UNIVERSIDADE ESTADUAL PAULISTA
INSTITUTO DE BIOCIÊNCIAS
CAMPUS DE BOTUCATU
Pós-graduação em Ciências Biológicas (Zoologia)

FLEXIBILIDADE FENOTÍPICA DO TRATO DIGESTÓRIO: EFEITOS DA RESTRIÇÃO ALIMENTAR E REALIMENTAÇÃO EM FRANGOS

Cristiane Regina do Amaral Duarte

Botucatu-SP
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Tese de doutorado apresentada ao Programa de Pós-graduação em Ciências Biológicas (Zoologia) do Instituto de Biociências da Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutor.

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Dedico este trabalho aos meus pais

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DEFICIÊNCIAS

“DEFICIENTE” é aquele que não consegue modificar sua vida, aceitando as imposições de outras pessoas ou da sociedade em que vive, sem ter consciência de que é dono do seu destino.

“LOUCO” é quem não procura ser feliz com o que possui.

“CEGO” é aquele que não vê seu próximo morrer de frio, de fome, de miséria. E só tem olhos para seus míseros problemas e pequenas dores.

“SURDO” é aquele que não tem tempo de ouvir um desabafo de um amigo, ou o apelo de um irmão. Pois está sempre apressado para o trabalho e quer garantir seus tostões no fim do mês.

“MUDO” é aquele que não consegue falar o que sente e se esconde por trás da máscara da hipocrisia.

“PARALÍTICO” é quem não consegue andar na direção daqueles que precisam de sua ajuda.

“DIABÉTICO” é quem não consegue ser doce.

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SUMÁRIO

Resumo

A restrição alimentar é comumente utilizada na produção de frangos para melhorar o desempenho e qualidade da carcaça e prevenir problemas reprodutivos, metabólicos e esqueléticos. Esta prática, no entanto, afeta diferentes sistemas do organismo, incluindo o trato gastrointestinal. Os efeitos da restrição alimentar aplicada durante a primeira ou segunda semana de vida em frangos sobre a morfologia do trato gastrointestinal são bem conhecidos. No entanto pouco se conhece sobre seus efeitos nos processos de digestão e absorção de nutrientes. Além disso, não há relatos na literatura sobre os efeitos da restrição alimentar e realimentação em outras fases do desenvolvimento de frangos. Assim, este estudo investigou os efeitos da restrição alimentar e da realimentação sobre o trato gastrointestinal de frangos nas fases inicial (7 dias de idade) e final (35 de idade) do desenvolvimento. Para tanto, utilizou-se 4 grupos experimentais: **C7**: alimentado *ad libitum* durante 7 dias, **R**: 70% restrito durante 7 dias, **C10**: alimentado *ad libitum* durante 10 dias e **RF**: 70% restrito durante 7 dias e realimentado por 3 dias. Ao final dos períodos de restrição e de realimentação os animais foram sacrificados e os seguintes parâmetros foram analisados: peso dos órgãos do trato gastrointestinal, atividade de enzimas pancreáticas e intestinais, expressão gênica de enzimas e de transportadores de nutrientes, quantidade da proteína SGLT1 (cotransportador Na^+/glicose) e de captação de glicose através da membrana apical pelo cotransportador SGLT1. Os resultados mostraram que o peso corporal de frangos restritos durante a fase inicial foi mantido e que o de animais restritos na fase final apresentou pequena perda. No entanto, animais restritos apresentaram diminuição do peso dos órgãos do trato gastrointestinal e da atividade de enzimas pancreáticas e intestinais. A expressão gênica de enzimas e transportadores intestinais aumentou nos animais restritos enquanto que a quantidade e atividade do transportador de glicose

SGLT1 diminuíram. Após realimentação por 3 dias, os parâmetros morfológicos e funcionais analisados apresentavam níveis semelhantes ao do grupo controle, com poucas exceções. Além disso, durante o período de realimentação, os animais previamente restritos apresentaram melhor eficiência alimentar e maior ganho de peso. Estes efeitos podem ser atribuídos à maior ingestão relativa de alimentos, à recuperação da atividade de enzimas e do transportador SGLT1, além da maior capacidade de estocagem de alimento pelo proventrículo e moela. Assim, as alterações observadas em resposta à restrição alimentar podem ser consideradas adaptativas para o animal, desde que poupam o gasto energético de manutenção do trato gastrointestinal no período de baixa ingestão de alimento. Tais alterações são rápidas e reversíveis, visto que após 3 dias de realimentação os parâmetros avaliados recuperaram os níveis apresentados pelo grupo controle.

Palavras-chave: digestório, frango, restrição alimentar, realimentação.

Abstract

Feed restriction is commonly used in chicken production to improve the performance and the carcass quality and to control reproductive, metabolic and skeletal disorders. However, this management affects different systems of the organism, including the gastrointestinal tract. The effects of feed restriction applied in the first or second week after hatching on the gastrointestinal morphology are well known, but there are few reports about their effects on the nutrient digestion and absorption. Moreover, there are not studies concerning the effects of feed restriction and realimentation in the other phases of chicken growth. Thus, this study investigated the effects of feed restriction and realimentation on the chicken gastrointestinal tract in the initial (7 days old) and final (35 days old) phase of growth. For this, the animals were divided into 4 experimental groups: **C7**: food *ad libitum* for 7 days, **R**: 70% food restriction for 7 days, **C10**: food *ad libitum* for 10 days and **RF**: 70 % food restriction for 7 days, followed by realimentation for 3 days. Animals were sacrificed in the end of each experimental period (7^{th} and 10^{th} days, according to the group) and the following parameters were evaluated: gastrointestinal organs weight, pancreatic and intestinal enzymes activities, gene expression of enzymes and nutrient transporters, abundance of Na^{+}-D-glucose cotransporter (SGLT1) and D-glucose uptake through the brushborder membrane by SGLT1. The results showed that the younger feed-restricted chickens maintained the body weight and that the older feed-restricted chickens slightly decreased the body weight. However, feed-restricted animals decreased the gastrointestinal organs weight and pancreatic and intestinal enzymes activities. The gene expression increased in the feed-restricted chickens whereas the SGLT1 abundance and activity decreased. After 3 days of reestablishment of feeding, the morphological and functional parameters presented similar level to the control group, with few exceptions. Moreover, during the

realimentation period, previously feed-restricted chickens presented a higher feed efficiency and body weight gain. These effects can be attributed to the higher relative feed intake, to the recovery of the enzymes activity and to the D-glucose uptake and to the higher food storage capacity of the proventriculus and gizzard. Thus, the presented changes in response to feed restriction can be considered adaptive for the animal, considering that they decrease the maintenance costs of the gastrointestinal tract during the feed restriction period. These changes are rapid and reversible, taking into account that three days after the reestablishment of feeding the evaluated parameters reach the level of the control group.

Key-words: chicken, digestive, feed restriction, realimentation

Introdução

Os animais enfrentam constantemente variações ambientais e fisiológicas e apresentam mecanismos de ajustes que garantam a homeostase e a sobrevivência. Esta capacidade de ajuste, chamada de flexibilidade fenotípica, é realizada através de mudanças comportamentais, morfológicas e fisiológicas (Overgaard *et al.*, 2002; Palacios & Bozinovic, 2003; Naya *et al.*, 2003).

Entre os desafios a serem enfrentados, estão as variações na disponibilidade e na qualidade dos alimentos. Nestas circunstâncias, os processos de digestão e absorção ajustam-se para manter a taxa de digestão, e, por conseguinte, o fornecimento de nutrientes e energia para todos os sistemas dos organismos (Diamond, 1991; Secor, 2001). De fato, a manutenção das funções do trato gastrointestinal é muito importante para crescimento, desenvolvimento e saúde dos animais.

A restrição alimentar e o jejum são eventos que podem ocorrer na vida dos animais naturalmente, em função da menor disponibilidade de alimentos na natureza, ou induzida pelo manejo, como o imposto a frangos e codornas com o objetivo de reduzir desordens metabólicas e problemas reprodutivos ou, ainda, para o controle do peso corporal (Nir *et* al., 1996; Tottori *et al*., 1997; Lee & Leeson, 2001; Camacho *et al*., 2004). No entanto, a restrição alimentar desencadeia respostas importantes nos processos de digestão e absorção de nutrientes, uma vez que afeta o trato gastrointestinal.

De fato, a restrição alimentar causa atrofia da mucosa, diminuição do número de células, da taxa de proliferação celular e aumento da apoptose celular (Susbilla *et al.*, 2003). Conseqüentemente, ocorre diminuição no peso dos órgãos do trato digestório, na

atividade de enzimas intestinais e pancreáticas e no número de transportadores intestinais (Palo *et al*., 1995a, b; Karasov & Pinshow, 1998).

A atividade enzimática tem sido comumente estudada durante a restrição alimentar. Porém, são encontradas diferentes respostas que podem ser atribuídas a variações na duração e intensidade da restrição alimentar, além da idade dos animais (Ferraris & Diamond, 1997). A restrição alimentar imposta a frangos durante 4 ou 7 dias reduz a atividade específica das enzimas pancreáticas, tripsina, amilase e lipase, e não afeta a atividade de sacarase e maltase no jejuno (Palo *et al*., 1995b). Pinheiro *et al*. (2004) observaram aumento da sacarase intestinal, amilase pancreática e lipase imediatamente após a restrição alimentar de 30% durante 7 dias, enquanto que Fassbinder-Orth & Karasov (2006) verificaram efeito da restrição alimentar de 54% durante 7 dias sobre a atividade da maltase no duodeno de frangos.

A realimentação, após o período de restrição alimentar, também tem despertado interesse, visto ser um período no qual se espera a recuperação da morfologia e função do trato gastrointestinal. Em codornas, observou-se recuperação da superfície mucosa a partir do terceiro dia de realimentação após jejum de 3 dias (Gallo, 2003). Também foi observado que a realimentação aumenta os órgãos do trato digestório, a expressão do transportador de glicose SGLT1 e recupera vários outros parâmetros diminuídos durante o período de restrição alimentar (Palo *et al.*; 1995a, b; Gallo, 2003; Pinheiro *et al*., 2004). Além disso, ocorre aumento na atividade de enzimas pancreáticas e intestinais (Palo, 1995b; Gallo, 2003; Fassbinder-Orth & Karasov, 2006).

Embora os efeitos da restrição alimentar sejam amplamente estudados nas fases iniciais do desenvolvimento dos frangos (Palo *et al*., 1995a, b; Lee & Leeson, 2001; Pinheiro *et* al., 2004; Fassbinder-Orth & Karasov, 2006), pouco se sabe sobre o efeito na fase final do crescimento desses animais.

Sabe-se que a demanda interna dos animais varia durante o desenvolvimento ontogenético, assim como a morfofisiologia do trato gastrointestinal. Na fase inicial do desenvolvimento de aves, as variações na morfologia e função do trato gastrointestinal são atribuídas à mudança na fonte de nutrientes após a eclosão (Overton & Shoup, 1964; Buddington & Diamond, 1989; Maiorka *et al*., 2006). De fato, o desenvolvimento do trato gastrointestinal na primeira semana de vida é crítico para o crescimento do animal. Contudo, o trato gastrointestinal continua a modificar-se após a segunda semana de vida, de modo a manter as taxas de digestão e absorção de nutrientes (Vazquez *et al.*, 1997; Gilbert *et al*., 2007).

Portanto, estudos sobre os efeitos da restrição alimentar em diferentes idades se justificam pela mudança constante da morfologia e função digestivas ao longo do desenvolvimento.

Nos últimos anos, o advento das técnicas de biologia molecular permitiu maior compreensão dos processos de digestão e absorção no trato gastrointestinal. Entretanto, encontramos apenas dois estudos em nível molecular sobre os efeitos da restrição alimentar na expressão gênica e atividade de enzimas e transportadores intestinais. Gilbert *et al*. (2008) avaliaram os efeitos da restrição alimentar na expressão gênica da aminopeptidase, do transportador de di e tripeptídeos (PEPT1), dos transportadores de glicose (SGLT1 e GLUT2) e de 4 transportadores intestinais de aminoácidos no jejuno de frangos. Dentre todos os genes estudados, apenas o transportador de di e tripeptídeos (PEPT1) foi afetado pela restrição alimentar, aumentando a abundância de RNAm.

No outro estudo, foram avaliadas, em conjunto, a expressão gênica e atividade do transportador SGLT1 em frangos jejuados durante 4 dias (Gal-Garber *et al*., 2000). Estudos como este são importantes para se determinar a correlação entre níveis de RNAm e atividade de transportadores e enzimas. No estudo de Gal-Garber *et al*. (2000)

não foi observada correlação entre a expressão gênica e atividade do transportador SGLT1. Segundo Ferraris & Diamond (1997), a abundância de transportadores SGLT1 na membrana apical do enterócito é independente do nível de RNAm do transportador SGLT1 em várias espécies. No entanto, para outros genes de enzimas e transportadores intestinais, como aminopeptidase, GLUT2 e GLUT5, uma variação na expressão de RNAm está correlacionada à sua expressão protéica.

Assim, é de extrema importância o desenvolvimento de estudos que correlacionem as respostas moleculares e bioquímicas e que possam complementá-las com alterações morfológicas e com seus efeitos sobre o organismo como um todo.

Portanto, este estudo teve como objetivo estudar os efeitos da restrição alimentar sobre o trato gastrointestinal de frangos, em nível morfológico, bioquímico e molecular. A restrição alimentar foi imposta nas fases precoce e tardia do crescimento. Para tanto, foram realizados 3 experimentos apresentados nos capítulos de 1 a 3, em artigos redigidos de acordo com as normas para publicação na revista ***British Journal of Nutrition***:

I. Feed restriction and realimentation affect the gastrointestinal tract and performance in chickens of different ages

II. Feed restriction affects the gene expression of intestinal enzymes and nutrients transporters in chickens of different ages

I. Effects of feed restriction and realimentation on Na^+-D-glucose cotransporter in jejunum of domestic chicken (realizado durante estágio sanduíche realizado na

Faculdade de Farmácia, da Universidade de Barcelona, sob supervisão da Profa. Dra. Joana María Planas Rosselló)

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Revisão bibliográfica

1. Estrutura e características gerais do trato gastrointestinal de aves

O sistema digestório desempenha funções importantes na manutenção da homeostase, destacando-se entre elas o fornecimento contínuo de água, eletrólitos e nutrientes ao organismo. Esta função é possibilitada pela adequada captação e posterior digestão e absorção de nutrientes procedentes da alimentação.

Os processos bioquímicos finais para utilização dos nutrientes e produção de energia são bastante similares quando se compara diferentes animais. No entanto, existem grandes diferenças quando se considera as características estruturais dos tratos digestórios.

Sendo assim, podemos dizer que a fisiologia do sistema digestório de aves é bastante similar a dos mamíferos. No entanto, o trato digestório das aves apresenta órgãos diferenciados, como proventrículo, moela e cloaca. Em algumas aves pode ser encontrado também o papo. Além disso, as aves apresentam modificações, como o desaparecimento dos dentes, conversão da maxila dos mamíferos em bico, trituração realizada pela moela e desenvolvimento dos cecos (Schwarse, 1980). Fígado, pâncreas, vesícula biliar e intestino delgado apresentam similaridades estruturais e funcionais com os de mamíferos.

