer)}$ is the absolute enrichment of the tracer; $X^{E}_{(sample)}$ is the atom percent in excess and represents the enrichment in the steady state; MW is the molar weight of the tracer, which together the multiplication by 10^{6} is used to transform µmol to g; 24 is to transform hour in day (Adapted from Mathews et al., 1980).

S (g.kg^{-1}.d^{-1}) = Q - O

Protein synthesis (S) for ^{13}C leucine or ^{13}C phenylalanine was calculated from the flux (Q) and oxidation (O) data. In the case of urine for the ^{15}N glycine and ^{13}C phenylalanine tracers it equals the flux (Q), provided the other parameters are calculated in the enrichment plateau (Mathews et al., 1980).

B (g.kg^{-1}.d^{-1}) = Q - I

Protein breakdown (B) was calculated from the difference between the flux (Q) of ^{13}C leucine or ^{15}N glycine or ^{13}C phenylalanine and the intake of the amino acids (I) in the case of glycine or phenylalanine (when the animals were fed) or equals the isotope dose for ^{13}C leucine (when the animals were fasted) (Mathews et al., 1980).

O (g.kg^{-1}.d^{-1}) = $F^{13}CO_2 \left(\frac{1}{X^E_{(13C)tracer}} - \frac{1}{X^E_{(13C)sample}}\right) * 100$

The oxidation (O) of ^{13}C phenylalanine and ^{13}C leucine was calculated with this equation, where: $X^E_{(tracer)}$ is the absolute enrichment of the tracer; $X^E_{(sample)}$ is the atom percent in excess and represents the enrichment in the steady state; $F^{13}CO_2$ represents the rate of $^{13}CO_2$ released by the tracer oxidation (g $^{13}CO_2$.kg^{-1}.d^{-1}), calculated by the following equation:

$F^{13}CO_2$ (g.kg^{-1}.d^{-1}) = $\left(\frac{FCO_2 * ECO_2}{BW}\right) * \left(\frac{60 * 41.6}{100 * 1}\right) * (MW * 24)$

Where FCO_2 is the CO_2 production rate of the animal determined in respirometry chambers (mL.min^{-1}), ECO_2 is the $^{13}CO_2$ enrichment plateau in the expired gas (APE); BW is the weight of the animal (kg). The constants 41.6 µmol.ml^{-1} and 60 min.h^{-1} convert FCO_2 to mol.h^{-1} and the factor 100 changes the plateau to a fraction. The factor 1.0 accounts for the $^{13}CO_2$ retained in the body because of bicarbonate fixation (Hoerr et al., 1989). MW is the molar weight of the tracer, which together the multiplication by 10^4 is used to transform µmol to g. The 24 is to transform hour in day (Adapted from Mathews et al., 1980).

Body composition determination

The isotope solution was prepared at a concentration of 7%, mixing 930 mL of saline solution and 70 mL of deuterium oxide (Sercon Limited, Unit 3B Crewe Trade Park, Gateway, Crewe, Cheshire, UK; 99% purity). A dosage of 1 mL/kg BW was subcutaneous applied between the scapula, after 21 hours fasting and 2 hours without water (FERRIOLLI et al., 2008). To increase the accuracy on inoculation, the empty syringe, the syringe with the isotope solution, and the syringe after solution inoculation were weight in a scale, according to recommendations of Ferrioli et al (2008).

Blood samples were collected before inoculation and after 2 h of isotope application (GOLONI, 2018). The isotope concentration on body fluids was evaluated at the Mass Spectrometry Laboratory of the Ribeirão Preto Medical School, São Paulo, Brazil. Deuterium was analyzed by isotope ratio mass spectrometry (ANCA 20-20, Europe Scientific, UK), following procedures described by Ferriolli et al., (2008).

Samples were processed in triplicate (150 µL per replicate) with platinum in vacutainers, after six hours resting.

The results for deuterium to determine the total body water, was calculated according Schoeller et al. (1996); Racette et al. (1994) and Ellis and Wong (1998). The lean mass of the dogs was calculated considering the hydration constant of 73.2% for mammals, by the equation: Lean mass (kg) = body water (kg)/0.732. The fat body mass (FM; kg) was estimated as: Total body mass (kg) – lean mass (kg) of the animal (PACE & RATHBUN, 1945).

Statistical analysis

Results were applied to equations and protein flux, synthesis and breakdown were calculated. After confirming the presupposition normality of residue and variance homoscedasticity, data were submitted to ANOVA in a completely randomized design in a factorial arrangement, with 4 methods x 2 ages (8 treatments) and 10 experimental units (dogs) per treatment. When differences were verified on F test means were compared by Tukey's test. To better compare results of the different methods, the confidence intervals of agreement between then were determined as the bias (mean difference) ± 1.96 SD, according to Bland and Altman (1986), and the means value was also compared using the Pearson correlation. The Bland and Altman statistics and Pearson correlation was performed on R Software (PBC, Boston, MA, 2020), and the remaining analyses were performed on SAS software 9.1 using the Proc MIXED (SAS Institute, Cary, NC, USA, 2003). Values of $P<0.05$ was considered significant.

Results and Discussion

The digestibility trial had adequate and similar food consumption between young and old (Table 2), standardized to 110 kcal.kg-0.75.d-1. The apparent digestibility coefficients showed similar values between ages ($P>0.05$).

Table 4. Intake (mean ± standard deviation) during the digestibility test, apparent digestibility coefficients and metabolizable energy of foods.

	Young	Aging	P-Value

Intake (g/kg^0.75/day)			
Dry matter	23.6±0.8	25.0±1.0	0.1897
Organic matter	17.9±0.1	18.2±0.3	0.1865
Crude protein	4.93±0.4	5.09±0.2	0.2865
Acid hydrolysis ethereal extract	2.16±0.1	2.17±0.4	0.9411
Raw fiber	4.32±0.3	4.48±0.3	0.1984
Apparent digestibility coefficients (%)			
Dry matter	85.18±2.6	86.65±2.9	0.2537
Organic matter	87.87±2.2	88.88±2.3	0.3298
Crude protein	85.50±3.3	86.42±2.8	0.5121
Acid hydrolysis ethereal extract	90.39±2.4	91.42±1.9	0.2988
Raw fiber	27.77±15.9	30.78±6.2	0.6242
Gross energy	87.77±2.2	88.74±2.3	0.3564
Metabolizable energy (kcal/g in MN)	*3987±125*	*4030±114*	*0.2952*

The production of feces was similar (Table 3) between the ages, but showed a difference in dry matter and score, showing different characteristics between young and old.

Table 5. Production and characteristics of feces of young and old dogs.

	Young	Aging	P-value
Feces			
g.kg.$^{-0.75}$.day^{-1}, in natural matter	11.8±2.3	10.9±2.4	0.3970
g.kg.$^{-0.75}$.day^{-1}, in dry matter	4.0±0.8	3.5±0.7	0.1454
Dry matter (%)	34.1±2.8	32.0±1.3	0.0474
score	4.1±0.1	3.9±0.2	0.0308

Nitrogen balance (Table 4) was calculated and showed no difference between ages during the collection period (P>0.05). For this collection, it is assumed that all ingested nitrogen comes from dietary protein, so it is measured, rather than protein, through nitrogen intake and losses in urine and faeces, while other losses (skin, fur, and sweat) are ignored. or estimated (Campbell et al., 2008; Earle, 1988). One of the

criticisms of this study is that, although it is possible to use different levels of protein in the diet, the animals can adapt to the low protein intake before the nitrogen balance study is carried out, as the body adapts to decrease "usual" protein intake (Laflamme, 2013).

