UNIVERSIDADE FEDERAL DE MINAS GERAIS

FISIOLOGIA, METABOLISMO E DESEMPENHO DE LINHAGENS DE TILÁPIA ALIMENTADAS COM DIETAS CONTENDO NÍVEIS DE PROTEÍNA

WELLIENE MOREIRA DOS SANTOS

Belo Horizonte
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Tese apresentada ao Programa de Pós-Graduação em Zootecnia da Escola de Veterinária da Universidade Federal de Minas Gerais como requisito parcial para obtenção do grau de Doutor em Zootecnia.

Área de concentração: Nutrição e Alimentação Animal

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“A sabedoria não nos é dada. É preciso descobri-la por nós mesmos, depois de uma viagem que ninguém nos pode poupar ou fazer por nós. ”

(Marcel Proust)

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LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

ALT	Alanina aminotransferase
ANOVA	Análise de variância
AOAC	Association of Official Analytical Chemists
AST	Aspartato aminotransferase
BApNA	Nα-benzoyl-L-arginine-4-nitroanilide
BHT	Hidroxitolueno butilado
BSA	Bovine serum albumin
BTEE	N-benzoyl-L-tyrosine ethyl ester
$CaCl_2$	Cloreto de cálcio
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CCK	Colecistoquinina
cck	Gene da colecistoquinina
cDNA	Ácido desoxirribonucléico complementar
CEUA	Comissão de Ética no Uso de Animais
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CP	Crude protein
DEPC	Dietilpirocarbonato
EDTA	Ethylenediamine tetraacetic acid
ef1α	Elongation factor 1 alpha
ERR	Energy retention rate
EUA	Estados Unidos da América
Fw	Foward
g	Grama
GH	Hormônio do crescimento
HCl	Ácido clorídrico
LAQUA	Laboratório de Aquacultura
mg	Miligrama
mM	Milimolar
mRNA	Ácido ribonucleico mensageiro
Na+	Sódio

nm	Nanômetro
nM	Nanomolar
npy	Gene do neuropeptídeo Y
NPY	Neuropeptídeo Y
p/v	Peso/Volume
PB	Proteína bruta
PCR	Polymerase chain reaction
pH	Potencial hidrogeniônico
PRR	Protein retention rate
pyy	Gene do peptídeo YY
PYY	Peptídeo YY
qPCR	Quantitative polymerase chain reaction
RNA	Ácido ribonucleico
Rv	Reverse
SGR	Specific growth rate
SPSS	Statistical Package for the Social Sciences
TCA	Trichloroacetic acid
TRE	Taxa de retenção energética
TRP	Taxa de retenção proteica
UFLA	Universidade Federal de Lavras
UFMG	Universidade Federal de Minas Gerais
Vit	Vitamina
µg	Micrograma
18s	18s ribosomal RNA

RESUMO

Objetivou-se avaliar o desempenho, a atividade de enzimas proteolíticas e a expressão gênica de enzimas digestivas e hormônios reguladores do apetite em duas linhagens de tilápia alimentadas com dietas contendo níveis de proteína bruta (PB). No experimento I, 240 juvenis de tilápia nilótica (*Oreochromis niloticus)* (16,56 ± 1,21 g) foram alimentados três vezes ao dia, por 65 dias. No experimento II, 240 juvenis de tilápia vermelha (*Oreochromis sp.*) (29,32 ± 5,19 g) foram alimentados duas vezes ao dia, por 42 dias. Nos dois experimentos, quatro dietas isoenergéticas foram formuladas para ter 24, 30, 36 ou 42% de PB. Cada dieta foi fornecida a cinco tanques diferentes (100 L; 12 peixes/tanque), sendo utilizado um total de 20 tanques em cada experimento. Os dados foram submetidos à análise de variância, análise de regressão polinomial (experimento I) e teste de Tukey (experimento II). No experimento I, o aumento de PB resultou na diminuição linear do desempenho animal e das taxas de retenção proteica e energética, e um aumento linear na atividade da alanina aminotransferase hepática. Houve efeito quadrático positivo da PB sobre a expressão de *pepsinogênio* ($P < 0,05$), mas a atividade da protease ácida não foi alterada ($P > 0,05$). A expressão de *tripsinogênio* seguiu um padrão similar ao da atividade de tripsina no intestino, uma vez que apresentaram efeito quadrático positivo frente aos níveis proteicos ($P < 0,05$). Houve aumento linear da expressão de *quimotripsinogênio* no intestino, mas a atividade da quimotripsina apresentou resposta quadrática positiva no intestino ($P < 0,05$). A expressão de colecistocinina (*cck*) e peptídeo yy (*pyy*) aumentou linearmente com o aumento da PB ($P < 0.05$). Os resultados do experimento II indicaram que a dieta com 42% de PB apresentou a maior expressão de *pepsinogênio* e a menor atividade de protease ácida ($P < 0,05$). A expressão de *tripsinogênio* hepatopancreático aumentou conforme os níveis de PB na dieta aumentaram até 36% ($P < 0,05$), enquanto a atividade de tripsina apresentou redução significativa com 42% de PB ($P < 0,05$). A dieta com 42% de PB apresentou a menor expressão de *quimotripsinogênio* intestinal e a menor atividade de quimotripsina ($P < 0,05$). A expressão de *α-amilase* diminuiu com o aumento ($P < 0,05$) nos níveis de PB da dieta até 36%. Não foram observadas diferenças significativas nas expressões de *procarboxypeptidase, lipase* e *leptina* entre todos os grupos ($P > 0,05$). A dieta com 42% de PB causou uma diminuição ($P < 0,05$) na expressão de *grelina* e *insulina* e um aumento ($P < 0,05$) na expressão de *cck* e *pyy*. Genes envolvidos na regulação da digestão e do apetite são modulados pela proteína da dieta de forma semelhante nas duas linhagens de tilápia.