Independente da diversidade, em todas as aves os processos mais importantes de digestão química e de absorção de nutrientes ocorrem no intestino delgado. O intestino delgado das aves, assim como o de mamíferos, pode ser dividido em três regiões: duodeno, jejuno e íleo. O duodeno inicia-se imediatamente após a moela e estende-se até o final da alça duodenal, seguido pelo jejuno que termina no

divertículo de Meckel, que é um vestígio do saco vitelínico. Imediatamente após, encontra-se o íleo que termina quando se iniciam os cecos.

2. Epitélio intestinal

A parede do intestino delgado apresenta uma estrutura especializada, o epitélio, pelo qual ocorre absorção e parte da digestão dos nutrientes. O epitélio é formado por muitos tipos celulares, entre os quais se destacam as células caliciformes, que secretam muco, as células endócrinas, que secretam diferentes hormônios, e os enterócitos.

Os enterócitos são as células responsáveis pelos processos de digestão e absorção de nutrientes. São células colunares polarizadas, nas quais são observadas uma membrana apical e uma membrana basolateral. A membrana apical, em contato com o lúmen intestinal, apresenta projeções digitiformes chamadas de microvilosidades, que aumentam em 14 a 40 vezes a superfície absortiva. Esta membrana contém enzimas, chamadas de membrana ou de parede, importantes na digestão dos nutrientes presentes na luz intestinal. Os enterócitos apresentam também poros, canais ou proteínas transportadoras localizadas nas membranas apical e basolateral que realizam a absorção dos nutrientes.

Além das vilosidades, também são encontradas no epitélio as criptas de Lieberkühn, onde se originam as células indiferenciadas que se desenvolverão em algum dos tipos celulares apresentados acima. À medida que estas células se diferenciam, elas desempenham sua função e migram em direção ao ápice da vilosidade, sendo eliminadas na luz intestinal por descamação.

3. Digestão e absorção de carboidratos

Os carboidratos correspondem a maior parte dos nutrientes na alimentação de frangos (Batal & Parsons, 2004) e fornecem energia para os processos vitais do organismo, como a manutenção da temperatura corporal e das funções essenciais, para formação de tecido corporal e compostos como hormônios, enzimas, proteínas sanguíneas, entre outras.

A digestão mecânica dos carboidratos é efetuada pela maceração e a trituração do alimento no papo e na moela. A digestão química do amido, em suas duas formas, amilose e amilopectina, ocorre principalmente no intestino delgado, por atuação da amilase pancreática. A amilase hidrolisa a amilose, um polissacarídeo que apresenta apenas ligações α1-4, formando maltotriose e maltose. Devido à incapacidade da amilase de romper ligações α1-6, sua ação sobre a amilopectina forma também as α-dextrinas limites, através da ruptura das uniões α1-4 que se encontram ao redor da α1-6 (Moran Jr, 1985). Os oligo e dissacarídeos formados na luz intestinal são, posteriormente, digeridos pela glicoamilase e pelas dissacaridases presentes na membrana apical dos enterócitos formando monossacarídeos, como a frutose, a glicose e a galactose.

Em frangos, as principais dissacaridases presentes na membrana apical dos enterócitos são a sacarase e a maltase (Uni *et al.*, 2003). As enzimas lactase e trehalase podem estar presentes apenas em certas fases do desenvolvimento (Siddons, 1969; Chotinsky *et al.*, 2001). As diversas regiões do intestino possuem diferentes níveis de atividade enzimática, sendo o jejuno o que tem maior capacidade de digerir dissacarídeos, seguido do íleo e, por último, o duodeno (Uni *et al.*, 1998).

A sacarase (complexo sacarase-isomaltase) é a enzima responsável pela hidrólise de sacarose, isomaltose e, isomaltotriose. A sacarose é um dissacarídeo que

está presente principalmente em frutas, enquanto que a isomaltose se encontra em polissacarídeos de origem animal e vegetal. Esta enzima apresenta atividade já em embriões de frango, aumentado após o nascimento (Brown, 1971). O complexo sacarase-isomaltase (SI) hidrolisa a união α1-6 dos oligo e dissacarídeos resultantes da digestão realizada pela amilase pancreática, formando glicose e frutose.

A maltase (maltase-glucoamilases) hidrolisa as uniões α1-4 e α1-6 dos di e oligossacarídeos liberando glicose. Esta dissacaridase é a mais abundante na membrana apical dos enterócitos (Galand & Fostner, 1974).

A trealase é responsável pela hidrólise da trealose em duas moléculas de glicose. A trealose é menos freqüente na alimentação dos frangos, visto que é encontrada em algas, fungos, insetos e invertebrados marinhos. Sua atividade é baixa ao nascer e depois do sétimo dia de vida encontram-se somente indícios de sua ação (Chotinsky *et al.*, 2001).

A lactase é a enzima que hidrolisa os derivados do leite em glicose e galactose. Existem indícios de sua atividade durante o desenvolvimento embrionário (Kedinger *et al.*, 1981) e após a eclosão (Siddons, 1969), mas sua atividade diminui com a idade (Chotinsky *et al.*, 2001).

O principal produto da digestão de carboidratos é a glicose. A absorção deste monossacarídeo através do epitélio intestinal pode ser realizada por via paracelular, através das uniões intercelulares ou, principalmente, por via transcelular, através dos enterócitos. A absorção transcelular de monossacarídeos pode ocorrer por difusão simples ou por transporte ativo, com a interação do substrato com proteínas específicas da membrana do enterócito. Na membrana apical do enterócito ocorre um mecanismo de transporte ativo, capaz de acumular monossacarídeos dentro da célula contra o seu gradiente de concentração. Trata-se de um mecanismo de cotransporte pela proteína

SGLT1, pelo qual a entrada de açucares está acoplada ao gradiente eletroquímico de íons sódio. Na membrana basolateral o transporte de glicose para o interstício ocorre por difusão facilitada pela proteína transportadora GLUT2.

4. Digestão e absorção das proteínas

As proteínas da dieta são as principais fontes de aminoácidos e peptídeos para a síntese de proteínas, obtenção de energia e regulação das diversas funções celulares através da síntese de hormônios e neurotransmissores. Uma vez ingeridas, as proteínas são em grande parte digeridas, sendo absorvidas principalmente na forma de aminoácidos ou de pequenos peptídeos (2 a 4 aminoácidos).

A digestão das proteínas inicia-se no proventrículo, com a secreção de pepsinogênio e ácido clorídrico. No proventrículo ocorre a conversão do pepsinogênio em pepsina, por ação do ácido clorídrico. O processo final de digestão das proteínas ocorre no intestino, com ação das proteases pancreáticas, que são secretadas na forma de proenzimas. Estas são ativadas inicialmente por enteroquinases, enzimas presentes na membrana apical do enterócito, cuja atividade é estimulada pelo tripsinogênio. As enzimas ativas resultantes incluem as endopeptidases (tripsina, quimiotripsina, elastase) e exopeptidases (carboxipeptidases A e B). Os produtos resultantes da ação dessas enzimas são aminoácidos e peptídeos.

A hidrólise dos peptídeos resultantes da digestão intraluminal ocorre na membrana apical dos enterócitos. Este processo é muito complexo, visto que envolve peptidases específicas para reconhecer os 20 aminoácidos existentes, com diferentes propriedades físicoquímicas, como solubilidade, carga, peso molecular e estrutura. As

peptidases presentes na membrana apical pertencem a 4 classes: endopeptidases, aminopeptidases, carboxipeptidases e dipeptidases.

Dentre as várias peptidases da membrana apical, a aminopeptidase N é a principal exopetidase na membrana apical de mamíferos e aves (Schondube & Martinez Del Rio, 2004). A aminopeptidase cliva aminoácidos de cadeia N-terminal dos peptídeos, preferencialmente aminoácidos neutros ou básicos (Taylor, 1993; Riemann *et al*., 1999).

Os produtos finais da digestão de proteína são aminoácidos livres, di e tripeptídeos. O epitélio intestinal possui mecanismos de transporte eficiente para absorver não apenas aminoácidos livres, mas também di e tripeptídeos. Os aminoácidos livres são absorvidos através da membrana apical dos enterócitos por sistemas de transportes específicos para determinados grupos de aminoácidos. Os di e tripeptídeos são transportados intactos através da membrana apical dos enterócitos por sistemas de transportes específicos para peptídeos, enquanto que os transportadores de aminoácidos livres exibem especificidade por substratos. Os transportadores de peptídeos podem transportar todos os 400 a 8000 tripeptídeos resultantes da combinação dos 20 diferentes aminoácidos da dieta (Daniel, 2004). Além disso, o transporte de aminoácidos na forma de peptídeos é mais rápido por unidade de tempo que aminoácidos na forma livre (Adibi & Phillips, 1968).

Di e tripeptídeos são transportados para dentro da célula por transportadores de peptídeos, PEPT1, um membro da família de cotransportadores de próton e oligopeptídeos. Além do transporte pelo PEPT1, peptídeos podem também ser absorvidos por rotas alternativas incluindo movimento paracelular e por peptídeos penetradores de células, que são capazes de movimentar carga através da membrana

plasmática. O transporte intestinal de di e tripeptídeos tem sido bem caracterizado em frangos (Chen *et al*., 2002, 2005; Gilbert *et al*., 2008).

O transportador de di e tripeptídeos PEPT1 está presente na membrana apical e basolateral dos enterócitos e sua atividade é dependente do gradiente de H^+, favorecido pelo microclima da membrana apical e pela atividade da bomba de H^+/Na^+ da membrana.

Os di e tripeptídeos que são absorvidos intactos são subseqüentemente hidrolisados pela ação de peptidases intracelulares, o que resulta na formação de aminoácidos livres que são, então, disponibilizados para a corrente sangüínea.

5. Digestão e absorção de lipídeos

Os principais lipídeos da dieta são os triglicerídeos, que constituem 90% da gordura ingerida. Os ácidos graxos provenientes da dieta não podem ser sintetizados pelo organismo a partir de outras substâncias e são imprescindíveis para assegurar um estado nutricional ótimo. A dieta também contém pequenas quantidades de colesterol, éster de colesterol de fonte animal, ceras de fonte vegetal e fosfolipídio de fonte animal e vegetal. O colesterol é o constituinte básico das membranas celulares, precursor da vitamina D e de hormônios esteróides. Além disso, os lipídeos desempenham várias funções importantes dentro do organismo, como reserva de energia, participa de sistemas enzimáticos e de transporte de lipídeos dentro do organismo.

Os lipídeos são pouco solúveis em água, e esta propriedade determina processos diferenciados de digestão e absorção. A digestão de gorduras inicia-se no estômago, com o aquecimento dos lipídeos à temperatura corpórea, mistura e emulsificação em gotículas menores. A gordura assim modificada passa para o intestino

delgado, onde a emulsificação é completada com a ação de ácidos biliares e fosfolipídeos. Esses produtos da bile reduzem a tensão de superfície dos lipídeos e permitem que as gotículas de gordura reduzam em tamanho, tornando-se acessíveis às membranas lipolíticas. Os lipídeos cobertos por sais biliares estão sujeitos à ação das enzimas hidrolíticas. A hidrólise de triglicerídeos ocorre devido à ação combinada de enzimas pancreáticas lipase e co-lipase. A lipase cliva os ácidos graxos de cada extremidade da molécula de triglicerídeo, resultando na formação de dois ácidos graxos livres ou não-esterificados e um monoglicerídeo.

Os produtos finais da digestão dos lipídeos se difundem pelo lúmen intestinal, através da camada estável de água e são absorvidas por difusão simples.

6. Flexibilidade fenotípica do trato digestório

Os animais possuem a capacidade de ajustar seus fenótipos em resposta à seleção genética, alterações na disponibilidade e qualidade dos alimentos, processos patológicos e demanda interna do animal. Essa capacidade é chamada flexibilidade fenotípica ou plasticidade fenotípica (Moran, 1992; Piersma & Lindstrom, 1997; Piersma & Drent, 2003) e pode ser realizada através de mudanças comportamentais e fisiológicas (Overgaard *et al.*, 2002; Palacios & Bozinovic, 2003; Naya *et al.*, 2003). A flexibilidade fenotípica permite que o animal otimize as funções vitais de acordo com as mudanças ambientais (Starck, 1996), garantindo a homeostase.

O trato gastrointestinal apresenta capacidade de responder rapidamente às demandas ambientais e fisiológicas ajustando-se para manter a taxa de digestão, e conseqüentemente o fornecimento de nutrientes e energia para todo o organismo (Diamond, 1991; Secor, 2001). Os ajustes realizados aumentam as possibilidades de

sobrevivência dos indivíduos uma vez que contribuem para a redução do gasto energético de manutenção do epitélio intestinal, haja vista o alto gasto energético da renovação celular intestinal e da síntese protéica (Diamond, 1991; Secor *et al*., 1994; Secor, 2001).

As variações ambientais que afetam o trato digestório podem ser alterações na disponibilidade de alimento ou na composição nutricional (McWilliams & Karasov, 2001) e os ajustes realizados pelo trato digestório podem ser morfológicos ou funcionais. Várias espécies de insetos, peixes, répteis, pássaros e mamíferos podem alterar o comprimento do intestino em reposta a variações na qualidade e quantidade de alimento (Starck, 1996; Piersma & Lindström, 1997; Siems & Sikes, 1998). As dimensões das microvilosidades e vilosidades intestinais, as taxas de proliferação e de migração celular, além de características importantes das membranas das células, como a permeabilidade e a atividade das proteínas transportadoras podem alterar-se (Misch *et al*., 1980; Yamauchi *et al*., 1996; Waheed & Gupta, 1997; Ferraris & Carey, 2000).

As mudanças ocorridas no epitélio intestinal podem, por conseguinte, afetar a digestão e a absorção intestinal dos nutrientes. Em aves, a variação na disponibilidade de um determinado nutriente pode levar a uma alteração na maquinaria necessária para sua digestão e absorção. Por exemplo, frangos alimentados com dieta livre de carboidratos exibem menos enzimas maltase e sacarase por área intestinal que frangos alimentados com dieta rica em carboidratos (Biviano *et al*., 1993).

A demanda interna do animal durante o seu desenvolvimento ontogenético também afeta a morfofisiologia do trato digestório. Em aves, o período de transição de embrião para os estágios pós-eclosão é crítico para o desenvolvimento normal dos frangos. Embora o trato digestório seja anatomicamente completo no período final de incubação, a demanda nutricional durante o estágio precoce é acompanhada por

mudanças adaptativas na morfologia e função do trato digestório. A superfície absortiva muda consideravelmente após a eclosão e a taxa de proliferação aumenta, fazendo com que o peso relativo dos intestinos seja maior após a eclosão, alcançado o máximo de 3 a 7 dias após e, então, declinando levemente.

Além disso, ocorre uma mudança abrupta na fonte de nutrientes no 1º dia de vida das aves, quando a gema, rica em lipídeos é substituída por uma dieta exógena rica em carboidratos e proteínas (Buddington & Diamond, 1989). Vários estudos têm evidenciado hiperplasia, hipoplasia e diferenciação celular após a eclosão (Overton & Shoup, 1964; Maiorka *et al*., 2006).

Nos primeiros dias após a eclosão a absorção de monossacarídeos, assim como a densidade de transportadores SGLT1, na membrana apical dos enterócitos é aumentada (Vázquez *et al*., 1997; Barfull *et al*., 2002). Já foi demonstrado também aumento na concentração das enzimas pancreáticas amilase e tripsina (Krogdahl & Sell, 1989).