Table 6. Nitrogen Balance in Young and aging dogs.

	Young	*Aging*	*P-valor*
Nitrogen balance (mg.Kg$^{-0.75}$.day^{-1})			
N intake	908.40±43.7	945.80±79.9	0.2104
N feces	123.30±50.4	147.50±33.0	0.2202
N urine	490.20±179.5	549.70±132.4	0.4100
N withheld	295.10±153.4	248.60±180.0	0.5419
Urea (mg.kg.$^{-0.75}$.day^{-1})	214.31±64.03	207.08±56.19	0.7913

Nitrogen balance and protein turnover have a direct relationship due to common metabolism. Wannemacher and McCoy (1966) when measuring nitrogen balance and protein turnover, observed that young beagles need 2.7 times more protein to get out of nitrogen balance and maximize protein turnover rates, while the elderly, in addition to having a greater basal need, need 3 times more in relation to equilibrium to maximize the parameters of synthesis and degradation.

The healthy body is in dynamic homeostasis, that is, all metabolic functions happen in a state of rotation, there is always something being synthesized, divided or converted into different compounds. In relation to the muscle protein pool in healthy adults, relative constancy is observed because the continuous breakdown of muscle protein is accompanied by synthesis and deposition, in cases of sarcopenia, for example, the constancy of the pool is respected, however, the size of the pool can be modified (Kim et al., 2016).

The protein turnover methodologies (Table 5) showed similar results when comparing the methods and difference between the ages, which was expected due to the sarcopenia process (to be proven by the analysis of body composition through the analysis of doubly labeled water).

Table 7. Body protein synthesis and breakdown rates obtained by four methodologies using the stable isotope tool.

	Young	Aging
	g protein.kg$^{-0,75}$.day^{-1}	
Synthesis		
^{13}C-Phe urine	3.11±1.16	3.10±1.13
^{13}C -Phe gs	3.72±0.68	2.87±0.76
^{13}C -Leu	3.91±1.44	3.27±0.53
^{15}N-Gly	3.83±1.02	2.93±0.37
Breakdown		
^{13}C-Phe urine	3.10±1.16	3.09±1.14
^{13}C -Phe gs	3.68±0.68	2.65±0.67
^{13}C -Leu	3.84±1.44	3.19±0.53
^{15}N-Gly	3.54±0.99	2.68±0.37
P-value	*Synthesis*	*Breakdown*
Method	0.6782	0.6874
Age	0.0488	0.0318
Method x Age	0.5136	0.4483

The different enrichment pathways and incorporation routes bring similar answers because they are the same objective, but it is necessary to critically compare the strengths and weaknesses of each of the isotope incorporation classes in relation to cost and data analysis, as well as as execution difficulty (Fan et al., 2016). Although the enrichment of the diet with 15N is relatively cheap, the isotopic dilution resulting from this method is complex, since it works on two compounds, urea and ammonia, which can cause complications in the interpretation of the results. Regarding 13C-labeled amino acids, which result in greater mass changes with a less complex isotopic dilution, they have a higher cost compared to 15N, but in all cases it is necessary to acclimate the animal to the diet to prevent isotopic exchanges in the food is confused with the compound (Basisty, Meyer and Schilling, 2018).

When the tracer used is 13C and has a continuous infusion, blood or gas samples, prior to enrichment and during the plateau, it brings information from the

absorption of oxidized substrate via the Krebs cycle, which allows determining the rate of oxidation of the substrate directly bound. to protein metabolism (Kim et al., 2016).

In comparative studies of the methods with humans, it was observed that, although the tracers provide similar qualitative and even quantitative information, there are some clear exceptions, because when used simultaneously, some differences were observed, not only in the final result, but also in the interpretation. physiological (Biolo et al. 1992; Wolfe et al. 1992; Marchini et al. 1993; Pacy et al. 1994).

Table 8. Body weight and body composition of young and aging dogs.

	Young	Aging	P-value
Weight (kg)	10.3±1.41	12.1±0.7	0.0029
Water (mL)	6516±964	6927±665	0.2820
Water (%)	63.3±4.2	57.4±2.9	0.0019
Fat (kg)	1.4±0.6	2.6±0.4	0.0001
Fat (%)	13.6±5.7	21.6±4.0	0.0019
Lean mass (kg)	8.9±1.3	9.5±0.9	0.2820
Lean Mass (%)	86.4±5.7	78.4±4.0	0.0019
ECC[1]	5±0,4	5±0,7	0,9999[3]
ECM[2]	3±0,2	2,5±0,1	0,0435[3]

[1] Body condition score of 9 pooints, where 5 is normal (Laflamme, 1997)

[2] Muscle mass score, where 3 represents normal muscle mass (Freeman. et al., 2011)

[3] Non-parametric Wilcoxon test.

In humans, strength and muscle mass are maintained until middle age, after which the process of loss in both is accelerated. Studies with continuous infusion of L-[1-13C]-Leucine with skeletal muscle biopsy determined that aging led to a reduction in synthesis rates, in the variety of muscle and mitochondrial proteins, as well as in the myosin chain, which can be the basis of age-related decline in muscle mass (Short et al., 2004; Balagopal et al., 2001; Hasten et al., 2000 Rooyackers et al., 1996).

The condition of sarcopenia is multifactorial in occurrence and the decline in muscle mass indicates a reduction in muscle protein content. Several studies in humans relate this phenomenon more to the reduction of specific muscle protein

synthesis than to general muscle proteolysis, which occurs naturally during life (Proctor et al., 1998; Nair, 2005; Augustin and Partridge, 2009).

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Capítulo 4 – Branched-chain amino acids, arginine and docosahexaenoic acid supplementation increase protein turnover and may improve body composition in old dogs

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Abstract

Dogs develop progressive reduction of lean mass with aging, resulting in sarcopenia. This condition is poorly studied in dogs, but as verified to humans may result in loss of muscle mass and strength, metabolic alterations, and reduction in life quality and life span. The present study investigated the effects of diet supplementation with a combination of leucine, isoleucine, valine, arginine, and docosahexaenoic acid on protein turnover and body composition of old and adult dogs. Two diets were formulated, a control and a test food (similar chemical composition but supplemented with the desired nutrients) and supplied to 10 adult (2.3±1.2 years) and 10 old (12.7±2.6 years) beagle dogs. Body composition was measured at start and end (deuterium oxide method), and protein synthesis and degradation at the end of 6 months of diet intake by the ^{13}C-Phenylalanine isotope method. The study followed a 2 (ages) x 2 (diets) factorial arrangement with 4 diets, organized in a cross-over design. Results were submitted to variance analysis considering the period, age, diet, and age * diet interaction effects, and means was compared with the Tukey test ($P<0.05$). Dogs presented the same body condition score ($P>0.05$), but old animals showed lower lean mass % and higher fatty mass % than adults ($P<0.01$), characterizing sarcopenia. The consumption of the test food stimulated protein renovation, with rates of protein synthesis 63% higher for adults and 10% higher for old dogs in comparison with the intake of the control food ($P<0.05$). This might explain the tendency for higher lean mass (kg) in old dogs fed the test diet in comparison to the control food ($P=0.06$), and the preservation of body composition of adult dogs fed the test diet, as when fed the control food these animals presented an increase in fatty mass % and reduction in lean mass % ($P<0.05$). By conclusion the studied old dog population presented sarcopenia

and the supplemented nutrient combination stimulated protein renovation and lean body mass preservation.