Palavras-chave: tilápia do Nilo, tilápia vermelha, proteína bruta, digestão, mRNA, apetite

ABSTRACT

The objective of this study was to evaluate the performance, activity of proteolytic enzymes and the gene expression of digestive enzymes and appetite regulating hormones in two tilapia strains fed diets containing levels of crude protein (CP). In experiment I, 240 Nile tilapia juveniles (*Oreochromis niloticus*) (16.56 ± 1.21 g) were fed three times a day for 65 days. In experiment II, 240 juveniles of red tilapia (*Oreochromis sp*.) (29.32 ± 5.19 g) were fed twice a day for 42 days. In both experiments, four isoenergetic diets were formulated to have 24, 30, 36 or 42% of CP. Each diet was fed to five different tanks (100 L; 12 fish/tank), using a total of twenty tanks in each experiment. Data were submitted to analysis of variance, polynomial regression analysis (experiment I) and Tukey's test (experiment II). In experiment I, the increment of CP resulted in a linear decrease in animal performance and protein and energy retention rates, and a linear increased in hepatic alanine aminotransferase activity. A positive quadratic effect of CP on *pepsinogen* ($P < 0.05$) was observed, although acid protease activity was not affected ($P > 0.05$). *Trypsinogen* and trypsin activity in the intestine presented similar patterns, showing a positive quadratic response to dietary protein levels ($P < 0.05$). A linear increase in intestinal *chymotrypsinogen* expression was observed, but the activity of chymotrypsin showed a positive quadratic response ($P < 0,05$). The expression of cholecystokinin (*cck*) and peptide yy (*pyy*) increased linearly with increasing CP ($P < 0.05$). The results of experiment II indicated that the diet with 42 % CP was associated with the highest expression of *pepsinogen* and the lowest activity of acid protease ($P < 0.05$). The expression of hepatopancreatic *trypsinogen* increased as CP levels in the diet increased up to 36% ($P < 0.05$), whereas trypsin activity showed a significant reduction with 42 % CP ($P < 0.05$). The diet with 42 % CP was associated with the lowest intestinal *chymotrypsinogen* expression and the lowest chymotrypsin activity ($P < 0.05$). The *α-amylase* expression decreased with increasing ($P < 0.05$) CP levels up to 36 %. No significant differences were observed in the expression of *procarboxypeptidase*, *lipase* and *leptin* among all the groups ($P > 0.05$). The diet with 42 % CP resulted in a decrease ($P < 0.05$) in the expression of *ghrelin* and *insulin* and an increase ($P < 0.05$) in the expression of *cck* and *pyy*. Genes involved in regulating digestion and appetite are modulated by dietary protein in a similar way in both tilapia strains.

Keywords: Nile tilapia, red tilapia, crude protein, digestion, mRNA, appetite

1 INTRODUÇÃO GERAL

Dentre as espécies de peixes mais estudadas no mundo, estão os representantes da família Cichlidae, sendo as tilápias as mais conhecidas e pesquisadas (Amaral Jr. et al., 2010). A tilápia do Nilo e a tilápia vermelha estão entre as duas mais conhecidas. Embora a tilápia do Nilo seja a mais existente no mundo, o interesse pela tilápia vermelha cresceu bastante nos últimos anos, devido a sua cor atrativa e ao seu apelo no mercado (Zhu et al., 2016; Lovshin, 2000). As tilápias reúnem vários atributos que são considerados importantes para a aquicultura, como rusticidade, rápido crescimento em cultivo intensivo e qualidade de carne (El-Sayed, 2006). Em função da importância da referida espécie no cenário mundial aquícola, novas abordagens que visem a melhoria de sua produção se fazem necessárias.

A nutrição tem um papel significativo na produção animal, uma vez que cerca de 70% dos custos da atividade aquícola se devem aos gastos com ração (Li et al., 2013). Além do quesito financeiro, do ponto de vista técnico, mesmo em sistemas de produção com condições ótimas, se os animais não forem alimentados e nutridos adequadamente a viabilidade do empreendimento fica comprometida. As proteínas são nutrientes essenciais ao desenvolvimento pleno dos animais, participando desde a deposição muscular até a formação e manutenção de sistemas vitais, envolvendo complexos enzimáticos, hormonais e do sistema imunológico (Tu et al., 2015). Os principais pontos críticos da nutrição de peixes incluem o conhecimento das exigências nutricionais, da composição dos alimentos e do consumo de ração. Dietas inadequadas não são aproveitadas eficientemente pelos animais, acarretando prejuízos econômicos e de qualidade de água, o que reduz o potencial de produção. Neste contexto, o desenvolvimento de estratégias que melhorem a eficiência alimentar, por meio de um maior aproveitamento e maior consumo de ração, podem determinar o sucesso da atividade.