Alguns estudos têm mostrado adaptações de estrutura e função do sistema digestório ocorridas em aves migratórias em períodos de escassez de alimento. (McWilliams *et al.*, 2004). São observadas alterações do tamanho intestinal, taxa de absorção, atividade hidrolítica de enzimas digestivas, eficiência digestiva e tempo de retenção dos nutrientes (McWillians & Karasov, 2001). Diferentemente do que ocorre com o intestino delgado, fígado e pâncreas, apenas o estômago não apresenta modificação de peso ou tamanho em resposta à restrição alimentar (Karasov *et al*., 2004).

Em frangos, a restrição alimentar e o jejum têm sido comumente utilizados para reduzir desordens metabólicas e problemas reprodutivos, assim como para controlar o peso corporal (Lee & Leeson, 2001; Camacho *et al*., 2004). A restrição

alimentar inibe os processos de hiperplasia e hipertrofia e promove melhoria na eficiência alimentar (Leeson *et al*., 1992). Tem sido observada também redução na taxa de mortalidade, aumento da resistência dos animais a doenças infecciosas e à exposição a altas temperaturas (O´Sullivan *et al*., 1991).

A restrição alimentar adotada no manejo das aves é aplicada entre a primeira e segunda semana de idade das aves. Na primeira semana, o animal é considerado muito frágil para suportar o estresse da restrição alimentar e após 21 dias de idade não há tempo suficiente para recuperação do peso até o momento do abate (Plavnik & Hurwitz, 1988). O restabelecimento do alimento após o período de restrição alimentar promove aumento na taxa de crescimento corpóreo, denominada de ganho compensatório (Yu *et al*., 1990). Animais submetidos à restrição alimentar com posterior realimentação atingem valores de peso igual ou superior ao de animais não submetidos à restrição alimentar em uma mesma idade (Plavinik e Hurwitz,1985, 1989, 1991; Plavinik *et al*., 1986; Yu *et al*., 1990; Fontana *et al*., 1992). O ganho compensatório, no entanto, é influenciado pelo sexo do animal, natureza, severidade e duração da restrição e o tempo de realimentação (Wilson & Osbourn, 1960). Assim, pode também ocorrer redução do peso corpóreo após restrição alimentar (Pokniak & Cornejo, 1982; Sims & Hooge, 1990; Zubair & Leeson, 1994).

Durante a restrição alimentar pode ocorrer melhor conversão alimentar em aves e em outros animais. Esta resposta tem sido atribuída, em parte, à alta eficiência metabólica associada a uma pequena massa corporal (Dickerson, 1978; Zubair & Leeson, 1994). Além disso, a velocidade de passagem de alimento pelo trato digestório é menor, o que aumenta o tempo disponível para o processo de digestão dos alimentos. De fato, uma taxa de passagem mais lenta pode melhorar a digestibilidade e, conseqüentemente, a eficiência de utilização dos alimentos (Sainz *et al*., 1995).

A presença de alimento é um estímulo importante para o crescimento da mucosa intestinal, embora o intestino se ajuste rapidamente ao estresse nutricional (Francis & Schleiffer, 1996). A mucosa gastrointestinal de frangos de corte pode ser influenciada por fatores tróficos, relacionados com a ingestão e absorção de alimentos e por suas características químicas e físicas (Macari, 1995). Estudos realizados em aves e outras espécies mostram que a restrição alimentar aumenta o comprimento de determinados segmentos do trato digestório (Pinchasov *et al*., 1985; Cruz *et al*., 2000), o peso relativo dos órgãos relacionados com a digestão (Furlan, 1996) e a expressão gênica do transportador de glicose SGLT1 (Gal-Garber *et al*., 2000). Além disso, pode reduzir o desenvolvimento da mucosa (Palo *et al*., 1995), a altura das vilosidades, a atividade da tripsina, amilase e lipase (Palo *et al*., 1995; Susbilla *et al*., 2003) e a profundidade das criptas (Pinheiro *et al*., 2000). Nir *et al.* (1987) e Katanbaf *et al*. (1989) mostraram que galinhas submetidas a períodos de restrição apresentam hipertrofia dos órgãos que armazenam alimentos e aumentam o peso relativo do pâncreas, jejuno e íleo.

O reestabelecimento da alimentação após período de restrição, por sua vez, promove a recuperação da mucosa intestinal em aves na fase inicial do desenvolvimento (Palo *et al*., 1995; Shamoto *et al*., 1999; Shamoto & Yamauchi, 2000), as quais apresentam aumento da altura das vilosidades e criptas intestinais (Nakage, 2000; Pinheiro *et al*., 2000) e peso dos órgãos do trato digestório. Após realimentação, os processos de digestão e absorção de nutrientes nestes animais jovens também são afetados, com aumento da atividade da maltase e sacarase intestinais (Palo *et al*., 1995) e expressão do transportador de glicose SGLT1 (Gal-Garber *et al*., 2000).

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FEED RESTRICTION AND REALIMENTATION AFFECT THE GASTROINTESTINAL TRACT AND PERFORMANCE IN CHICKENS OF DIFFERENT AGES

Abstract

The gastrointestinal tract of young chicken shows an adaptive response to feed restriction modifying morphology and functions, but its response is not known in older chickens. Thus, this study investigated the effect of feed restriction on enzymes activity and gastrointestinal organs weight in chickens of two ages: 7 and 35 days old. For this, the animals were submitted to 70% feed restriction followed by realimentation *ad libitum* for 3 days. The control groups were fed *ad libitum* during equal periods. Animals were sacrificed in the end of feed restriction or realimentation period (7^{th} or 10^{th} days, respectively). Gastrointestinal organs (proventriculus, gizzard, small and large intestine), pancreas and liver were weighed. Pancreatic chymotrypsin, trypsin, amylase, lipase and intestinal sucrase and maltase activity were evaluated. Feed intake, feed efficiency and percentage of body weight gain were determined during realimentation period. Feed restriction decreased the organs weight and enzymes activity in both ages, with few exceptions. After realimentation for three days, these parameters returned to values similar to control group. Feed efficiency, percentage of body weight gain and feed intake increased during realimentation period. The present study showed that during feed restriction occurs reduction of maintenance costs through the decrease of organs weight and of the activity of pancreatic and intestinal enzymes. After the realimentation, the increase of feed intake, accompanied by the recovery of enzymatic activity to the control level, provided an improvement of chicken performance.

Key words: enzymes, feed restriction, performance, realimentation

Introduction

Feed restriction is a common management strategy applied in chicken production in order to control the body weight and reproductive, metabolic and skeletal disorders[1-3]. In fact, many reports on feed restriction in chickens show a decrease of body weight and carcass fat and an improvement of feed efficiency with compensatory growth after realimentation[4,5]. This practice can affect the physiology of systems, including the gastrointestinal tract. Indeed, feed-restricted chickens show a decrease of gastrointestinal organs weight and activity of jejunal alkaline phosphatase, pancreatic trypsin, amylase and lipase[6,7]. However, in others studies, feed restriction increased the intestinal sucrase activity[8] whereas decreased the maltase activity[9]. The difference among results can be attributed, at least partially, to different feed restriction protocols employed in these investigations. Realimentation after feed restriction, in turn, caused the recovery of the weight of all gastrointestinal organs and the increase of duodenal maltase[6,7,9].

The most studies focus on the effects of feed restriction on the gastrointestinal morphology and function in the first or second week after hatching[2,6,7,9,10]. However, the knowledge about the effect of feed restriction and realimentation during other phases of chickens growth is scarce.

It is known that internal demand varies during the chicken development, which can consequently affect the food intake and the digestive physiology[11,12]. In fact, many authors have shown age-related changes in the gastrointestinal system, as increase of pancreatic enzymes and intestinal disaccharidases and decrease of intestinal passage time and jejunal Na^+-dependent D-glucose transport[12-16].

Thus, the present study aimed to compare the effects of feed restriction and realimentation on the gastrointestinal system, including organs weight and activities of

intestinal and pancreatic enzymes in two stages of chicken growth. It was also evaluated the body weight gain, feed intake and feed efficiency during realimentation.

Material and Methods

A total of 64 male broiler chicks (Cobb) were obtained from a commercial hatchery (Frigorífico Mabella Ltda, Tatuí, Brazil) on the hatching day. Animals were housed in metal batteries in a room with controlled illumination, temperature and humidity. Until 14 days of age the animals were fed with a common starter diet, and thereafter, with a grower diet (table 1). Water was offered *ad libitum* during all experiment. All procedures were approved by University Ethics Committee for Animal Research.

Feeding regimens were applied in chickens from 7 to 17d old (groups identified by small letters) and 35 to 45d old (groups identified by capital letters). At 7 or 35d old, chicks were weighed and divided into four groups, with 8 chickens per one: **r** and **R** - 70% food restriction for 7 days; **c7** and **C7** - food *ad libitum* for 7 days; **rf** and **RF** - 70 % food restriction for 7 days, followed by refeeding for 3 days; **c10** and **C10** - food *ad libitum* for 10 days (figure 1).

Animals were weighed daily and the food amount supplied to feed-restricted chickens was equivalent to 30% of the quantity consumed by the fed *ad libitum* group on the previous day. The effects of treatments were determined at 14 and 17d old and at 42 and 45d old, according to the group. For this, the animals were weighed and killed by beheading.

Feed intake and performance

Feed intake, feed efficiency (ratio of weight gain to feed intake) and percentage of body weight gain were measured during the realimentation periods (from 14 to 17d and from 42 to 45d old).

Organs weight

Immediately following euthanasia, gastrointestinal organs (proventriculus, gizzard, small and large intestine), pancreas and liver were excised from each chicken. The organs were cleaned with ice-cold physiologic saline solution, dried with filter paper and weighed. The organs weight was expressed as absolute and relative to the respective body weight (grams/100 g BW).

Enzyme analysis

Pancreas and jejunum, free of residual food, were frozen in liquid nitrogen and stored in freezer -80ºC until required for assay.

The activity of the pancreatic enzymes was determined after the whole organ was homogenized (1:20 wt/vol) in 50mmol/l Tris-HCl buffer, pH 8, containing 50 mmol/l $CaCl_2$. For trypsinogen determination[17], enterokinase was added to the homogenate and allowed to convert the enzyme into trypsin. Trypsin activity was then measured from the hydrolysis of p-nitroaniline from benzoyl-DL-arginine-p-nitroanilide (DL-BAPNA) at pH 8.2. Units are expressed as nanomoles of p-nitroanilide released per minute per milligram of protein. A similar method was used for determination of chymiotrypsin[18], with BAPNA replacing N-glutaryl-L-phenylalanine-p-nitroanilide (GPNA). The reaction was stopped with 3% acetic acid solution. Amylase was determined by the iodometric method modified by Caraway[19] (In vitro Diagnostica,

Brazil) and the activity was expressed as amylase units (AU) per microgram of protein. One amylase units is the amount of enzyme that will hydrolyze 10 mg of starch in 30 min. Lipase activity was obtained by BALB-DNTP method (In vitro Diagnostica, Brazil). Accordingly, lipase hydrolyzes the thioester, producing a thioalcohol that reacts with nitrobenzoic acid, yielding a yellow anion. The color intensity is proportional to enzyme concentration. The enzyme activity was expressed as International Units (IU) per microgram of protein.

To determine the activity of intestinal disaccharidases by Dahlquist method[20], each jejunum segment was opened longitudinally and the mucosa was scraped off with a glass microscope coverslip. This tissue was mechanically homogenized after the addition of 4 parts of ice-cod deionized water. Maltase and sucrase activity were assayed by incubating aliquots of the homogenates with the appropriate substrate in malate buffer at pH 6.4. Released glucose was determined by the glucose-oxidase method (Laborlab, Brazil). The enzymes activity was expressed as units per gram of protein, which was determined by the method of Bradford[21].

Statistical analysis

Values were expressed as mean ± sem. Statistical differences between feed-restricted and control groups (r x c7 and R x C7), realimented and control groups (rf x c10 and RF x C10) and feed-restricted and realimented groups (r x rf and R x RF) were established by Student's t-test. Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, Inc, USA). Probability of $P < 0.05$ was taken as significant.

Results

Feed intake and performance (Figure 2)

During the reestablishment of *ad libitum* feeding, chickens previously feed-restricted of both ages (rf and RF) presented higher percentage of body weight gain and feed efficiency ($P<0.05$) than control animals (c10 and C10). In both ages, the relative feed intake (feed intake normalized to body weight) during the realimentation period was higher in previously restricted chickens than control group ($P<0.05$).

Absolute organs weight (Figure 3)

In feed-restricted chickens of both ages (r and R), the absolute weight of all organs was significantly lower ($P<0.05$) than control animals (c7 and C7). The absolute organs weight in younger realimented chickens was lower than control group (c10) and greater than feed-restricted group (r). No statistical differences ($P<0.05$) were found in absolute organs weight between control (C10) and realimented group in older animals (RF). The older realimented group of both ages (rf and RF) presented absolute organs weight greater than feed-restricted group (r and R).

Relative organs weight (Figure 4)

In both ages, the relative weight of proventriculus was greater ($P<0.05$) and the relative weight of liver was lower in feed-restricted group (r and R) compared to the control group (c7 and C7). The gizzard relative weight of feed-restricted animals (r) was greater ($P<0.05$) only in younger chickens and the small intestine relative weight was lower in older feed-restricted animals (R) compared to respective control group (C7). Pancreas and large intestine were not affected by feed restriction in the both ages.

After realimentation for 3 days, relative weight of proventriculus, gizzard, small intestine and liver of previously feed-restricted groups of both ages (rf and RF) was greater ($P<0.05$) compared to the respective control groups (c10 and C10). Relative weight of large intestine and pancreas were greater ($P<0.05$) only in older animals (RF).

In comparison to the feed-restricted groups, the younger realimented group (rf) showed a greater relative weight of gizzard, small intestine and liver ($P<0.05$). In the older realimented animals (RF) all organs were greater ($P<0.05$) compared to the feed-restricted group (R).

Pancreatic digestive enzymes (Figure 5)

Younger feed-restricted chickens (r) presented trypsin and amylase activity lower ($P<0.05$) than control group (c7). In older feed-restricted chickens (R) only chymotrypsin activity was lower ($P<0.05$) compared to the control group (C7). After realimentation, the pancreatic enzymes in animals of both ages (rf and RF) were similar ($P>0.05$) to control groups (c10 and C10). When compared to feed-restricted group, the trypsin and chymotrypsin activity were higher ($P<0.05$) only in younger group (rf). The feed regimens did not affect the lipase activity in chickens of both ages ($P>0.05$).

Intestinal digestive enzymes (Figure 5)

Disaccharidases of younger chickens were affected ($P>0.05$) neither by feed restriction (r) nor by realimentation (rf). Sucrase and maltase activity was lower ($P<0.05$) in older feed-restricted chickens (R) compared to control group (C7). After realimentation, the disaccharidases activities were similar to the control group in both ages ($P>0.05$).

Discussion

In the present study, chickens were 70% feed-restricted in two phases of growth, from 7 to 14d old and from 35 to 42d old. Thereafter, both groups were given *ad libitum* access to food for three days. Animals of both ages showed capacity for rapid and reversible changes in digestive enzymes and in organs weight in response to alterations in feed regimens. In fact, the digestive enzymes activity, which decreased during feed restriction, reached the level of control group 3 days after the reestablishment of feeding. This response was accompanied by an increase in feed efficiency, relative feed intake, percentage of body weight gain and relative organs weight.