KEYWORDS: lean body mass; leucine; protein turnover; sarcopenia

Introduction

The increase in the care and improvement of dog nutrition has led to an increase in the life expectancy of these animals (Adams, Morgan and Watson, 2018). With aging, the dog population began to demonstrate physiological changes of age, many of which require dietary changes to ensure health and quality of life (Churchill and Eirmann, 2021). Among the changes in elderly dogs, cachexia, resulting from disease, and sarcopenia, resulting from aging processes, stand out (Saker, 2021).

Sarcopenia is a natural process characterized by the reduction of skeletal muscle tissue by the senescence process of organisms (Lewandowicz et al., 2020). There are several mechanisms that can explain the change in muscle mass, including lack of regular physical activity, natural changes in protein metabolism with an imbalance between synthesis and degradation, decrease in the concentration of hormones (growth, testosterone and IGF-1), increased cortisol and cytokine production, loss of neuromuscular function and muscle contraction strength, altered gene expression, and apoptosis (Rudman, 1985; Herbst et al., 2007).

In humans, the risks of sarcopenia are associated with changes in the individual's body composition, resulting from protein loss (Mitchell et al., 2012). Commonly, there is greater exposure to infections, fractures caused by weakening muscle contractions and reduced muscle extension, which lead to problems with bone quality and fracture by compression of the vertebrae due to lack of muscle support in the spine. In humans, these changes can lead to a decrease of up to 50% in work capacity and an increase in mortality (Dutta, 1997; Tipton, 2001; Short et al., 2004; Rizzoli et al., 2013). In dogs, studies that raise the occurrence and characterize sarcopenia are very scarce. It has been suggested that the loss of lean mass is a natural process with advancing age and that it would present the same symptomatic and physiological characteristics as seen in humans (Meyer and Stadtfeld, 1980; Lawler et al., 2009). Unfortunately, however, the characterization and consequences

of sarcopenia are still poorly studied for dogs, with no clear view of its pathogenesis and implications (Freeman, 2018).

In the past, protein restriction has been suggested for older dogs because of possible benefit in preserving renal function (Finco, 1992). However, it is currently suggested that this restriction is unnecessary in healthy elderly dogs that do not have renal impairment (Churchill, 2021), and that it may even be harmful due to the possible worsening of sarcopenia. It is recognized that with aging there is a decrease in the daily energy expenditure (DG) of dogs (Churchill, 2021; Larsen and Farcas, 2014). This reduction in energy expenditure results in lower consumption of food for constant weight and corresponding lower intake of proteins, amino acids and other nutrients. For this reason alone, increases in the protein content of the ration are already justified to compensate and maintain adequate nutritional intake in the elderly.

In addition, nutritional tables proposed for adults are used for the elderly (NRC, 2006), with no consolidated nutritional propositions for this age group so far. A study showed, however, a need for protein about 50% greater in elderly dogs compared to adults (Wannemacher and McCoy, 1966), and this possible greater need for protein or specific amino acids may be part of the development of sarcopenia. This is reinforced by the fact that senescence does not induce changes in protein digestibility in dogs, which remains unchanged with advancing age (Maria et al., 2017), with post-absorption utilization seeming to be a potentially relevant aspect in sarcopenia. A more recent publication employing the glycine-stable isotope end-product method, however, did not confirm this age effect with adult-like protein degradation and synthesis rates (Williams et al., 2001), so the matter is open and needs further investigation.

Among the possible alternatives to mitigate the effect of sarcopenia include branched-chain amino acids (ACR) and docosahexaenoic acid (DHA), already investigated in animal models and for humans (Lee, Jeon and Lee, 2020; , Murphy et al., 2010; Uojima et al., 2017). Among the ACR, leucine is the most studied, mainly aiming to improve the regenerative capacity of muscles, by regulating the turnover of proteins in skeletal muscle, which can result in lean mass gain or decrease its loss (Dodd and Tee, 2012; Li et al., 2011; Baptista et al., 2010). This also seems to attenuate the loss of muscle mass in elderly animals by possible decrease in nuclear apoptosis (Hao et al., 2011) and by increased proliferation of satellite cells (Alway et

al., 2014). It is known that mediators produced from DHA induce lower inflammation response and promote immune functions, facilitating defense and decreasing the pathological effect of organic inflammation (Bannenberg and Serhan, 2010; Serhan, Yacoubian and Yang, 2008). It is possible, therefore, that these alter the fluidity of the cell membrane, the functioning of its receptors and that the reduction of inflammation may have been beneficial in the recovery of muscle function in the elderly (Murphy et al., 2010, Wang et al., 2013, 2013).). Arginine, in turn, is an essential amino acid for dogs, with few studies in the species that have addressed the deleterious effects of diets free of the amino acid, both for growing and adult animals (Burns, Milner and Corbin, 1981; Hai, Milner et al. Corbin, 1978). Little is known about its benefits and synergism with other nutrients, however, arginine is essential for the occurrence of the urea cycle, which includes the maintenance, growth and renewal of protein-dependent tissues (NRC, 2006).

Given the above, and aiming to compare the body composition and protein metabolism of adult and elderly dogs, as well as to evaluate the effect of nutritional intervention on the protein metabolism of these animals, the objective of the present study was to evaluate the protein synthesis and degradation of dogs adults and elderly fed with two diets with similar chemical compositions, one control and the other supplemented with a blend of ACR, arginine and DHA.

Material and Methods

Local

The experiment was conducted at the Research Laboratory on Nutrition and Nutritional Diseases of Dogs and Cats “Prof. Dr. Flávio Prada”, from the Faculty of Agrarian and Veterinary Sciences, Universidade Estadual Paulista, Jaboticabal campus. All experimental procedures were previously judged by the Ethics Committee on the Use of Animals (CEUA) of the same institution, and approved under protocol n.º 009537/18.

Animals, housing and experimental design

Twenty beagle dogs (10.8±2.24 kg) belonging to the kennel of that laboratory were used, divided into 10 adults (2.3±1.2 years) and 10 elderly (12.7±2.6 years).

These were previously evaluated by a veterinarian, including physical examination and complementary blood count, urinalysis and serum biochemistry (alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, albumin, urea and creatinine), and were considered healthy for the study. The dogs' body condition score (BCS) was similar between groups (P=0.999), with a mean value at the beginning of the experiment of 5.0±0.4 for adults and 5.0±0.7 for the elderly, on stairs from 1 to 9 where 5 is ideal (Laflamme, 1997). The muscle condition score of the dogs differed, being lower in the elderly, with a mean value of 2.5±0.1 than for adults, with a mean value of 3.0±0.2 (P=0.043) on a scale where 3 represents absence of muscle loss (Freeman. et al., 2011). Thus, possible sarcopenia is evident in the elderly group at the beginning of the experimental period.

The study followed a 2 (ages) x 2 (diet) factorial arrangement, totaling 4 experimental treatments. This followed a cross-over design, so in each period half of the dogs randomly received a diet, which was inverted in the following period, totaling 10 dogs per combination of age and food. Each cross-over period lasted 6 months. Before the start of the cross-over period, the dogs' body composition was determined by the deuterium isotope method. These then received the designated experimental food for 6 months. At the end of the period, the body composition was determined again, as well as the rates of protein degradation synthesis by the stable isotope method of 13C-Phenylalanine. A washout period lasting 3 months was established between the crossover periods. Additionally, the coefficients of apparent digestibility of the nutrients of the experimental diets were calculated for each block, after two weeks of adaptation.

A control diet was formulated for dog maintenance (FEDIAF, 2018) and a test diet, with similar chemical composition but with 1.74-fold elevation of leucine, 1.24-fold isoleucine, 1.2-fold valine, 1-fold arginine. .37-fold and DHA 2.87-fold compared to the control. The analyzed composition of the rations is shown in Table 1. These were processed at the Feed Factory of the Faculty of Agrarian and Veterinary Sciences, FCAV/UNESP, Jaboticabal, SP, in its experimental extruder. All diets were mixed and ground with a hammer mill (Moinhos Tigre, São Paulo, Brazil) fitted with a 1-mm screen sieve before extrusion in a single-screw extruder with a processing capacity of 250 kg/h (MEX 250; Manzoni, Campinas, Brazil). The extrusion conditions were kept the

same for all treatments. During the extrusion process, the extruder motor amperage was constantly monitored and recorded every 10 minutes. After the extrusion system stabilized, bulk density (g/L) was determined every 10 minutes. The preconditioning temperature was maintained over 90°C through direct steam injection. To have good processed foods, water and steam additions in the extruder screw were adjusted according to each diet. After extrusion, kibbles were dried for 20 minutes in a forced air dryer at 105°C and coated with fat and palatability enhancers.

Table 9. Composição química analisada das dietas controle e teste fornecidas aos cães jovens e idosos (valores sobre a matéria seca).

Item	Diets	
	Control[1]	Test [2, 3]
Moisture (%)	7.2 ± 0.6 [4]	7.1 ± 0.3
Crude protein (%)	22.8 ± 0.7	21.4 ± 0.6
Ethereal extract acid hydrolysis (%)	15.5 ± 1.0	15.7 ± 0.6
Crude fiber (%)	2.8 ± 0.2	2.9 ± 0.5
Mineral matter (%)	6.2 ± 0.5	5.4 ± 0.6
Calcium (%)	1.2 ± 0.1	0.9 ± 0.2
Phosphor (%)	0.8± 0.1	0.7 ± 0.1

[1] - Lista de ingredientes: Arroz, milho, farinha de vísceras de frango, gordura de aves, glúten de milho 60%, polpa de beterraba, palatabilizante, fosfato bicálcico, celulose, óleo de peixe, cloreto de potássio, levedura de cerveja, DL-metionina, premix mineral e vitamínico, L-lisina, colina, sal comum, mananoligosacarídeo, inulina, propionato de cálcio, frutoligossacarídeo, L-triptofano, Vitamina C.

[2] – Lista de ingredientes: Arroz, milho, farinha de vísceras de frango, gordura de aves, proteína do soro de leite, glúten de milho 60%, polpa de beterraba, aditivo palatabilizante, fosfato bicálcico, Alga Prime (Schizochytrium sp, source of DHA), celulose, óleo de peixe, cloreto de potássio, levedura de cerveja, DL-metionina, premix mineral e vitamínico, L-lisina, L-arginina, L-leucina, L-valina, L-isoleucina, colina, sal comum, mananoligosacarídeo, inulina, propionato de cálcio, frutoligossacarídeo, L-triptofano, Vitamina C.

[3] – Teores de leucina 1,74 vezes maiores, isoleucina 1,24 vezes maiores, valina 1,2 vezes maiores, arginina 1,37 vezes maiores e DHA 2,87 vezes em relação à controle (valores analisados)

[4] – Média ± desvio padrão de dois lotes de produção

During the study the dogs were fed to maintain body weight. For this, they were initially fed based on their individual energy needs, established by the food history of each animal in the kennel, and considering the metabolizable energy of the food. The metabolizable energy of the food was established by its chemical composition and with the formula that considers the crude fiber contents (NRC, 2006). The daily amount of food was provided in a single daily meal. The amount supplied and leftovers were weighed, and consumption was recorded. The animals were then weighed every two weeks, always in the morning before the first meal, and the amount of feed provided was adjusted so that the dogs had a constant body weight throughout the experimental period.

The dogs were housed in pairs in pens measuring 1.5m x 4.0m with a solarium. They were released daily for 4 hours on a collective lawn for interaction and voluntary physical exercise. On days 16 to 20 of each period, to evaluate the digestibility of nutrients and nitrogen balance, the dogs remained restricted to individual stainless steel metabolic cages, measuring 0.9m x 0.9m x 0.9m, with a separation system. of feces and urine for collection. At the end of each period, the dogs remained restricted to the pens with access to the solarium to conduct the 13C-Phenylalanine method.

Apparent digestibility of nutrients, nitrogen balance and 24h renal urea excretion

The digestibility and nitrogen balance assay was performed using the total collection method of feces and urine, according to FEDIAF recommendations (2018). Feces and urine were quantitatively collected at least twice a day for 5 days (120 hours). Feces were weighed and frozen (-15°C). Urine was collected in a collection flask containing 4mL of 6N hydrochloric acid solution, the volume was quantified and stored frozen (-15°C) until analysis. At the end of the collection period, feces and urine were thawed and homogenized, composing one sample per animal. The stool samples were pre-dried in a forced ventilation oven (320-SE, FANEM, São Paulo) at 55°C for 72h. The pre-dried samples of feces and feed were ground in a knife mill (MOD 340, ART LAB, São Paulo), with a 1mm sieve. Feed and faeces were then analyzed according to the procedures described by the AOAC (2010) for dry matter, mineral matter, crude protein (LECO Corporation, St. Joseph, MI, USA) and ether extract in

acid hydrolysis. The gross energy content of diets and feces was determined using a bomb calorimeter (IKA Calorimeter System C 200, IKA-Werke GmbH & Co. KG, Staufen, Germany). Based on these results, the apparent digestibility of dry matter, organic matter, crude protein, ether extract acid hydrolysis and gross energy of the rations were calculated for both adult and elderly dogs, considering the calculation procedures described by FEDIAF (2018).

During collection, fecal samples were also scored according to the following system: 0 = watery liquid, which can be poured; 1 = soft, unformed; 2 = soft, malformed stool, which assumes the shape of a container; 3 = soft, formed, and moist, which retains shape; 4 = well-formed and consistent stool, which does not adhere to the floor; and 5 = hard, dry pellets, which are small and hard mass (Carciofi, et al., 2008).

To quantify urine nitrogen, the samples were thawed, homogenized and centrifuged at 3500 rpm for 15 minutes to precipitate possible feces and hair residues. Subsequently, they were analyzed in tin capsules (Tin Capsules 501-059, LECO Corporation, St. Joseph, MI, USA) in LECO equipment (AOAC, 1995; method 990.03; FP528, LECO Corporation, St. Joseph, MI, USA). Based on this procedure, the nitrogen balance was calculated by computing the N ingested – N feces – N urine. Urinary urea determination was performed using a commercial kit (Urea Nitrogen kit no. 535-B, Sigma, St. Louis, MO) in a spectrophotometer (Labquest, Labtest Diagnóstica S.A, Lagoa Santa, MG, Brazil).

Indirect method of amino acid oxidation (L-[1-13C] Phenylalanine)

To conduct the method, it is necessary to know the volume of CO2 produced by the animal. A respirometry system (Animal respirometry system, Sable System International, Las Vegas, USA) was used to determine the volume produced of CO_2 (VCO_2) of each dog. Dogs were housed in hermetically sealed chambers with internal control of temperature and moisture, with the dimensions of 1m x 1m x 1m (ALB 1000 CG Respirometry Chamber, Inbras Equipamentos para Saúde, Ribeirão Preto, Brazil). The chamber temperature was adjusted to 26±1 °C and the relative humidity of the air in the interval of 55% to 60%. Dogs were previously adapted to stay and be fed inside the chamber, avoiding stress that could interfere on results. Dogs were fasted for 24h, placed on the cages and fed with their usual amount of food. Water was provided ad

libitum. Each run latest 8 hours. According to previous data of pilot studies, 2 hours was waited to equilibrate the gasses inside the chambers.