Feed-restricted chickens of both ages decreased the absolute weight of all organs studied. Similar response was showed by Palo *et al.*[6], applying early feed restriction in chickens from 11 to 14 days old and from 7 to 14 days old. Other authors also verified decrease of gastrointestinal organs weight in response to feed restriction[9,10,22,23]. This response is expected, considering that nutrients have trophic effects on the gastrointestinal tract, stimulating, for example, the development of villus and crypts[24]. In fact, many authors showed decrease in villus height and cell area, cell proliferation and mitosis rate caused by feed restriction[25-28].

However, when the organ weight was normalized to the body weight, it was verified that the organs were differently affected by feed restriction. Whereas proventriculus increased in chickens of both ages, the liver decreased. Pancreas and large intestine were not affected. Gizzard and small intestine showed responses that varied according to the animal age. The gizzard relative weight increased in younger chickens but not in older animals. The small intestine decreased in older chickens and did not change in chicks.

It is interesting to note that many authors have verified hypertrophy of proventriculus and gizzard in response to feed restriction[6,23,29,30]. These organs play important function as storage organs and it is probable that feed-restricted animals give priority to development of this kind of organs, including the stomach, at the expense of the demand tissues like breast and thigh[31]. Moreover, increased capacity of these organs, accompanied by slow evacuation, provides the nutrients supply to the intestine during the periods that the food is not available[32].

The results showed that pancreas appears to be the organ less affected by feed restriction in the both ages. Palo *et al*.[6-7] showed a similar response in the relative weight of pancreas and small intestine applying feed restriction from 11 to 14 days old and from 7 to 14 days old. Pancreas plays important function in the digestion of protein, carbohydrate and fat, by secretion of enzymes into the duodenum. Despite the maintenance of pancreas relative weight during feed restriction, the activity of chymotrypsin, trypsin and amylase was decreased. These results are consistent with the reduction of the proteolytic activity of the proventriculus and pancreas in feed-restricted chickens observed by Susbilla *et al*.[10].

Early feed restriction decreased the trypsin and amylase activity while later feed restriction decreased the activity of chymotrypsin and jejunal maltase and sucrase. The response verified in younger chickens is coherent with the results observed by Palo *et al*.[7] and Fassbinder-Orth & Karasov[9]. But, we could not found other studies about the effect of feed restriction in older chickens in order to compare with the observed results.

A decrease in enzymes activity was observed, although different enzymes have been affected in both ages. It is known that pancreatic and intestinal enzymes activities change in response to the age and dietary composition[14,33-35]. But, the effects cannot be

explained by variations during the development. In general, amylase activity increases rapidly during the first 14 days, trypsin and lipase activity increase from 14 days old, and chymotrypsin reaches the maximum level at 11 days and then decrease[33,34]. Maltase activity gradually decreases from hatching to 21 days old and sucrase activity increases after hatching and then remained unchanged until d 35 and thereafter declines[36].

However, the response to feed restriction must be caused by low quantity of food in the gastrointestinal tract. In fact, pancreatic proteases, amylase and disaccharidases activities are proportional to the amount of the specific substrate[7,14,37,38]. Corring[37] considers that the biosynthesis of all digestive enzymes is markedly decreased in response to moderate feed restriction.

After the reestablishment of feeding for three days, older chickens, but not the younger, were able to reach the absolute organs weight verified in control group. Variations according to the age can be explained by the organs growth rate during development. Indeed, during early post-hatching the gastrointestinal segments present a faster growth compared to the other organs and tissues[39,40]. However, in both ages, the relative organs weight of realimented group was increased, with exception of small intestine and pancreas in younger chicks. Thus, the most studied organs showed a more rapid recovery than the body weight.

Previously feed-restricted animals presented a higher feed efficiency and body weight gain during the reestablishment of feeding and this improvement in chicken performance was accompanied by a return of the enzymatic activity to the level of control group. The improvement of growth and feed efficiency can be attributed to high feed intake and hypertrophy of the gastrointestinal tract that occurs following refeeding[22,41-,44]. Also, according to Zubair & Leeson[44], it can be explained to a

reduced overall maintenance requirements caused by a transient decrease in basal metabolic rate.

In our study, the relative feed intake by realimented animals must have contributed to higher feed efficiency. In fact, although the absolute feed intake has been higher only in older animals (data not shown), the relative feed intake was higher in chickens of both ages. Increased relative weight of proventriculus and gizzard in realimented chickens, as observed herein, could improve the food storage capacity.

Feed efficiency is also related to digestive process of food. The efficient digestive process can have been attained by a recovery of enzymatic activity to the control group level, parallel to greater relative weight of small intestine. Moreover, Ferraris & Carey[13] reviewed the intestinal sugar transport and hypothesized that feed restriction increases the ratio of transporting to nontransporting cells, increasing the sugar transport. Thus, it is probable that this ratio remains increased after the reestablishment of feeding, explaining the higher feed efficiency in realimented chickens of both ages.

Although previously feed-restricted animals have shown a higher performance during realimentation period, compensatory growth was not observed in this study (data not shown). Compensatory growth was observed by many authors after feed restriction period[2,8,45]. Maybe the three days of realimentation were not sufficient to observe this response.

Thus, the responses presented in this study showed adaptive changes in the gastrointestinal system provoked by feed restriction and realimentation. There was a decrease in organs weight and enzymes activity, with few exceptions. These changes were rapid and reversible, considering that 3 days after realimentation the organs weight and the digestive enzymes activity returned to the values presented by the control group.

These changes can be considered as an adaptive response of the chickens, taking into account the decreasing of the maintenance costs of the organs and the synthesis of digestive enzymes in pancreas and small intestine during feed restriction.

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Table 1. Composition and calculated dietary analysis

	Starter[1] *1 to 14 d*	*Grower*[2] *15 to 45 d*
Soybean meal	34.30	30.70
Corn	59.34	62.03
Calcium calcite	0.90	0.89
Dicalcium phosphate	1.80	1.68
Soy oil	2.30	3.29
Salt	0.47	0.47
L-Lysine	0.20	0.25
L-treonine	0.05	0.05
DL-Methionine	0.24	0.24
Mineral-vitamin mix1	0.40	0.40
Total	100.00	100.00
Calculated analysis		
ME (MJ/kg)	12.56	12.98
Crude protein (%)	20.80	19.43
Crude fiber (%)	2.88	2.73
Calcium (%)	0.89	0.85
Available phosphorus (%)	0.44	0.42
Methionine (%)	0.53	0.51
Methionine + cysteine (%)	0.81	0.78
Lysine (%)	1.16	1.11

[1]Mineral and vitamin mix (1 to 14 d) supplied per kilogram of diet: Cu, 1.25 mg; Fe, 62.62 mg; I, 0.025 mg; Mn, 67.5 mg; Se, 0.225 mg; Zn, 68.75 mg; folic acid 1.25 mg; pantothenic acid, 12.5 mg; BHT 2.5 mg; biotin, 0.125 mg; choline chloride, 750 mg; niacin, 37.5 mg; vitamin A, 12,500 IU; vitamin B_1, 2.5 mg; vitamin B_{12}, 25 mg; vitamin B_2, 5 mg; vitamin B_6, 5 mg; vitamin D3, 2,500 IU; vitamin E, 25 mg; vitamin K_3, 2.5 mg; monensin, 125 mg.

[2]Mineral and vitamin mix (15 to 45 d) supplied per kilogram of diet: Cu, 5 mg; Fe, 25.05 mg; I, 0.01 mg; Mn, 27 mg; Se, 0.09 mg; Zn, 55 mg; folic acid 1 mg; pantothenic acid, 10 mg; BHT 2.0 mg; biotin, 0.1 mg; choline chloride, 600 mg; niacin, 30 mg; vitamin A, 10,000 IU; vitamin B_1, 2 mg; vitamin B_{12}, 20 mg; vitamin B_2, 4 mg; vitamin B_6, 4 mg; vitamin D3, 2,000 IU; vitamin E, 20 mg; vitamin K_3, 2 mg; monensin, 100 mg.

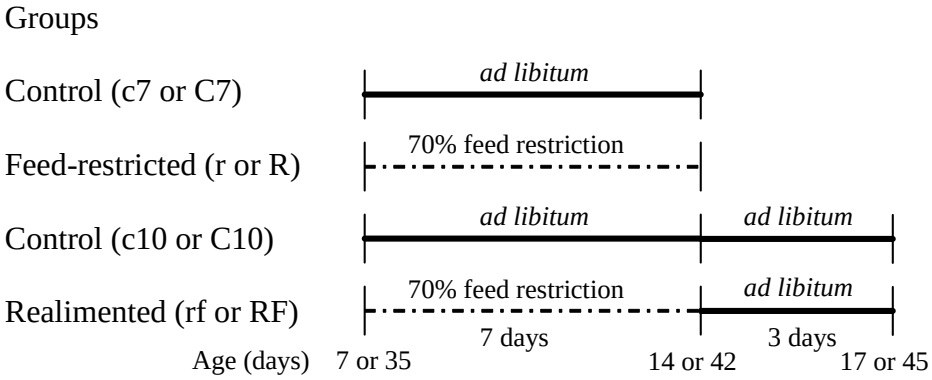

Figure 1. Experimental design showing the studied groups.

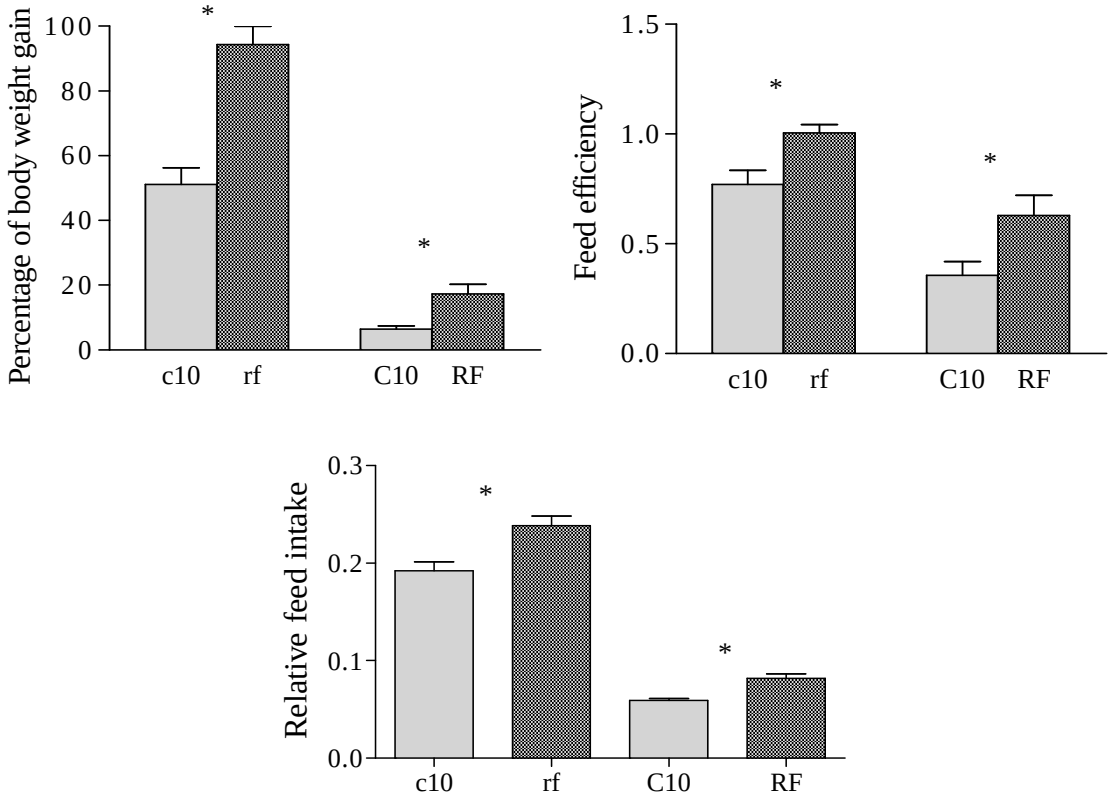

Figure 2. Effects of feed restriction on feed efficiency, percentage of body weight gain and relative feed intake during realimentation period. **c10** and **C10**: food *ad libitum* for 10 days and **rf** and **RF**: 70 % food restriction for 7 days followed by realimentation for 3 days. Data are the mean ± sem. * means statistical difference between realimented and control group, Student's t test, $P<0.05$.

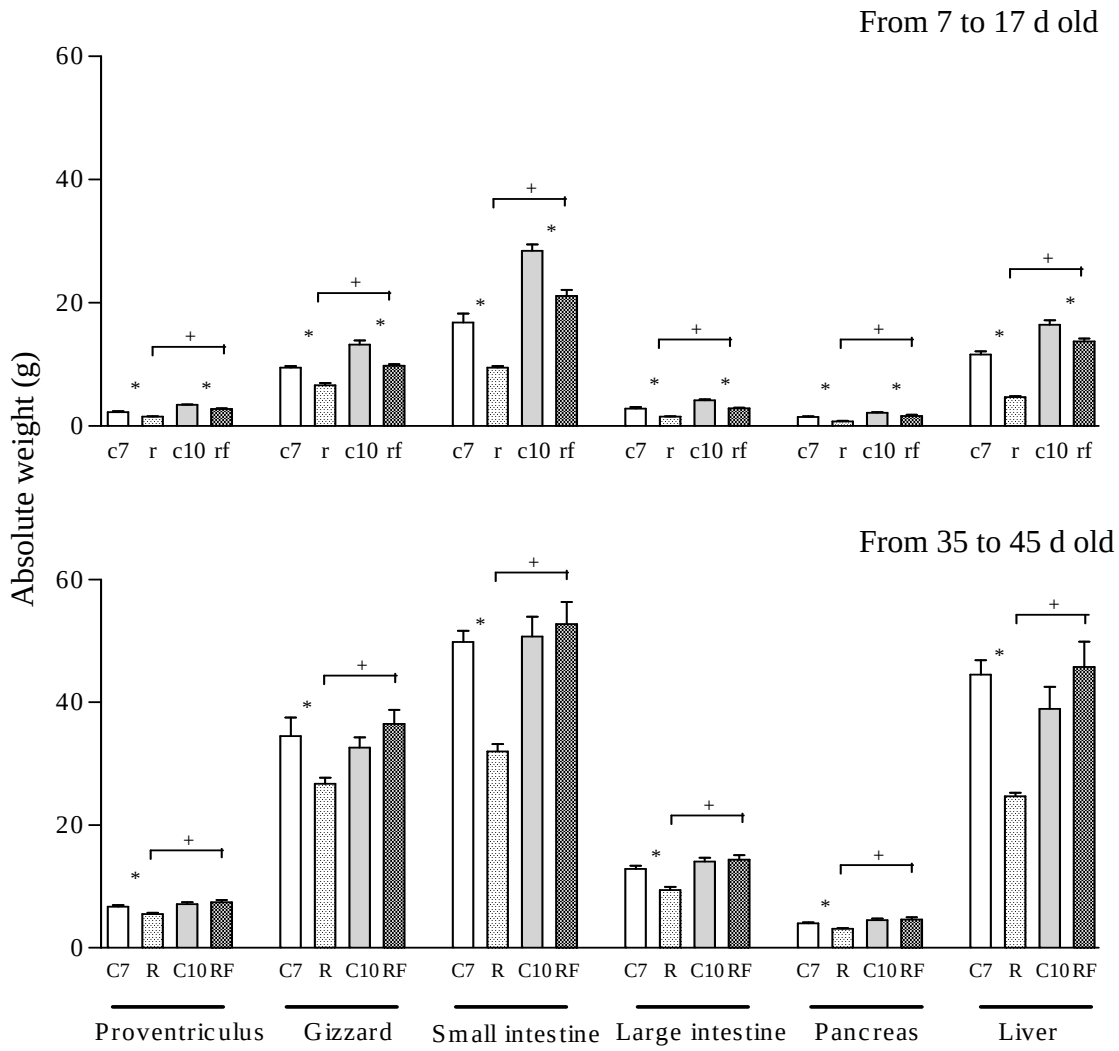

Figure 3. Effects of feed restriction (c7 x r and C7 x R) and realimentation (c10 x rf and C10 x RF) on absolute weight of digestive organs of chickens. **c7** and **C7**: food *ad libitum* for 7 days; **r** and **R**: 70% food restriction for 7 days; **c10** and **C10**: food *ad libitum* for 10 days and **rf** and **RF**: 70 % food restriction for 7 days followed by realimentation for 3 days. Data are the mean ± sem. * means statistical difference between experimental and control group and + means statistical difference between feed-restricted and realimented group, Student's t test, $P<0.05$.