Ambient air flows were introduced into the chambers by a negative pressure system, with an average flow of 10 L min^{-1}, with control and adjustments provided by a mass flow pump with a maximum flow capacity of 100.0 L min^{-1} (Sable Systems International, Las Vegas, Nevada, USA). The air flow volumes were established to maintain CO_2 saturations below 1.0% (average CO_2: 0.3±0.1% and average O_2: 20.4±0.2%) inside the chambers. Channel air sampling was performed by independent pressure gauges (RM-8 Gas Flow Multiplexer, Sable Systems International, Las Vegas, Nevada, USA). The air samples were routed by a subsampler (SS-4 Gas Analyzer Subsampler, Sable Systems International, Las Vegas, Nevada, USA) to a humidity meter (RH-300 Relative Humidity & Dew Point Analyzer, Sable Systems International, Las Vegas, Nevada, USA). The oxygen concentrations were measured by a paramagnetic analyser (PA-10, Sable System International, Las Vegas, Nevada, USA), and the CO_2 concentrations were measured with a carbon dioxide analyser (CA-10, Sable System International, Las Vegas, USA). Before measuring the gas concentrations, moisture was removed from the air by using anhydrous magnesium perchlorate (Exodus Científica, Sumaré, São Paulo, Brazil). The control and gauging of the gas metres were carried out using a standard air mixture (CO_2: 1.0031% and O_2: 21.00%; White Martins, Vinhedo, São Paulo, Brazil). The data obtained by the mass flow metre and gas analysers were collected by an ExpeData system (Sable System International, Las Vegas, Nevada, USA).

Before and at the end of the evaluations, the chambers and the respirometry system were validated for their gas recovery efficiency by burning Ethanol (Ethyl alcohol, Signma-Aldrich, Missouri, USA) for 430 minutes, assuming the combustion values proposed by Elia (1992). The volume of ethanol burned (initial weighing - final weighing), the inflow and the outflow of air was considered and the volumes of O_2 and CO_2 that should have been detected by the device were calculated. Based on these values, the recovery percentage was established. To validate the system a CO_2 recovery greater than 95% was established.

The VCO_2 produced by each dog was determined by the following equation (Lighton, 2008):

$$VCO_{2(mL \cdot min^{-1})} = FR_e \frac{[(F_eCO_2 - F_iCO_2) + F_iCO_2(F_iO_2 - F_eO_2)]}{(1 + F_iCO_2)}$$

Where FR_e is the excurrent flow rate, F_e is the fractional concentration of a gas in the excurrent airstream, and F_i in the incurrent airstream.

After obtaining the VCO_2, dogs were housed individually in metabolic cages equipped with apparatus to separate feces and urine for collection, measuring 0.9 m × 0.9 m × 0.9 m. After 24h fasting animals were fed with their usual amount of food divided in four equal meals, and fed each amount at every 20 min until complete the daily food intake. Only dogs that ate all offered food was tested. Dogs that did not complete the meal was tested on the other day.

Immediately after completed the meal, dogs received a prime dose of 0.7 mg/kg of ^{13}C Phenylalanine and 0.2 mg.kg^{-1} of ^{13}C Soidum Bicarbonate (Cambridge Isotope Laboratories, Tewksbury, US) supplied orally in gelatin capsules. After, dogs were orally dosed every 30 minutes four times with 1.3 mg/kg of ^{13}C Phenylalanine in gelatin capsules (Figure 2).

Item	Minutes after isotope administration							
	0	30	60	90	120	150	180	210
Food intake								
^{13}C-Phe infusion	●	●	●	●	●			
$NaH^{13}CO_3$ infusion	■							
Expired gas collection	♠	♠	♠	♠	♠	♠	♠	♠

Figure 4. Sampling protocol of the ^{13}C-Phenylalanine method.

Samples of expired gas were collected prior to enrichment, and 30, 60, 90, 120, 150, 180 and 210 min of the priming dose administration. At each sampling time expired gas was collected using a specific designed mask for at least 2 minutes, directly into a glass vial coupled to the mask and stored at room temperature for latter analysis.

Whole body protein turnover

The flux of labeled compounds and rates of protein synthesis and breakdown were calculated according to the model proposed by Picou and Taylor-Roberts (1969):

$$\mathbf{Q} = \mathbf{O} + \mathbf{S} = \mathbf{I} + \mathbf{B}$$

Where Q is tracer flux (g.kg^{-1}.d^{-1}); O is the oxidation of the amino acid measured through the expired gas (not considered in the other tracers); S is the protein synthesis rate (g.kg^{-1}.d^{-1}); I is the N intake from the diet (g.kg^{-1}.d^{-1}); and B is the protein breakdown rate (g.kg^{-1}.d^{-1}).

$$\mathbf{Q\ leucine}\ (\mathbf{g}.\boldsymbol{kg^{-1}}.\boldsymbol{d^{-1}}) = \left(\boldsymbol{D} * \left(\left(\frac{\boldsymbol{X^{E}_{(13C)Leucine}}}{\boldsymbol{X^{E}_{(13C)Plasma}} * \mathbf{1.33}} - \mathbf{1}\right)\right)\right)\boldsymbol{MW} * \mathbf{10^{6}} * \mathbf{24}$$

$$\mathbf{Q\ glycine}\ (\mathbf{g}.\boldsymbol{kg^{-1}}.\boldsymbol{d^{-1}}) = \left(\boldsymbol{D} * \left(\left(\frac{\boldsymbol{X^{E}_{(15N)Glycine}}}{\boldsymbol{X^{E}_{(15N)Urine}}} - \mathbf{1}\right)\right)\right)\boldsymbol{MW} * \mathbf{10^{6}} * \mathbf{24}$$

$$\mathbf{Q\ phenylalanine}\ (\mathbf{g}.\boldsymbol{kg^{-1}}.\boldsymbol{d^{-1}}) = \left(\boldsymbol{D} * \left(\left(\frac{\boldsymbol{X^{E}_{(13C)Phenylananine}}}{\boldsymbol{X^{E}_{(13C)Gas/Urine}}} - \mathbf{1}\right)\right)\right)\boldsymbol{MW} * \mathbf{10^{6}} * \mathbf{24}$$

Where Q is the tracer flux (g.kg^{-1}.d^{-1}), D is the tracer infusion rate (µmol.kg-1.h-1); $X^{E}_{(tracer)}$ is the absolute enrichment of the tracer; $X^{E}_{(sample)}$ is the atom percent in excess and represents the enrichment in the steady state; MW is the molar weight of the tracer, which together the multiplication by 10^{6} is used to transform µmol to g; 24 is to transform hour in day (Adapted from Mathews et al., 1980).

S (g.kg^{-1}.d^{-1}) = Q - O

Protein synthesis (S) for ^{13}C leucine or ^{13}C phenylalanine was calculated from the flux (Q) and oxidation (O) data. In the case of urine for the ^{15}N glycine and ^{13}C phenylalanine tracers it equals the flux (Q), provided the other parameters are calculated in the enrichment plateau (Mathews et al., 1980).

B (g.kg^{-1}.d^{-1}) = Q - I

Protein breakdown (B) was calculated from the difference between the flux (Q) of ^{13}C leucine or ^{15}N glycine or ^{13}C phenylalanine and the intake of the amino acids (I) in the case of glycine or phenylalanine (when the animals were fed) or equals the isotope dose for ^{13}C leucine (when the animals were fasted) (Mathews et al., 1980).

$$\mathbf{O\ (g.kg^{-1}.d^{-1})} = F^{13}CO_2 \left(\frac{1}{X^E_{(13C)tracer}} - \frac{1}{X^E_{(13C)sample}} \right) * 100$$

The oxidation (O) of ^{13}C phenylalanine and ^{13}C leucine was calculated with this equation, where: $X^E_{(tracer)}$ is the absolute enrichment of the tracer; $X^E_{(sample)}$ is the atom percent in excess and represents the enrichment in the steady state; $F^{13}CO_2$ represents the rate of $^{13}CO_2$ released by the tracer oxidation (g $^{13}CO_2.kg^{-1}.d^{-1}$), calculated by the following equation:

$$\mathbf{F^{13}CO_2\ (g.kg^{-1}.d^{-1})} = \left(\frac{FCO_2 * ECO_2}{BW} \right) * \left(\frac{60 * 41.6}{100 * 1} \right) * (MW * 24)$$

Where FCO_2 is the CO_2 production rate of the animal determined in respirometry chambers ($mL.min^{-1}$), ECO_2 is the $^{13}CO_2$ enrichment plateau in the expired gas (APE); BW is the weight of the animal (kg). The constants 41.6 $\mu mol.ml^{-1}$ and 60 $min.h^{-1}$ convert FCO_2 to $mol.h^{-1}$ and the factor 100 changes the plateau to a fraction. The factor 1.0 accounts for the $^{13}CO_2$ retained in the body because of bicarbonate fixation (Hoerr et al., 1989). MW is the molar weight of the tracer, which together the multiplication by 10^4 is used to transform µmol to g. The 24 is to transform hour in day (Adapted from Mathews et al., 1980).

Body composition determination

To determine body composition an isotope solution was prepared at a concentration of 7%, mixing 930 mL of saline solution and 70 mL of deuterium oxide (Sercon Limited, Unit 3B Crewe Trade Park, Gateway, Crewe, Cheshire, UK; 99% purity). A dosage of 1 mL/kg body weight was subcutaneous applied between the scapula, after 21 hours fasting and 2 hours without water (FERRIOLLI et al., 2008). To increase the accuracy on inoculation, the empty syringe, the syringe with the isotope solution, and the syringe after solution inoculation were weight in a scale.

Blood samples were collected before inoculation and after 2 h of isotope application (GOLONI, 2018). The isotope concentration on body fluids was evaluated at the Mass Spectrometry Laboratory of the Ribeirão Preto Medical School, São Paulo, Brazil. Deuterium was analyzed by isotope ratio mass spectrometry (ANCA 20-20, Europe Scientific, UK), following procedures described by Ferriolli et al., (2008).

Samples were processed in triplicate (150 µL per replicate) with platinum in vacutainers, after six hours resting.

The results for deuterium to determine the total body water, were calculated according Schoeller et al. (1996); Racette et al., (1994) and Ellis and Wong (1998). The lean mass of the dogs was calculated considering the hydration constant of 73.2% for mammals, by the equation: Lean mass (kg) = body water (kg)/0.732. The fat body mass (FM; kg) was estimated as: Total body mass (kg) – lean mass (kg) of the animal (Pace & Rathbun, 1945).

Statistical analysis

The study considered the individual dog as the experimental unit. The sampling size was established based on the results of analysis of variance for protein oxidation by the ^{13}C-Phenilalanie method obtained in chapter two, considering the factorial arrangement of treatments and the cross-over design. The test power was set at 0.8 (procedure Opdoe of the R software), $\alpha = 0.05$, standard deviation = 1.03, and a standard error of approximately 0.18. The analysis was performed with the sample size procedure of the R software, and a sample size of 8 dogs was obtained. The error normality and homoscedasticity presuppositions were previously verified (two dogs more per diet was included accounting for possible outliers or the necessity to remove dogs from the study). Data was evaluated in a 2 (diets) x 2 (ages) factorial arrangement, totaling 4 treatments, in a cross-over design. Means was submitted to variance analysis considering the effects of period, age, diet, and age x diet interaction. When differences were found on F test, means was compared by Tukey test ($P<0.05$). To compare the body weight and body composition of the dogs inside each diet at the start and end of each period, a paired T-test was performed ($P<0.05$). The analysis was performed on SAS software 9.1 using the Proc MIXED (SAS Institute, Cary, NC, USA, 2003).

Results

During the experiment animals did not present any problems related to the consumption of the diets. The two formulations were adequately accepted and

consumed by the dogs. The dogs were well adapted to the experimental and sampling procedures, not presenting any visible sign of stress. Considering the relatively long experiment, with two periods of six months, some dogs in both age groups presented isolated episodes of vomiting or food refusal, nothing that interfered with the experiment protocol. The analyzed composition of the diets was similar, and very close to the target for the experiment.

Nutrient digestibility and nitrogen balance

Food and nutrient intake during digestibility test was similar between foods and ages (P>0.05), as shown on Table 10. The total tract apparent digestibility of the nutrients was also similar between diets and age groups (P>0.05).

Table 10. Nutrient intake during the digestibility test and coefficient of total tract apparent digestibility of experimental diets to adult and old dogs.

Item	Diets		Mean	SEM[2]	P value		
	Control	Test [1]			Age	Diet	Age x Diet
Intake (g.Kg$^{-0.75}$.d^{-1})							
Dry matter							
Adult	35.3	34.6	35.0	1.41			
Old	35.5	37.6	36.5	1.03			
Mean	35.4	36.1			0.364	0.699	0.412
Organic matter							
Adult	20.0	21.8	20.9	0.31			
Old	20.3	21.6	20.9	0.29	0.912	0.686	0.506
Mean	20.2	21.7					
Crude fiber							
Adult	4.8	4.3	4.5	0.10			
Old	4.5	4.8	4.6	0.11	0.741	0.890	0.311
Mean	4.6	4.6					
Crude protein							
Adult	6.3	5.7	6.0	0.36			
Old	6.5	6.1	6.3	0.10	0.210	0.056	0.642
Mean	6.4	5.9					
Fat							
Adult	1.8	2.0	1.9	0.15			

	Old	1.9	2.0	1.9	0.24	0.910	0.295	0.910
	Mean	1.8	2.0					
Total tract apparent digestibility (%)								
Dry matter								
	Adult	86.4	86	86.2	0.29			
	Old	86	86.3	86.1	0.34	0.921	0.897	0.571
	Mean	86.2	86.1					
Organic matter								
	Adult	89.7	88.4	89	0.29			
	Old	89.2	88.7	88.9	0.31	0.883	0.083	0.483
	Mean	89.4	88.5					
Fat								
	Adult	93.9	93.7	93.8	0.33			
	Old	93.5	95.4	94.3	0.66	0.482	0.343	0.247
	Mean	93.7	94.5					
Crude protein								
	Adult	86.1	87	86.6	0.42			
	Old	85.2	86.5	85.8	0.48	0.364	0.188	0.803
	Mean	85.6	86.7					
Crude fiber								
	Adult	35.6	29.9	33	1.71			
	Old	37.9	35.8	36.9	1.65	0.200	0.225	0.573
	Mean	36.8	33.1					
Gross energy								
	Adult	89.5	88.8	89.1	0.28			
	Old	89.2	89.1	89.1	0.31	0.987	0.531	0.563
	Mean	89.3	88,9					

[1] – Values of leucine 1.74 times greater, isoleucine 1.24 times greather, valine 1.2 times greather, arginine 1.37 times greater and DHA 2.87 times greater than control (analyzed values).