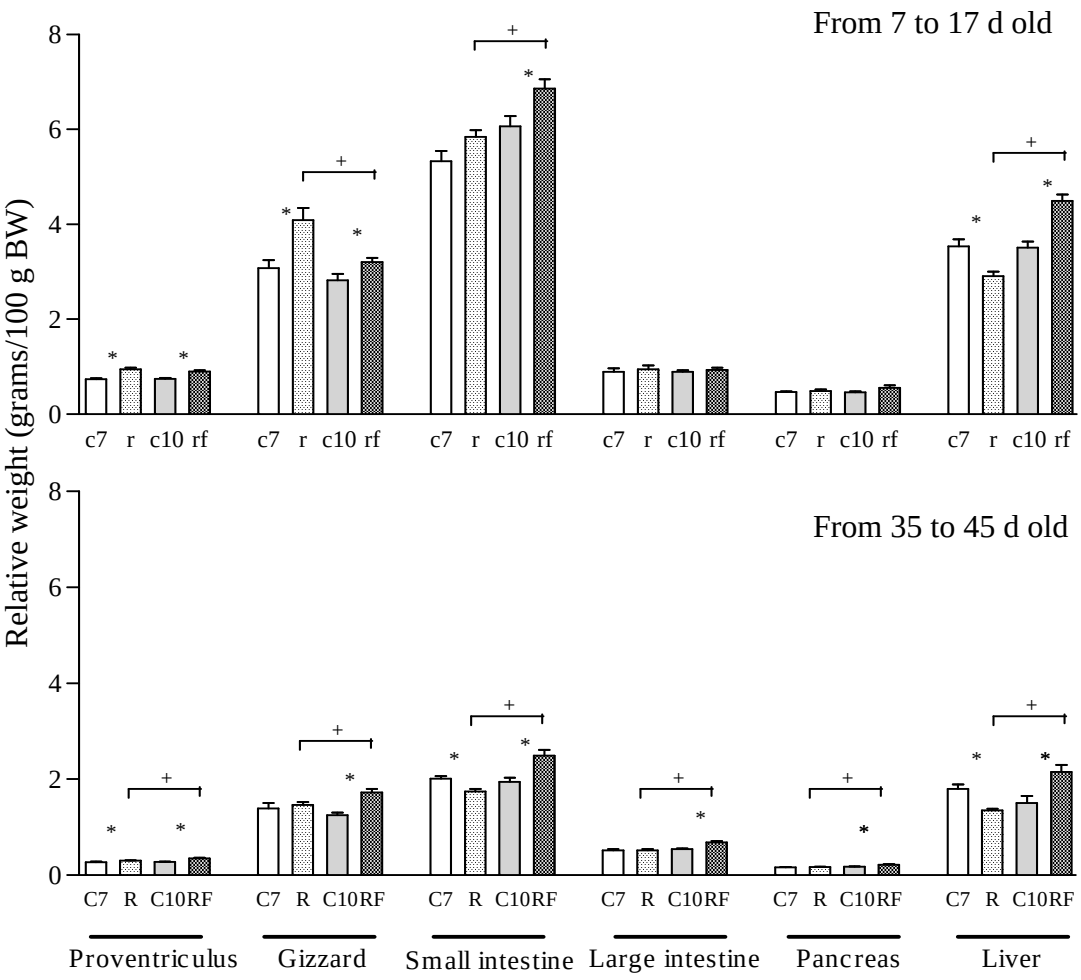

Figure 4. Effects of feed restriction and realimentation on relative weight of digestive organs of chickens. **c7** and **C7**: food *ad libitum* for 7 days; **r** and **R**: 70% food restriction for 7 days; **c10** and **C10**: food *ad libitum* for 10 days and **rf** and **RF**: 70 % food restriction for 7 days followed by realimentation for 3 days. Data are the mean ± sem. * means statistical difference between experimental and control group and + means statistical difference between feed-restricted and realimented group, Student's t test, $P<0.05$.

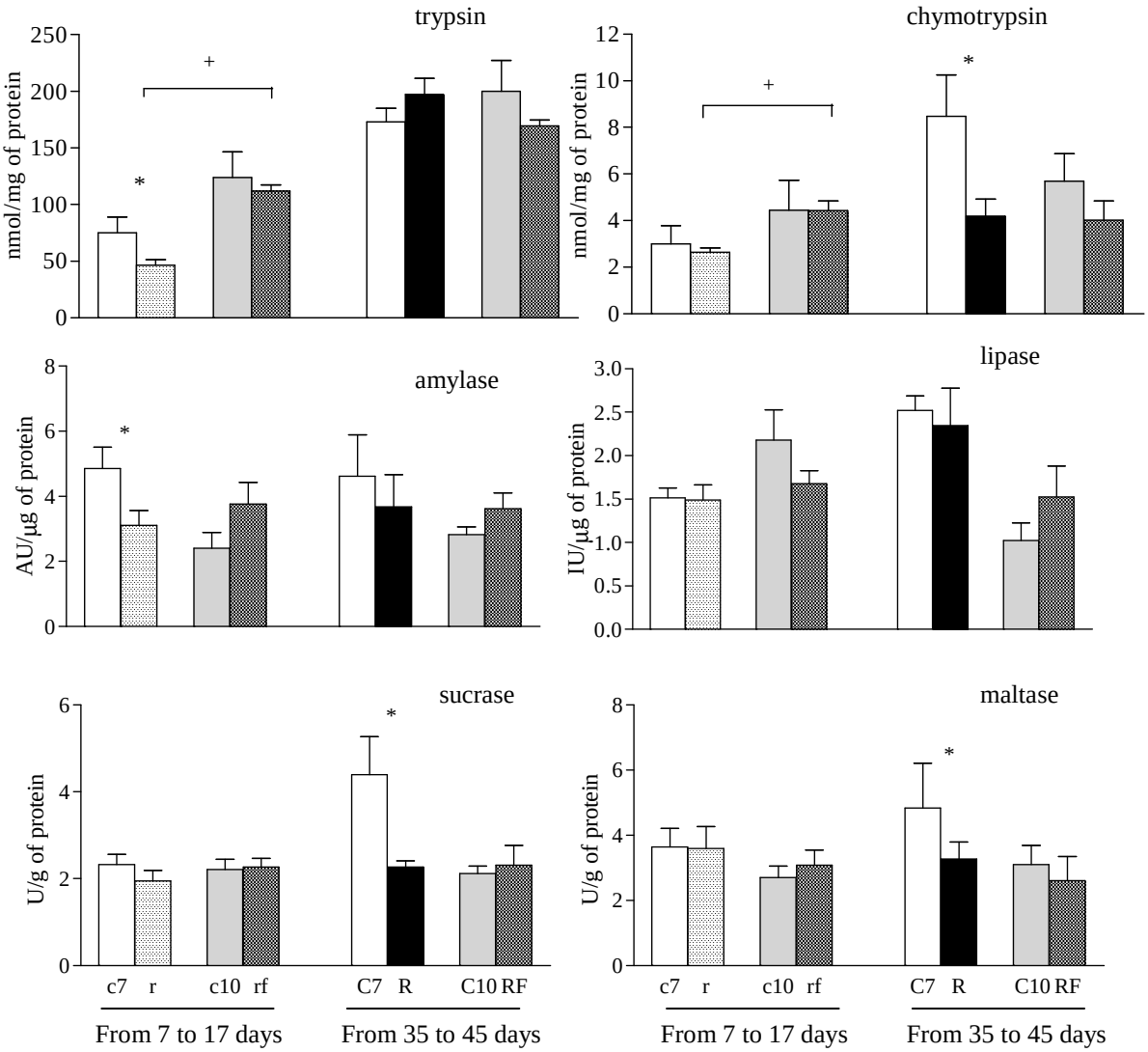

Figure 5. Effects of feed restriction and realimentation on pancreatic and intestinal activity in chickens. **c7** and **C7**: food *ad libitum* for 7 days; **r** and **R**: 70% food restriction for 7 days; **c10** and **C10**: food *ad libitum* for 10 days and **rf** and **RF**: 70 % food restriction for 7 days followed by realimentation for 3 days. Data are the mean ± sem. * means statistical difference between experimental and control group and + means statistical difference between feed-restricted and realimented group, Student's t test, $P<0.05$.

FEED RESTRICTION AFFECTS THE GENE EXPRESSION OF INTESTINAL ENZYMES AND NUTRIENTS TRANSPORTERS IN CHICKENS OF DIFFERENT AGES

Abstract

Feed restriction applied in chicken for rearing affects different systems, including the gastrointestinal tract. In fact, some studies show changes in enzymes and transporters activity in feed-restricted animals. However, there are few studies about the effect of feed restriction and realimentation on gene expression of enzymes and transporters. This study investigated these effects in chickens of two ages: 7 and 35 days old. Animals of each age were 70% restricted for 7 days and refed *ad libitum* for 3 days. The control groups were fed *ad libitum* during equal periods. Animals were daily weighed and were sacrificed in the end of each experimental period (7^{th} and 10^{th} days, according to the group). Total RNA of jejunal mucosa was extracted according to Trizol protocol and mRNA expression of SGLT1, GLUT2, PEPT1, aminopeptidase N (APN), sucrase-isomaltase complex (SI) and maltase was obtained by real time RT-PCR. During feed restriction, body weight was maintained or slightly decreased in the feed-restricted chickens of both ages. Feed restriction increased the expression of SGLT1, PEPT1, and APN in chickens of both ages and increased the expression of sucrase-isomaltase complex only in animals restricted from 35 days of age. Maltase and GLUT2 expression did not change in animals of both ages. After realimentation for 3 days, all parameters showed values similar to the control group. We concluded that feed restriction increases the enzymes and nutrient transporters expression, independently of the animal age, and these parameters return to the control values after realimentation.

Key words: feed restriction, intestinal enzymes, nutrient transporters, realimentation

Introduction

Feed restriction and fasting are common management strategies in chicken production, which have proven successful in reducing metabolic disorders, reproductive problems, skeletal deformities and controlling the body weight[1-3]. When submitted to feed restriction animals show adjustment of physiological functions in order to assure the survival. This capacity is denominated “phenotypic flexibility” and it is performed by behavioral, morphological and physiological changes[4-8].

Among the systems affected by feed restriction is the gastrointestinal system[9]. Changes in quality and quantity of nutrients affect the digestive and absorptive functions, mainly the activity of enzymes and transporters[10-12]. In fact, feed-restricted chickens from 7 to 14 days of age show increase in the activity of sucrase, amylase, lipase and trypsin immediately after the restriction period[13]. Also, the expression of SGLT1 mRNA in jejunum is increased in chickens of 42 days of age starved by 3 days[14].

However, there are discrepant results, which can be partially attributed to the diversity of experimental protocols, levels and duration of feed restriction[15]. Indeed, different effects on enzymes activities in response to feed restriction applied in chickens were reported in the literature, showing unchanged[16] or increased intestinal sucrase activity[13]. Furthermore, more discrepant results are found about the intestinal nutrient transport in response to semi-starvation, as showed in the review published by Ferraris & Diamond[15].

The gastrointestinal tract can also be affected by internal demand during the animal development. In fact, the gut surface and the activity of enzymes and nutrient transporters change during the development, resulting in variations of absorption and digestion[17-20]. Studies about the gene expression of transporters confirm age-related

variations[21]. However, studies about the gene expression of enzymes during the development and about effects of feed restriction on gene expression of enzymes and transporters in chickens are missing.

Thus, this work aimed to evaluate the jejunal expression of enzymes sucrase-isomaltase complex, maltase and aminopeptidase and nutrient transporters SGLT1, GLUT2 and PEPT1 in response to feed restriction and realimentation in two stages of chicken growth.

Material and Methods

A total of 64 male broiler chicks (Cobb) were obtained from a commercial hatchery (Frigorífico Mabella Ltda, Tatuí, Brazil) on the hatching day. Animals were housed in metal batteries in a room with controlled illumination, temperature and humidity. Water was offered *ad libitum* during all experiment. All procedures were approved by University Ethics Committee for Animal Research.

Feeding regimens were applied in chickens from 7 to 17 days old (groups identified by small letters) and 35 to 45 days old (groups identified by capital letters). At 7 or 35d old, chicks were weighed and divided into four treatment groups (figure 1), with 8 chickens per group: **r** and **R** - 70% food restriction for 7 days; **c7** and **C7** - food *ad libitum* for 7 days; **rf** and **RF** - 70 % food restriction for 7 days, followed by refeeding for 3 days, and **c10** and **C10** - food *ad libitum* for 10 days. Until 14 days of age the animals were fed with a common starter diet. After this, it was employed a grower diet (table 1).

Animals were weighed daily and the food amount supplied to feed-restricted chickens was equivalent to 30% of the quantity consumed by the fed *ad libitum* group on the previous day. The effects of treatments were determined at 14 and 17d old or at

42 and 45d old, according to the group. For this, the animals was weighed and killed by beheading.

Tissue sampling

To determine the expression of intestinal enzymes and nutrient transporters, jejunal mucosa was collected. For this, the jejunum segment (from the end of duodenal loop to the Meckel diverticulum) was opened longitudinally, mucosa was scraped off with a glass microscope coverslip and immediately frozen in -80ºC until required assay.

RNA extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA (n=5 per group) was extracted from jejunum mucosa using Trizol reagent (Invitrogen, Brazil) according to the manufacturer's protocol, and total RNA concentration was determinated using a spectrophotometer (NanoDrop ND 1000; NanoDrop Technologies, USA). One microgram of total RNA was incubated with DNAse I (1U/µg RNA; Invitrogen, , Brazil) and then reverse transcribed with SuperScript III (200U/µl; Invitrogen) and oligo-d(T) primer (Invitrogen). Reactions were performed at 42◦Cfor 15 min, 50◦C for 50 min and at 70◦ for 15 min for enzyme inactivation.

Relative qualitative (q) polymerase chain reaction (PCR) was performed using an ABI 7500 (Applied Biosystems, USA) with Power Sybr Green PCR Master Mix (Applied Biosystems). Reactions were performed in 25 µl volumes and the PCR cycling conditions were: 95ºC for 10 min followed by 40 cycles of denaturing at 95ºC for 10 s and then annealing at the temperatures given in Table 2 for 1 minute. The primer sequences, fragment size and annealing temperature for each gene are listed in table 2.