[2] – SEM: standard error of the means (n = 10 dogs per treatment).

Nitrogen balance results was also similar, not differing among age groups or diets, (P>0.05), as presented on Table 11.

Table 11. Nitrogen Balance of old and adult dogs fed the experimental diets.

Item	Diet		Mean	SEM[2]	P Value		
	Control	Test [1]			Age	Diet	Age x Diet
	mg.Kg$^{-0,75}$.day^{-1}						
N intake							

Adult	1037.7	922.3	980.0	39.0			
Old	990.1	982.5	985.0	32.6	0.906	0.317	0.256
Mean	1008.3	946.9					
N feces							
Adult	142.1	179.4	160.8	10.7			
Old	147.5	143.9	145.1	5.2	0.227	0.107	0.179
Mean	143.1	167.8					
N urine							
Adult	570.1	521.5	545.8	33.5			
Old	480.5	522.8	508.7	33.2	0.389	0.376	0.951
Mean	544.9	506.6					
N retido							
Adult	325.5	302.9	296.9	35.0			
Old	362.2	315.8	331.3	38.1	0.306	0.649	0.242
Mean	320.3	304.6					

[1] – Values of leucine 1.74 times greater, isoleucine 1.24 times greather, valine 1.2 times greather, arginine 1.37 times greater and DHA 2.87 times greater than control (analyzed values).

[2] – SEM: standard error of the means (n = 10 dogs per treatment).

Higher feces production on as-is basis and feces moisture (P<0.05) were observed for old dogs, and higher feces moisture was observed for dogs fed the test diet, regardless of the age group (P<0.05). Although feces score was adequate for all dogs, for both diets the mean scores were lower for old than adult dogs (P<0.05).

Table 12. Feces production and characteristics of adult and old dogs fed the experimental diets.

Item	Diets		Mean	SEM[2]	P value		
	Control	Test[1]			Age	Diet	Age x Diet
Fecal production (g.Kg$^{-0,75}$.day^{-1})							
Adult	10.23	10.88	10.5	0.40			
Old	11.11	12.53	11.8	0.42	0.031	0.071	0.496
Mean	10.7	11.7					
Fecal production (g.Kg$^{-0,75}$.day^{-1},dry matter)							
Adult	3.96	3.99	4.0	0.09			
Old	4.03	4.08	4.1	0.09	0.652	0.818	0.959

Mean	4.0	4.0					
Fecal dry matter (%)							
Adult	39.04	35.73	37.4	0.60			
Old	34.89	31.81	33.4	0.60	<0.001	<0.001	0.8978
Mean	36.8	33.4					
Score							
Adult	3.97	4.00	3.99	0.03			
Old	3.90	3.91	3.91	0.03	0.050	0.579	0.853
Mean	3.93	3.96					

[1] – Values of leucine 1.74 times greater, isoleucine 1.24 times greather, valine 1.2 times greather, arginine 1.37 times greater and DHA 2.87 times greater than control (analyzed values).

[2] – SEM: standard error of the means (n = 10 dogs per treatment).

The body weight and body composition of the dogs at start and after 6 months of diet intake are presented on Table 13. Old dogs presented a constant body weight, fat body mass and lean body mass along the study (P>0.05), however adult animals gained body weight and lean mass on both diets (P<0.05), and fatty mass on control diet (P<0.05). These resulted in no alteration on lean mass % or fatty mass % in old dogs (P>0.05), but for adults increased in fatty mass % and reduction in lean mass % was observed for control diet intake (P<0.05), but not for test diet consumption (P>0.05). When the diet effect was observed inside a group of dog age, no difference in body weight, fatty mass (kg), fatty mass %, or lean mass % was observed (P>0.05). However, a tendency for higher lean mass (kg) after the intake of the test diet was observed for old dogs in comparison to the intake of the control food (P=0.06), suggesting an increase on lean mass in comparison to the control diet in this age group. In addition, an age effect regardless of time was observed for body weight in the test diet, as old dogs were heavier than adults (P<0.05). The fat mass content (kg) and fatty mass % was higher in old than adult dogs, regardless of diet (P<0.01), and although the lean mass content (kg) was similar (P>0.05), the proportion of lean mass in % was lower for old dogs (P<0.01) characterizing the presence of sarcopenia in this age group.

Table 13. Body weight and body composition of old and adult dogs at the beginning (Initial) and after 6 months (Final) of intake of the experimental diets.

Item	Diet		SEM [2]	P value
	Control	Test [1]		
Body Weight (kg)				
Old Dogs				
Initial	11.92	12.28	0.23	*0.725*
Final	12.22	12.91	0.24	*0.123*
P value	*0.201*	*0.111*		
Adult Dogs				
Initial	10.31	10.84	0.35	*0.462*
Final	11.38	11.36	0.40	*0.985*
P value	*0.006*	*0.019*		
Age effect	*0.276*	*0.024*		
Fat Body Mass (kg)				
Old Dogs				
Initial	2.51	2.49	0.20	*0.408*
Final	2.66	2.57	0.15	*0.907*
P value	*0.326*	*0.342*		
Adult Dogs				
Initial	0.78	1.25	0.14	*0.086*
Final	1.29	1.40	0.13	*0.694*
P value	*0.023*	*0.608*		
Age effect	*<0.001*	*<0.001*		
Lean Body Mass (kg)				
Old Dogs				
Initial	9.43	9.64	0.25	*0.754*
Final	9.56	10.14	0.19	*0.063*
P value	*0.532*	*0.316*		
Adult Dogs				
Initial	9.53	9.59	0.38	*0.939*

Final	10.09	9.97	0.33	*0.857*
P value	*0.042*	*0.032*		
Age effect	*0.389*	*0.601*		
Fat Body Mass (%)				
Old Dogs				
Initial	*21.07*	*20.68*	*1.64*	*0.465*
Final	*21.64*	*19.84*	*1.06*	*0.691*
P value	*0.582*	*0.408*		
Adult Dogs				
Initial	*7.84*	*11.76*	*1.39*	*0.163*
Final	*11.12*	*12.03*	*0.96*	*0.648*
P value	*0.037*	*0.955*		
Age effect	*<0.001*	*<0.001*		
Lean Body Mass (%)				
Old Dogs				
Initial	*78.93*	*79.32*	*1.64*	*0.465*
Final	*78.36*	*80.16*	*1.06*	*0.691*
P value	*0.582*	*0.408*		
Adult Dogs				
Initial	*92.16*	*88.24*	*1.39*	*0.160*
Final	*88.88*	*87.97*	*0.96*	*0.647*
P value	*0.043*	*0.955*		
Age effect	*<0.001*	*0.003*		

[1] – Values of leucine 1.74 times greater, isoleucine 1.24 times greater, valine 1.2 times greater, arginine 1.37 times greater and DHA 2.87 times greater than control (analyzed values).

[2] – SEM: standard error of the means (n = 10 dogs per treatment).

The results of protein synthesis, degradation and flux are presented on Table 14. A diet effect with higher values for dogs fed the test food was observed (P<0.05), without effect of age (P>0.05). In the adult dogs group an increase of 58% on protein synthesis and 63% on breakdown was observed after the intake of the treatment diet. For old dogs protein synthesis and breakdown increased 10% comparing the test and

control diets. As no age*diet interaction was observed (P=0.217) the diet effect although less pronounced was also significant of old dogs.

Table 14. Protein synthesis and breakdown of old and adult dogs fed the experimental diets. Values estimated with the indirect amino acid oxidation method with ^{13}C-Phenylalanine.

	Diet			P value		
	Control	Test[1]	SEM[2]	Age	Diet	Age x Diet
Synthesis (g of protein.kg$^{-0,75}$.d^{-1})						
Adult	3.43	5.62	0.42			
Old	2.72	3.75	0.29	*0.0934*	*0.0461*	*0.5540*
Mean	3.10	4.10				
Breakdown (g of protein.kg$^{-0,75}$.d^{-1})						
Adult	3.39	5.58	0.45			
Old	2.68	3.71	0.29	*0.0939*	*0.0463*	*0.5565*
Mean	3.07	4.06				

[1] – Values of leucine 1.74 times greater, isoleucine 1.24 times greater, valine 1.2 times greater, arginine 1.37 times greater and DHA 2.87 times greater than control (analyzed values).

[2] – SEM: standard error of the means (n = 10 dogs per treatment).

Discussion

Although nitrogen balance is a classic standard for protein studies in dogs and cats, it is known that maintenance of lean body mass or measures of protein synthesis and degradation can provide better indicators for the physiological status of the animal, as well as the possibility adequacy of protein supply to the animal (Laflamme, 2005).

When dietary protein intake is insufficient, the immediate physiological response is to reduce the rates of protein synthesis and degradation and mobilize lean mass to support these rates, and recycling occurs. Animals under healthy conditions can adapt to low protein intake and maintain nitrogen balance even in a depleted state, but they have a reduced ability to respond to infections, toxic substances, and other negative stimuli (Wannemacher and McCoy, 1966).

In addition to the direct effect of protein intake, aging also has a negative effect on protein turnover. In a review published by Richardson and Birchenall-Sparks (1983), about 85% of the studies brought the relationship between age and the decline in endogenous protein synthesis. In healthy animals, mild protein deficiency or limitation of certain amino acids can impair immune function, effects that may be more pronounced in older dogs due to the natural loss of lean mass caused by sarcopenia (Lau and McMurray, 1999; Yoshino et al. , 2003).

The consumption of the ACR+DHA diet induced an increase of approximately 59% in the rates of protein synthesis and degradation in adult animals and an increase of 10% in these parameters in the elderly, indicating a greater stimulus to protein renewal in relation to the CO diet (P=0.045; Table 14). In the elderly group, although this increase was more discreet, there was no age*ration interaction (P=0.217), reinforcing the effect of the ACR+DHA diet also for this age group. Garlick (2005) described that infusion of branched-chain amino acids increases protein synthesis as efficiently as complete mixing of all amino acids, suggesting that, although the diets are isoprotein, the increase in ACR stimulated protein turnover and elevation. of synthesis and degradation rates.

However, the effect of mixing these three amino acids on protein turnover can be attributed mainly to leucine (Li and Jefferson, 1978). Pereira et al. (2014) demonstrated that leucine supplementation in young rats improves the recovery of myofiber size and contractile function of regenerating young skeletal muscles after cryoinjury, accompanied by a decrease in the accumulation of ubiquitinated proteins, but in relation to the elderly, this improvement was more subtle (Pereira et al., 2015). Leucine has also been shown to regulate protein turnover in skeletal muscle, promoting mass gain (Dodd and Tee, 2012; Li et al., 2011) and decreasing mass loss (Baptista et al., 2010).

Wei et al. (2013) studied the effect of DHA on protein metabolism in the fed and fasted states in humans. It has been shown that there are differences in the fed state, in which the effect of supplementation depends on hormonal and nutritional stimulation. This DHA carrier response is reinforced by what was found in our study in association with branched-chain amino acids.

When it comes to aging, there is an increase in the production of free radicals in various tissues, which can promote systemic cellular apoptosis and acceleration of physiological dysfunctions, variations in omega-3 status have a strong impact on muscle strength and functionality in the elderly (Liu, Chen, & Hong-Guang, 2018). Branched-chain amino acids help improve performance for high-performance athletes, however, their supplementation combined with DHA and arginine can assist in protein renewal by improving synthesis and degradation parameters for the maintenance of lean mass and aid in the longevity of these animals.

Conclusions

The studied old dog group presented sarcopenia, as animals demonstrated the same body condition score, but old animals lower lean mass % and higher fatty mass % than adults. For adult and old dogs, the consumption of the diet supplemented with branched-chain amino acids, arginine, and docosahexaenoic acid stimulated protein renovation, with higher rates of protein synthesis and breakdown. This might explain the tendency for higher lean mass (kg) in old dogs fed the test diet, and the preservation of body composition in adult dogs fed the test diet, as when fed the control food these animals presented an increase in fatty mass % and reduction in lean mass %. Long term studies with different dosages of the nutrient combination tested might allow a better comprehension of its role in delaying sarcopenia development in old dogs.

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Capítulo 5 – Considerações Finais

A sarcopenia é uma condição natural que ocorre com o envelhecimento, caracterizada pela perda de massa magra independente de doença, pouco estudada em cães, visto que a população tem se tornado longeva há pouco tempo devido aos avanços em nutrição, saúde e tecnologias. A nutrição é uma importante ferramenta para a manutenção de massa magra, aminoácidos-chave como leucina, isoleucina, valina e arginina podem ser utilizados a fim de estimular a renovação de proteína e favorecer essa condição. Estudos com metabolismo de proteína com cães são escassos e separados através das décadas e pouco se entende sobre as diferenças entre jovens adultos e animais idosos. Muito se fala sobre necessidades de aminoácidos, nutrição de precisão e proteína ideal, porém, a aplicação desses conceitos ainda se encontra distante devido às ferramentas não-invasivas para tais determinações estarem ao alcance da pesquisa nos últimos anos.

Este cenário motivou a proposta da presente pesquisa. Iniciando por um piloto para verificar quais os gargalos a serem resolvidos para os estudos principais, onde foram utilizados 6 animais idosos e foram aplicados os 4 métodos de avaliação de turnover proteico utilizando 3 traçadores (^{13}C-Phe, ^{13}C-Leu e ^{15}N-Gly). Os resultados foram bastante interessantes, os resultados de síntese e degradação foram semelhantes em todas as metodologias, com algumas diferenças numéricas, creditadas à forma de coleta.

No segundo estudo, já melhor desenhado após o piloto, foram abordadas duas idades (jovens adultos e idosos) consumindo a mesma dieta, utilizando os mesmos 4 métodos de avaliação de turnover proteico com 3 traçadores (^{13}C-Phe, ^{13}C-Leu e ^{15}N-Gly). Foi observada uma diferença entre idades, mas sem diferença entre métodos, demonstrando que qualquer uma das metodologias pode ser utilizada para estudos. Outro resultado interessante foi que, mesmo com a diferença observada entre idades, não foi observada diferença no balanço de nitrogênio, comprovando que essa última metodologia pode não ter sensibilidade para definir parâmetros nutricionais entre jovens e idosos.

No terceiro estudo, o turnover proteico foi medido por apenas uma metodologia e um traçador (^{13}C-Phe), com dieta suplementada com aminoácidos de cadeia ramificada (Leu:Iso:Val), Arg e ácido docosaexaenoico. Foi observado que essa dieta

ajudou na manutenção de massa magra ao longo do tempo tanto para jovens quando idosos e melhorou a renovação de proteína de maneira expressiva para jovens adultos e sutilmente para idosos.

Como informações relevantes, esse trabalho traz um compilado metodológico para definições de estudos com proteína em cães demonstrando sua sensibilidade perante mudanças na dieta e idade do animal.