Primers for SGLT1, GLUT2, PEPT1 and APN were based on the sequences used by Gilbert *et al.*[21] and primer for ß-actina by De Boever *et at.*[22]. Primers for sucrase-isomaltase complex and maltase were designed based on the predicted chicken sequences (Genbank accession No XM_001235991.1 and XM_422811.2, respectively).

Reactions were optimised to provide maximum amplification efficiency for each gene (which were >90%). Each sample was analyzed in duplicate, and the specificity of each PCR product was determined by melting curve analysis and amplicon size determination in agarose gels. Negative controls (water replacing DNA) were run on every plate.

To determine which housekeeping gene should be used for data normalization, the geNorm program was used[23]. The results indicated that ß-actina was the most stable endogenous control for jejunal mucosa. The relative expression of each gene was calculated using $^{\Delta\Delta}$Ct method with efficiency correction[24]. Mean efficiency values for each gene were calculated from the amplification profiles of individual sample with LinRegPCR software[25].

Statistical analysis

Values were expressed as mean ± sem. Differences in the body weight between control and experimental groups were examined by Student's t-test. Effects in the body weight within group during both feed restriction period and realimentation period were analyzed by repeated measures ANOVA, followed by Dunnett´s test, considering as control the initial body weight of each period.

Differences in the gene expression between the feed-restricted and control groups (r x c7 and R x C7), realimented and control groups (rf x c10 and RF x C10) and feed-restricted and realimented groups (r x rf and R x RF) were established by Student's

t-test. Statistical analysis were performed using GraphPad Prism 5.0 (GraphPad Software, Inc). Probability of $P < 0.05$ was taken as significant.

Results

Body weight (Figure 2)

In both ages, initial body weight was similar among the four experimental groups. The body weight of feed-restricted birds from the first day of feed restriction to the end of realimentation period was significantly less than control groups.

Younger feed-restricted animals showed decrease of the body weight, but this difference was significant only in the 2nd and 3rd days of restriction (9 and 10d old, respectively), thereafter the body weight increased again, restoring its initial value (Dunnett´s test, $P>0.05$). The body weight of older feed-restricted animals showed a significant decrease (5.6%) during all restriction period when compared with the initial value (Dunnett´s test, $P<0.05$).

Animals fed *ad libitum* of either age increased the body weight. Younger animals showed 93.8 % increase from 2nd day to the final of experiment. The body weight of older animals increased by 25.8% during the same period.

During the realimentation period (3 days), the feed-restricted animals of both ages augmented the body weight (Dunnett´s test, $P<0.05$). The weight gain was greater than respective control to the youngest animals (92.5 x 49.6%), as well as the oldest chickens (16.3 x 6.1%). In spite of this response, the body weight of animals previously restricted did not reach the value of control group.

Gene expression (Figure 3)

Immediately following the period of feed restriction, the relative expression of SGLT1, APN and PEPT1 in jejunum of feed-restricted groups was higher than their control, in animals of both ages. The sucrase-isomaltase complex expression was higher only in older feed-restricted chickens (Student's t-test, $P<0.05$).

The increase of gene expression in younger feed-restricted animals was 181.4% for APN, 116.7% for SGLT1 and 80.4% for PEPT1. However, in older feed-restricted animals the PEPT1 transporter presented the highest increase (195.9%), followed by sucrase-isomaltase complex, APN and SGLT1 (159.1, 143.5 and 84.2%, respectively). Although maltase had presented an increase of expression in feed-restricted groups, it was not different from control group. The GLUT2 expression was not affected by feeding restriction and realimentation.

There was no significant difference between the realimented and control groups in chickens of both ages (Student's t-test, $P>0.05$). In comparison with feed-restricted animals, younger realimented chickens presented a decrease of the relative expression of sucrase-isomaltase complex (SI), maltase, APN, SGLT1 and PEPT1 expression, while older realimented chickens presented a decrease of SGLT1 and APN relative expression.

Discussion

The molecular aspects of intestinal digestion and absorption in feed-restricted and realimented chickens were evaluated in this study. Feeding regimens was applied in two ages, 07 and 35 days, to comparing the ability of adjustment in the expression of intestinal enzymes and nutrient transporters in two different phases of development. It was observed different responses of mRNA expression among the studied enzymes and

transporters. In fact, there was an increase in RNAm expression for SGLT1, PEPT1 and aminopeptidase (APN), but not for GLUT2 and maltase in feed-restricted chickens of both ages. Also, the sucrase-isomaltase complex expression augmented only in feed-restricted animals in the final phase of growth. The increase in gene expression was not maintained after the reestablishment of feeding.

Up-regulation in the expression of genes involved in nutrient digestion and absorption in response to feed restriction or fasting has been observed in fishes, birds and mammals[26-30]. In chickens, Gilbert *et al.*[29] studied the influence of feed restriction on peptide, amino acid and monosaccharide transporter and aminopeptidase mRNA abundance and they observed an increase only in peptides transporter (PEPT1), whose expression was higher than in chickens given free access to food. Ihara *et al.*[27] found similar response to APN in rat intestine after starvation.

Aminopeptidase, PEPT1 and SGLT1 play important role in the final digestion and absorption of dietary nutrients in the brushborder membrane, providing amino acids and glucose for cellular metabolism. An increase of production of SGLT1, PEPT1 and APN mRNA for feed-restricted birds, and a consequent increase in absorption and digestion, could contribute to the maintenance or a slight decrease of body weight, as observed in this study for feed-restricted chickens of both ages.

The increase in enzymes and nutrients transporters expression showed in feed-restricted chickens was related to a decrease in the intestinal weight (Duarte *et al.*, unpublished results). The enhancement of gene expression occurs in parallel with a decrease in the intestinal absorptive surface area that shows less villus height, cell area and cell mitosis[31]. According to Ferraris & Diamond[15], the intestine of feed-restricted animals presents a change in the ratio of transporting and non-transporting cells in the

villus in response to feed-restriction, maintaining in its mucosa mainly mature cells capable of transport.

However, a close correlation between gene and protein expression is not observed for all enzymes and nutrient transporters. In fact, in previous study we showed that feed restriction decreases the amount of SGLT1 protein and α-methyl-D-glucoside uptake in the brushborder membrane vesicles[32, 33]. This discrepancy was also noted by Gal-Garber *et al.*[14] that observed a high expression of SGLT1 in feed-restricted chicks parallel with low affinity and low activity of the transporter. These results indicate that the brushborder glucose transport and SGLT1 protein expression are independent of SGLT1 mRNA and can be explained by a post-transcriptional regulation of this gene[15]. However, for other enzymes and nutrients transporters presents in the enterocyte (*e.g.*, APN, GLUT5 and GLUT2), changes in mRNA occur in parallel with changes in number of transporters, suggesting a transcriptional regulation of some genes[15, 27].

The over-expression of genes involved in nutrient digestion and absorption in response to feed restriction might be an adaptive mechanism that occurs to maximize the ability to absorb carbohydrates and proteins once refeeding occurs, as suggested by Ihara *et al.*[27]. In general, the presence of specific substrates is an important factor regulating enzyme activity and transporter expression, and this regulation depends on the turnover rate of intestinal cells[15]. It is known that jejunal epithelium of chickens is replaced in 48 hours[34]. Thus, it is probable that a high insertion of enzymes and nutrient transporters occurs in the villus in the first day after the reestablishment of feeding. However, three days after starting realimentation, the expression of all genes is restored to the control group level. This response is expected, considering that mRNA is degraded in the upper villus cells[9].

After realimentation for 3 days, the relative expression of APN and SGLT1 was decreased in chickens of both ages when compared to feed-restricted groups. Moreover, realimentation applied from 14 to 17 was able to decrease PEPT1, maltase and sucrase-isomaltase complex expression compared to feed-restricted group. Our results differ from the pattern described by Gal-Garber *et al.*[14], that showed a higher increase in the SGLT1 expression in refed animals for 2 days compared to control and starved animals for 4 days. The discrepancies between our results and the presented by Gal-Garber *et al.*[14] can be explained by the difference on feed restriction and realimentation protocols and housekeeping gene used for normalization.

The evolution of body weight during feed restriction was different considering animals of both ages. Animals submitted to feed restriction from 7 to 14 days maintained the body weight during the feed restriction, while animals restricted from 35 to 42 days decreased slightly the body weight. Although, 70% feed restriction can be considered severe[35], Palo *et al.*[36] observed that chickens from 7 to 14 days old submitted to this level of restriction gain approximately 6g per day. After realimentation, feed-restricted animals of both ages showed a higher increase of the body weight compared to respective control group. Although compensatory growth following feed restriction has been showed by many authors[37, 38], it did not occur in this experiment, maybe because of the short time of realimentation. Moreover, different responses are expected for different feeding protocols, considering that compensatory growth can be influenced by animal age and severity of feeding restriction[39].

The results of the present study demonstrated that feed restriction increases the jejunal expression of enzymes and transporters involved in the digestion and absorption of nutrients. Although the mechanisms are not clarified, it seems that this increase in

gene expression is an adaptive response that provides a lower body weight loss during feed restriction and a faster body weight gain during realimentation.

Acknowledgments

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Table 1. Composition and calculated dietary analysis

	Starter[1] *1 to 14 d*	*Grower*[2] *15 to 45 d*
Soybean meal	34.30	30.70
Corn	59.34	62.03
Calcium calcite	0.90	0.89
Dicalcium phosphate	1.80	1.68
Soy oil	2.30	3.29
Salt	0.47	0.47
L-Lysine	0.20	0.25
L-treonine	0.05	0.05
DL-Methionine	0.24	0.24
Mineral-vitamin mix1	0.40	0.40
Total	100.00	100.00
Calculated analysis		
ME (MJ/kg)	12.56	12.98
Crude protein (%)	20.80	19.43
Crude fiber (%)	2.88	2.73
Calcium (%)	0.89	0.85
Available phosphorus (%)	0.44	0.42
Methionine (%)	0.53	0.51
Methionine + cysteine (%)	0.81	0.78
Lysine (%)	1.16	1.11

[1]Mineral and vitamin mix (1 to 14 d) supplied per kilogram of diet: Cu, 1.25 mg; Fe, 62.62 mg; I, 0.025 mg; Mn, 67.5 mg; Se, 0.225 mg; Zn, 68.75 mg; folic acid 1.25 mg; pantothenic acid, 12.5 mg; BHT 2.5 mg; biotin, 0.125 mg; choline chloride, 750 mg; niacin, 37.5 mg; vitamin A, 12,500 IU; vitamin B_1, 2.5 mg; vitamin B_{12}, 25 mg; vitamin B_2, 5 mg; vitamin B_6, 5 mg; vitamin D_3, 2,500 IU; vitamin E, 25 mg; vitamin K_3, 2.5 mg; monensin, 125 mg.

[2]Mineral and vitamin mix (15 to 45 d) supplied per kilogram of diet: Cu, 5 mg; Fe, 25.05 mg; I, 0.01 mg; Mn, 27 mg; Se, 0.09 mg; Zn, 55 mg; folic acid 1 mg; pantothenic acid, 10 mg; BHT 2.0 mg; biotin, 0.1 mg; choline chloride, 600 mg; niacin, 30 mg; vitamin A, 10,000 IU; vitamin B_1, 2 mg; vitamin B_{12}, 20 mg; vitamin B_2, 4 mg; vitamin B_6, 4 mg; vitamin D_3, 2,000 IU; vitamin E, 20 mg; vitamin K_3, 2 mg; monensin, 100 mg.

Table 2. Information of specific primers used for amplification in real-time polymerase chain reaction

Gene	*Primer sequence*	*Size (bp)*	*Annealing temperature (ºC)*
SGLT1	S 5´ GCCGTGGCCAGGGCTTA 3´ A 5´ CAATAACCTGATCGTTGCACCAGTA 3´	66	61
GLUT2	S 5´ CACACTATGGGCGCATGCT 3´ A 5´ ATTGTCCCTGGAGGTGTTGGT 3´	68	63
PEPT1	S 5´ CCCCTGAGGAGGATCACTGTTGGCATGTT 3´ A 5´ CAAAAGAGCAGCAGCAACGA 3´	66	60
APN	S 5´ AATACGCGCTCGAGAAAACC 3´ A 5´ AGCGGGTACGCCGTGTT 3´	70	59
SI complex	S 5´ ACAGCAAATCGCTTCCGGTT 3´ A 5´ AAAGCACTTTCCCGCTCACT 3´	175	59
Maltase	S 5´ TTGCCTCCCGGATACTCAGTGTTT 3´ S 5´ TTAGCAGCGCATCCAGGAAGTT 3´	170	59
ß-actina	S 5´ CACAGATCATGTTTGAGACTT 3´ A 5´ CATCACAATACCAGTGGTACG 3´	101	55

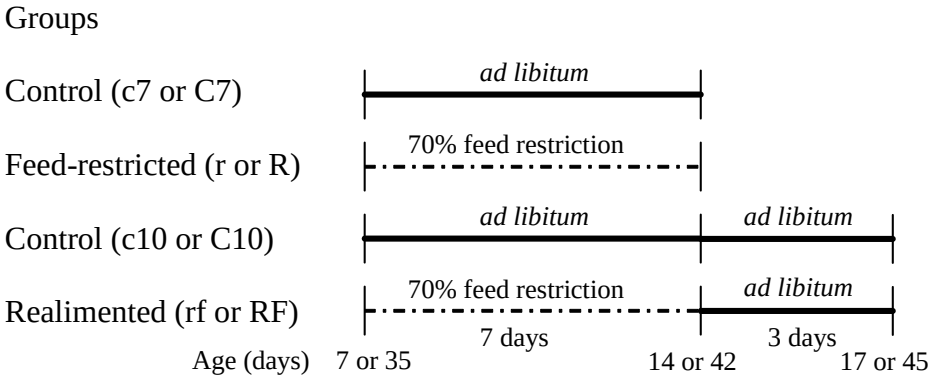

Figure 1. Experimental design showing the groups.

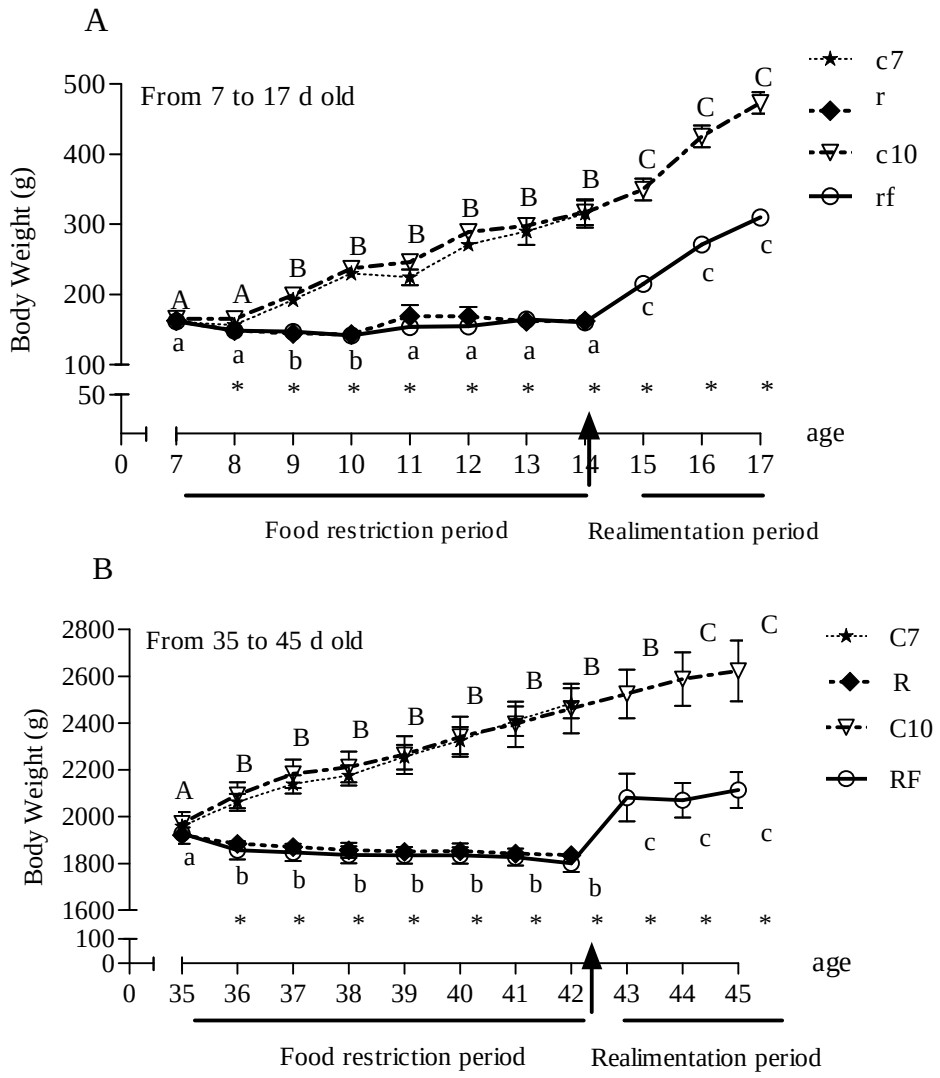

Figure 2. Effect of feed restriction and realimentation on the body weight (means ± sem) from 7 to 17d of age (A) and from 35 to 45d of age (B). **c7** and **C7**: food *ad libitum* for 7 days; **r** and **R**: 70% food restriction for 7 days; **c10** and **C10**: food *ad libitum* for 10 days and **rf** and **RF**: 70 % food restriction for 7 days followed by realimentation for 3 days. ↑ indicates the end of feed restriction period. * indicates significant difference between control and feed-restricted animals (Student´s t-test, $P<0.05$). Capital letters indicate significant differences within control group and small letters show significant differences within feed-restricted group (Repeated measures ANOVA and Dunnett´s test, $P<0.05$, considering the initial body weight of the period as control, d 0 and d 7).

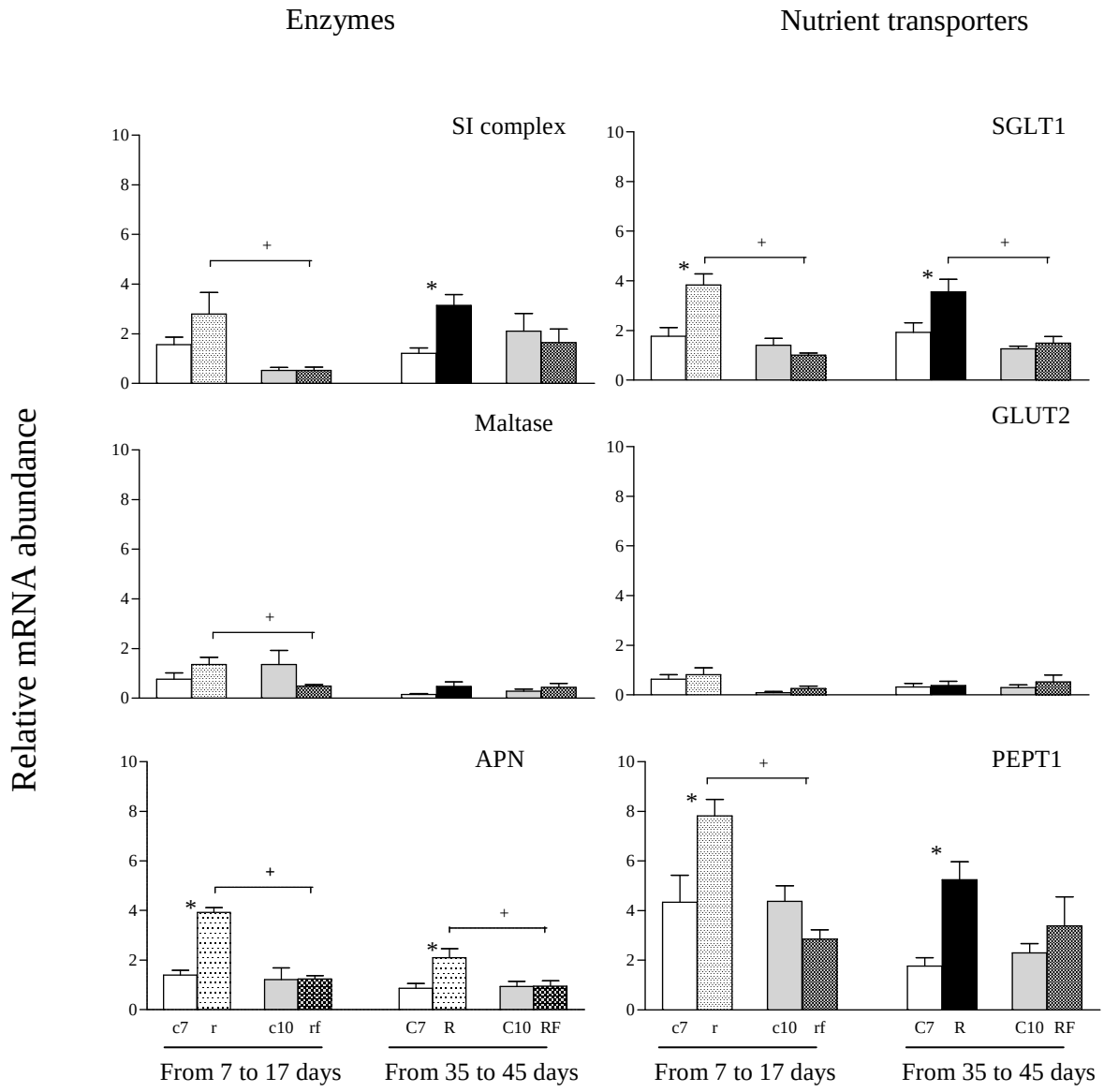

Figure 3. Effects of feed restriction (c7 x r and C7 x R) and realimentation (c10 x rf and C10 x RF) in the transcript levels of Sucrase-isomaltase complex, maltase, Aminopeptidase (APN), SGLT1, GLUT2 and PEPT1 gene, determined by real-time polymerase chain reaction in jejunal mucosa of chickens. **c7** and **C7**: food *ad libitum* for 7 days; **r** and **R**: 70% food restriction for 7 days; **c10** and **C10**: food *ad libitum* for 10 days and **rf** and **RF**: 70 % food restriction for 7 days followed by realimentation for 3 days. Data are the mean ± sem. Data were normalized against the ß-actina, with results expressed relative to the control sample using the $^{\Delta\Delta}$Ct method with efficiency correction. * means statistical difference between experimental and control group and + means statistical difference between feed-restricted (R) and realimented group (RF), Student's t test, $P<0.05$.

EFFECTS OF FEED RESTRICTION AND REALIMENTATION ON NA^+-D-GLUCOSE COTRANSPORTER IN JEJUNUM OF DOMESTIC CHICKEN

Introduction

The process of assimilation of carbohydrates in the small intestine of chickens is very important since starch is the major carbohydrate of their diet. This nutrient source is broken down to sugars by the action of enzymes within the gut lumen and in the brushborder membrane. The end-products generated by this process are absorbed into the cell mediated by transporter proteins. These proteins recognize and transport the substrates from the lumen to the inside of the cell across the brushborder membrane and thereafter across the basolateral membrane to the blood[1,2]. Glucose is the most important end-product of the starch digestion and it is transferred into the enterocyte by active transport via a sodium-coupled glucose transporter, SGLT1[3]. Thereafter, glucose is transported from the cytosol through basolateral membrane to the blood by facilitated diffusion via GLUT2.

The SGLT1 was the first member identified from a large gene family (SLC5) and it is one of the most intensively studied membrane transporters. According to Wright *et al.*[4], SGLT1 is a multifunctional protein that plays the role of Na^+/glucose cotransporter, Na^+ uniporter, water channel, water cotransporter and urea cotransporter.

There are many evidences that intestinal nutrient transporters are adaptively regulated by the dietary levels of their substrates[6-11]. This regulation can be made by specific mechanisms such as changes in density of glucose transporters, and by nonspecific mechanisms such as changes in plasma membrane lipid composition[9]. In fact, rats fed with a high-carbohydrate diet show an increase in intestinal glucose transport, in site density of glucose transporters as well as in SGLT1 mRNA levels[12,13]. Moreover, the nutrient transport systems are influenced by the lipid composition and physical state of the membrane during gut development[14,15].

In chicken production, feed restriction and fasting are common strategies to reduce metabolic disorders, reproductive problems, skeletal deformities and to control the body weight[16-18]. This management can alter intestinal digestion and absorption, considering that the presence of luminal nutrients regulates these processes in the intestine. In fact, glucose uptake in chickens is increased as a response to acute fasting[19]. Moreover, the expression of SGLT1 mRNA is enhanced in the fasted chickens in parallel to low glucose uptake in BBMV[20]. These responses show that changes in SGLT1 protein and in glucose transport rate seem to be independent of changes in the level of SGLT1 mRNA. In fact, according to Ferraris and Diamond[9], this discrepancy can be attributed to a post-transcriptional regulation of this gene.

However, different results were observed by Gal-Garber *et al*.[20] when studied realimented chickens after fasting period. In these animals the glucose uptake and the SGLT1 expression were higher than control group.

Taken together, these studies indicate that the absence of food in the gastrointestinal tract causes a decrease of transporters, which could be explained by a low energy supply to organism. However, there are few studies about the effects of feed restriction and realimentation on the intestinal D-glucose transport by SGLT1 in chickens. Thus, this study aimed to evaluate the uptake of α-methyl-D-glucopyranoside and the abundance of Na^+/D-glucose cotransporter (SGLT1) in jejunal brushborder in response to feed restriction and realimentation in chicken.

Material and Methods

A total of 18 male label chickens (*Gallus gallus domesticus*) were obtained from a commercial hatchery (Lliçà D´Amunt, España) at 21 days old. Animals were housed individually in metal batteries under controlled temperature (at 34ºC during the

1st week after hatching and at 25ºC thereafter) and humidity, with an 18:6 h light-dark cycle. Water was offered *ad libitum* during all experiment. The chickens were fed with a grower chick diet (pollos Label Crecimiento, Nanta S.A., España) containing 18.5% of crude protein. The metabolizable energy content was 12.98 MJ/kg diet. All procedures were approved by University Ethics Committee for Animal Research.

Animals were acclimatized to the conditions for 14 days, before conducting the following feeding experiments. At 35 d of age, chickens were weighted and divided in four experimental groups: **R** - 70% food restriction for 7 days (n=6); **C7** - food *ad libitum* for 7 days (n=3); **RF** - 70 % food restriction for 7 days, followed by realimentation for 3 days (n=6); and **C10** - food *ad libitum* for 10 days (n=3).

Animals were weighed daily and the food amount supplied to feed-restricted chickens was equivalent to 30% of the quantity consumed by the *ad libitum* fed chickens on the previous day. The animals were sacrificed in the morning of seventh or tenth day of the experiment, according to the respective experimental group, by beheading without previous withholding of food.

Tissue sampling

The jejunum (from the end of the duodenal loop to Meckel´s diverticulum) was removed, freed from adherent mesenteric tissue and immediately flushed with ice-cold saline solution in the presence of phenylmethylsulfonyl fluoride (0.2mmol/l), LiN_3 (0.41 µmol/l) and benzamidine (0.1 mmol/l). The segment was then placed on an ice-cold glass plate and slit longitudinally so that muscle of the jejunum flattened out on to the cold plate. Mucosal scrapings were frozen in liquid nitrogen and stored at -80ºC.

BBMV preparation

BBMV were isolated from jejunal mucosal scrapings by an $MgCl_2$ precipitation method[15]. All the steps were carried out at 4ºC. The mucosal scrapings were homogenized for 30 s in 30ml of a buffer containing 100 mmol/l mannitol, 2 mmol/l N-HEPES/Tris, (pH 7.1), 0.2 mmol/l phenylmethylsulfonyl fluoride, 0.2 mmol/l benzamidine and 0.41 µmol/l LiN_3. The sample was then placed in a Waring blender at maximal speed and the homogenate was filtered through nylon stocking material and homogenized for 30 s at high speed. $MgCl_2$ was added to reach a final concentration of 10 mmol/l. After stirring for 20 min, the suspension was centrifuged at 3,000 g for 30 min. The supernatant was centrifuged at 30,000 g for 35 min. The resultant pellet was resuspended in 100 mmol/l mannitol, 2 mmol/l HEPES-Tris (pH 7.4), 0.1 mmol/l $MgSO_4$, and 0.41 µmol/l LiN_3. This suspension was homogenized with a glass-Teflon homogenizer (40 up-down strokes) and centrifuged at 30,000 g for 35 min. The final pellet containing purified BBMV was resuspended in a medium of 300 mmol/l mannitol, 0.1 mmol/l $MgSO_4$, 0.41 µmol/l LiN_3, and 20 mmol/l HEPES-Tris (pH 7.5) and homogenized with a 27-gauge needle. The vesicles were frozen, stored in liquid nitrogen in 150 µl aliquots, and used during a period of 14 days.

Measurement of α-methyl-D-glucopyranoside transport

The α-methyl-D-glucopyranoside (αGLc1Me) was used as substrate since it is a D-glucose specific only for SGLT1. The uptake of α-methyl-D-glucopyranoside was measured at 38ºC by a rapid filtration technique for periods of time ranging from 1s to 30 min. For each uptake, 10µl BBMV suspension were rapidly mixed with 40 µl of the incubation medium containing 125mmol/l mannitol, 125 mmol/l NaCl, 0.1 mmol/l Mg SO_4, 0.41 µmol/l LiN_3, and 25 mmol/l HEPES-Tris (pH 7.4), 1 mmol/l of α-methyl-D-

glucopyranoside, an aliquot of α-methyl-D-[^{14}C]glucopyranoside. At selected incubation times, the uptake was stopped by the addition of 1ml of an ice-cold stop solution containing 300mmol/l mannitol, 0.41 µmol/l LiN_3, 0.1 mmol/l Mg SO_4, 20 mmol/l HEPES-Tris (pH 7.4). The resulting suspension was rapidly filtered under negative pressure through 0.22 µ pore size cellulose nitrate filters (Millipore, USA) and washed with 2 ml ice-cold stop solution. The filters were dissolved in Biogreen-6-cocktail (Sharlau, Spain), and the radioactivity retained was measured by scintillation counter (Packard Tricarb, USA). Nonspecific radioactivity binding to the filters was obtained by adding the stop solution to reaction tubes immediately after addition of the vesicles. This nonspecific binding was subtracted from the total radioactivity of each sample. All the experiments were performed in duplicate.

SDS-Page and Western blot analysis

Measurements of sodium-glucose transporter isoform 1 (SGLT1) abundance in BBMV of chicken jejunum were examined by Western blot analysis as previously described[21]. For this, similar amounts of BBMV protein (30 µg) were solubilized by boiling in the presence of SDS and 2-mercaptoethanol and were subjected to SDS-PAGE with a 10% linear polyacrylamide gel. After electrophoresis, the proteins were electrophoretically transferred from the unstained gel to nitrocellulose membranes in a transfer buffer containing 20 mmol/l Tris, 150 mmol/l glycine, and 20% methanol; transfer was done for 1 h at constant voltage of 100 V at 4 ºC using a Trans-Blot apparatus (Bio-Rad, USA). Non-specific binding sites were first blocked with PBS containing 0.05% Tween 20 (TBT) and 4% BSA. Blots were incubated overnight at 4 ºC with a rabbit polyclonal antibody (generously donated by Dr. M. Kasahara, Teikyo University, Tokyo, Japan) raised against a synthetic peptide corresponding to amino

acids 564 to 575 of the deduced amino acid sequence of rabbit intestinal SGLT1[22] diluted to 1:5,000. The membranes were washed (3 × 10 min) with TBT. Then, anti-SGLT1 antibody was detected by ECL chemiluminiscence (Biorad, USA) using a peroxidase-conjugated antirabbit IgG as a secondary monoclonal antibody (1:3,000). Membranes were also incubated with a mouse anti-actin monoclonal antibody following the same protocol. After detection, the samples were measured by scanning densitometry using Image J analysis software (NIH, USA).

Statistical analysis

Values were expressed as mean ± sem. Significant differences between means of feed-restricted and control group (R x C7), realimented and control group (RF x C10) and realimented and feed-restricted group (RF x R) were established by Student's t-test, with $P<0.05$ considered to indicate significant differences.

Results

Na^+-dependent uptake of methyl-α-D-glucopyranoside across BBMV isolated from enterocytes (Figures 1 and 2)

In the presence of 125 mmol/l extravesicular-to-intravesicular Na^+ gradient, all animal groups presented an overshoot at 10 s, that is a typical transient increase in the intravesicular concentration of sugar which indicates the capacity of the BBMV to accumulate hexoses against a concentration gradient. The uptake of α-methyl-D-glucopyranoside at equilibrium (30min) was identical in all groups.

Feed-restricted animals (R) showed a lower ($P<0.05$) value in the magnitude of the overshoot compared to the control group. This difference represented a decrease

by 55.1% in response to feed restriction. There was no significant difference ($P>0.05$) in the overshoot level between realimented (RF) and control group (C10). Realimented animals (RF) presented an increase ($P<0.05$) in the α-methyl-D-glucopyranoside overshoot in comparison to feed-restricted animals by 61.4%.

Abundance of SGLT1 (Figure 3)

Figure 3 shows a typical Western blot with rabbit polyclonal antibody raised against the 564-575 amino acid sequence of rabbit SGLT1. The antibody recognized a single band of 75 kDa. The densitometric analysis indicated the same pattern of responses found for α-methyl-D-glucopyranoside uptake into BBMV preparations. The relative abundance of SGLT1 in feed-restricted (R) animals decreased ($P<0.05$) by 66.6% compared to the control group (C7). Realimented group (RF) presented similar value ($P>0.05$) to the control group (C10), but in comparison to feed-restricted animals, realimented group showed an increase ($P<0.05$) by 483.05% in relative abundance of SGLT1.

Discussion

It is known that the digestive tract is able to change in response to differences in the amount of food ingested to maintain the homeostasis and the energy supply for cells. In this study, we investigated the effects of 70% feed restriction and realimentation on the molecular level of sugar intestinal absorption. Our results demonstrated that chickens show alterations in the intestinal glucose absorption in response to feed restriction and realimentation.

Jejunal Na^+-dependent α-methyl-D-glucopyranoside uptake and SGLT1 abundance decreased in BBMV of feed-restricted chickens. After realimentation,

chickens show an increase of Na^+-dependent α-methyl-D-glucopyranoside uptake and SGLT1 protein expression compared to feed-restricted animals. This increase was sufficient to reach the values presented by fed *ad-libitum* chickens, indicating that the functions involved in D-glucose absorption were restored.

Initial rates and maximal overshoot levels of Na^+-dependent glucose transport were lower in the feed-restricted group compared to the control and realimented groups. The differences in Na^+-dependent D-glucose transport found between feed-restricted and the other groups in response to feed restriction was not due to variations in the purity or size of BBMV preparations, considering the transport at equilibrium (30 min) was similar in all groups.

The protocol of feed restriction applied in this study caused a decreased on α-methyl-D-glucopyranoside transport per milligram of BBM protein. This result is consistent with the data of Gal-Garber *et al.*[20], which verified that starved chickens show a lower glucose uptake in BBMV accompanied by higher expression on SGLT1 mRNA when compared to control and realimented groups. According to these authors, these responses are related to higher turnover and degradation rate at the microvillus membrane. However, others authors demonstrated that duodenal glucose absorption increases in response to acute and intermittent starvation in chickens *in vivo*[19]. In rats, some authors showed an increase in the affinity of the sugar transporters in the small intestine during feed restriction[23-25]. But, Hopfer[26] did not verify effect of feed restriction on the D-glucose active transport.

Furthermore, we observed that the relative abundance of SGLT1 of chicken jejunum measured by Western blot decreased after feed restriction. This response was well correlated with the decrease observed in methyl α-D-glucopyranoside transport. Thus, these results could explain the decrease in transport of methyl-α-D-

glucopyranside, since the molecular mass was similar between groups, indicating that there were no structure changes in SGLT1. Moreover, a decrease of the amount of intestinal SGLT1 in feed-restricted rats compared with fed rats was also observed by Pan *et al*.[27]. In contrast, Habold *et al*.,[28] and Das *et al*.,[29] found that fasting caused an overall increase on intestinal D-glucose absorption. Notwithstanding, it is very difficult compare these results because of great variations in the feed restriction protocols and in the methods to measure the intestinal transport.

The decrease in the amount of SGLT1 accompanied by a reduction in D-glucose transport in response to feed restriction can be attributed to changes in the morphology of the small intestine[30]. Indeed, according to Shamoto & Yamauchi[31], duodenal villus heights of chicks fasted for 3 days decreased and this change is correlated to the intestinal functions.

Thus, the realimentation for 3 days after feed restriction for 7 days was sufficient to restore the values of α-methyl-D-glucopyranoside uptake and SGLT1 abundance to the level of control group. The recovery of digestive structure and functions in realimented chickens after to be submitted to fasting or feed restriction were found ranging from few hours to some days[31,32]. In fact, three days after realimentation intestinal weight and enzymes activities of feed-restricted chickens were restored to the level corresponding to animals fed *ad libitum* (Duarte *et al*., unpublished data). Moreover, long-term fasted chickens increased the intestinal villus height, cell area and cell mitosis one day after realimentation[32].

In conclusion, the results demonstrated that the feed restriction affects the jejunal sugar transport, decreasing SGLT1 abundance and consequently the transport of α-methyl-D-glucopyranoside. The results found in this study and by Yamauchi *et al*.[31] can be interpreted as adaptive responses that provide benefits of reducing maintenance

costs of the small intestine during feed restriction. Moreover, this study showed that the reestablishment of feeding restores the glucose uptake in the small intestine.

This is the first study on the effects of feed restriction and posterior realimentation on the abundance of SGLT1 in the small intestine and it provides complementary informations about the adaptive changes in the small intestine in response to food intake.

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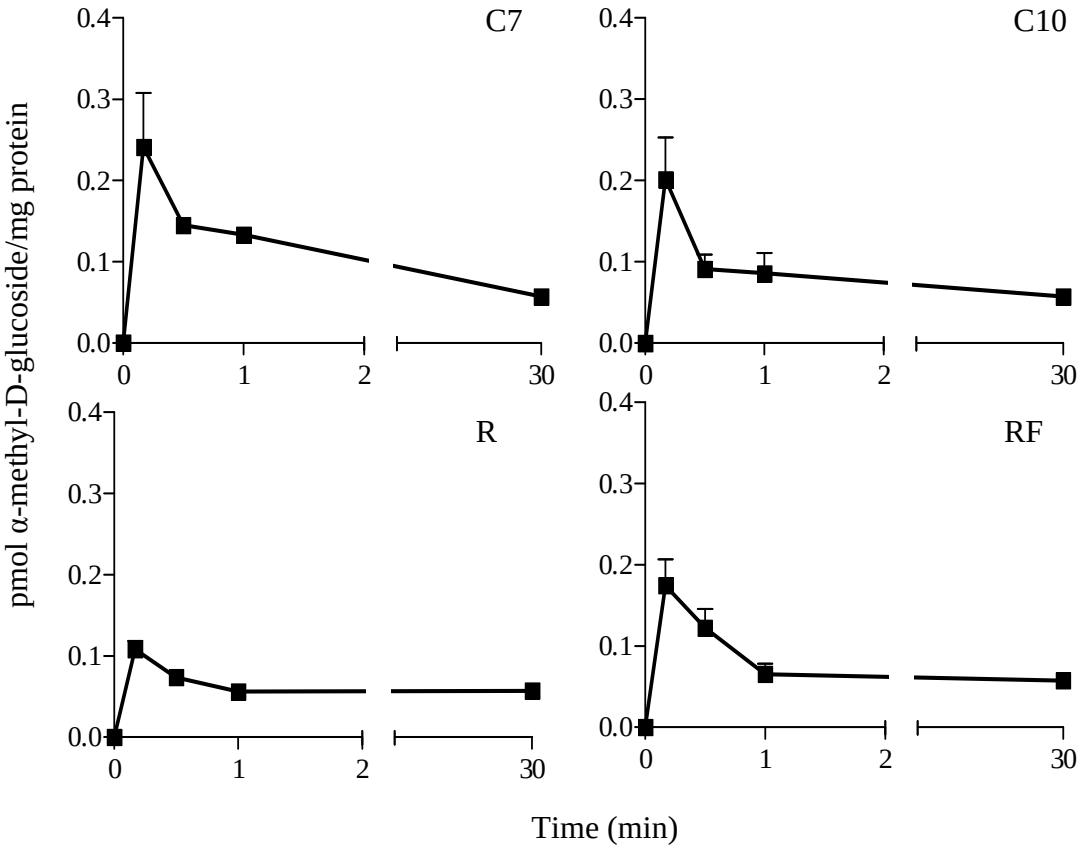

Figure 1. Time course of 1mmol/l of α-methyl-D-glucopyranoside uptake by brushborder membrane vesicles (BBMV) of chicken jejunum under different feed regimens: **C7**: food *ad libitum* for 7 days; **R**: 70% food restriction for 7 days; **C10**: food *ad libitum* for 10 days and **RF**: 70 % food restriction for 7 days followed by realimentation for 3 days. Data are shown for uptakes in presence of an initial 125mmol/l NaCl. Results are expressed as means ± sem.

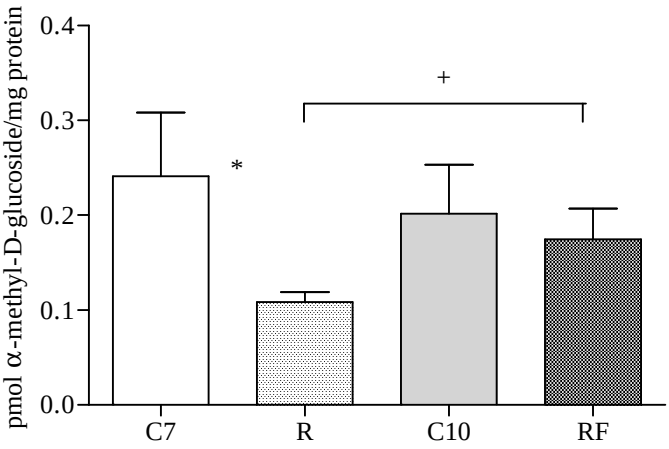

Figure 2. Initial rates (overshoot at 10 s) of 1mmol/l α-methyl-D-glucopyranoside uptake by brush-border membrane vesicles (BBMV) of chicken jejunum in response to feed-restriction and realimentation. **C7**: food *ad libitum* for 7 days; **R**: 70% food restriction for 7 days; **C10**: food *ad libitum* for 10 days and **RF**: 70 % food restriction for 7 days followed by realimentation for 3 days. Results are expressed as means ± sem. * means statistical difference between experimental and control group and + means statistical difference between feed-restricted and realimented group, Student's t test, $P<0.05$.

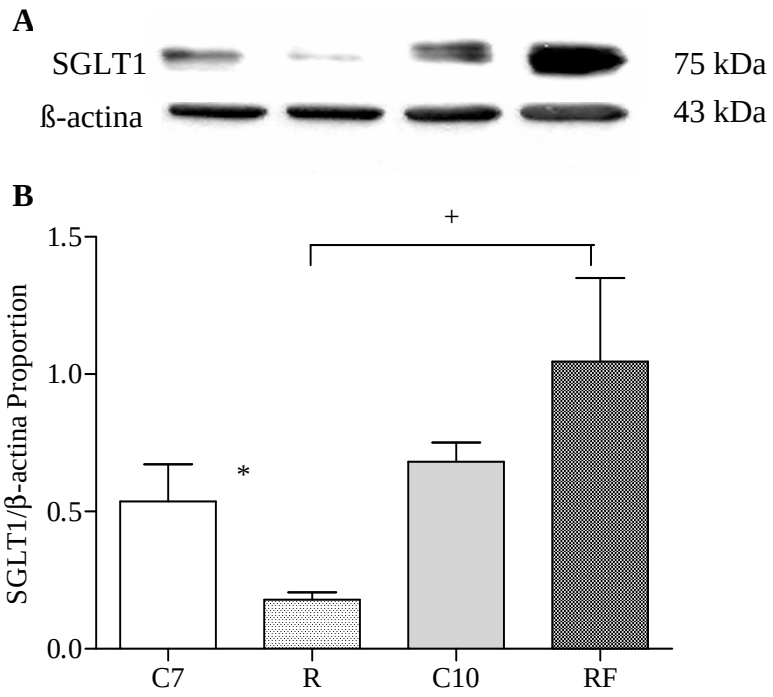

Figure 3. (Panel A) Western blot analysis of sodium-glucose transporter isoform 1 (SGLT1) and ß-actina, in the brushborder membrane vesicles (BBMV) of chicken jejunum from **C7**: food *ad libitum* for 7 days (lane 1), **R**: 70% food restriction for 7 days (lane 2), **C10**: food *ad libitum* for 10 days (lane 3) and **RF**: 70 % food restriction for 7 days followed by realimentation for 3 days (lane 4). Each lane contained 30µg of protein. A single 75 kDa band was detected for SGLT1 and a single 43 kDa band for ß-actina. (Panel B) Relative abundance of SGLT1 was measured by optical densitometry. Data show the means ± sem of the four experimental groups. * means statistical difference between experimental and control group and + means statistical difference between feed-restricted and realimented group, Student's t test, $P<0.05$.

CONCLUSÕES GERAIS

- A restrição alimentar restringe funções e peso de órgãos do trato gastrointestinal de frangos, porém diferentes parâmetros são afetados nas fases inicial e final do desenvolvimento;

- Alterações da atividade de enzimas e transportadores não são correlacionadas com alterações da expressão gênica, sugerindo regulação pós-transcricional dos genes envolvidos;

- Os efeitos da restrição são revertidos rapidamente, visto que em três dias de realimentação *ad libitum* os animais recuperam a maior parte dos parâmetros afetados, possibilitando melhor performance associada à maior ingestão.