A formulação eficiente de dietas requer o conhecimento da capacidade digestiva dos animais, que podem ser avaliadas através de enzimas digestivas (Canada et al., 2017). A digestão é um processo complexo que envolve uma série de eventos que requerem a coordenação de uma variedade de processos básicos no trato digestório, que é iniciada pela ingestão de alimentos, incluindo as secreções enzimáticas e de muco, digestão mecânica e enzimática, absorção, motilidade (incluindo a evacuação) e, finalmente, a regulação destes diferentes processos (Zambonino-Infante e Cahu 2001; Rønnestad et al. 2013; Morais e Almeida 2014). As regulações destes processos são controladas e otimizadas pelos sistemas nervoso e endócrino, bem como por fatores luminais, neurotransmissores, hormônios, fatores

parácrinos e sinais de transdução e transcrição (Rønnestad et al. 2007). O cérebro produz os principais fatores que estimulam (orexígenos) ou inibem (anorexígenos) a ingestão de alimentos (Volkoff, 2016). Entre os fatores orexígenos destacam-se o neuropeptídeo Y (NPY) e a grelina, enquanto a colecistoquinina (CCK), peptídeo YY (PYY), leptina e insulina são potentes fatores anorexígenos (Volkoff, 2016).

A composição da dieta pode influenciar a capacidade digestiva e as funções endócrinas dos peixes, levando a inúmeros efeitos fisiológicos, como maior crescimento somático e desenvolvimento (Kortner et al. 2011). Além disso, a composição da dieta pode modular a expressão das enzimas digestivas e hormônios reguladores do apetite a nível transcricional (Jin et al., 2015; Wang et al., 2006), e assim tornar os animais mais ou menos eficientes em converter alimentos em crescimento corporal.

A presente tese está sendo apresentada na forma de dois artigos científicos, tratados como manuscritos. O primeiro manuscrito apresenta as respostas fisiológicas e metabólicas em tilápia nilótica e o segundo em tilápia vermelha.

1.1 Objetivos

O presente trabalho teve como proposta central avaliar as respostas fisiológicas e metabólicas de duas linhagens de tilápia, em adaptação as variações da proteína bruta da dieta, mediante a avaliação da:

a) atividade de enzimas proteolíticas (protease ácida, tripsina e quimotripsina);

b) expressão gênica de enzimas digestivas: *pepsinogênio*, *tripsinogênio*, *quimotripsinogênio*, *procarboxipeptidase*, *α-amilase* e *lipase*;

c) expressão gênica dos peptídeos ligados à ingestão alimentar: *neuropeptídeo Y*, *leptina*, *peptídeo YY*, *colecistoquinina*, *grelina* e *insulina*;

d) atividade de alanina aminotransferase (ALT) e aspartato aminotransferase (AST);

e) desempenho produtivo e retenção de nutrientes dos juvenis de tilápia.

2 REVISÃO DE LITERATURA

2.1 Tilápia

A tilápia pertence à família Cichlidae, sendo nativa dos países africanos e da região que atualmente pertence à Palestina. As diferentes espécies estão adaptadas a uma ampla diversidade de habitats, refletindo condições ambientais muito variáveis, que incluem parâmetros físicos, químicos e biológicos (El-Sayed, 2006). Apesar de ser um peixe nativo do continente africano, a tilápia foi introduzida em muitos países tropicais, subtropicais e temperados durante a segunda metade do século 20 (Pillay e Kutty, 2005). A maioria desses peixes possui hábito alimentar herbívoro/onívoro, vive em lagos e rios em profundidades de até 15 metros, em temperaturas de 12 a 35ºC, pH de 6,5 a 8,0 e oxigênio dissolvido de 2 a 8 mg/L (Hussain, 2004; El-Sayed, 2006).

Atualmente, as tilápias são os peixes de cultivo mais importantes das regiões tropicais no mundo (Ng e Romano, 2013). São rústicos, de crescimento rápido, não requerem tecnologia sofisticada, possuem alta prolificidade, aceitam uma grande variedade de alimentos, têm boa conversão alimentar, são resistentes a muitas doenças e desovam durante todo o ano, além de possuírem excelente sabor e textura (Melo et al., 2006). A tilápia do Nilo (*Oreochromis niloticus*) é a principal espécie de tilápia cultivada no mundo, mas o interesse pela tilápia vermelha (*Oreochromis sp*.) cresceu bastante nos últimos anos, devido a sua cor atrativa que gera maior apelo no mercado (Lovshin, 2000). Segundo esses mesmos autores, a tilápia do Nilo produz quantidades mais consistentes de alevinos do que a tilápia vermelha. As sobrevivências dos ovos, alevinos e juvenis de tilápia do Nilo são também maiores, assim como são mais tolerantes às baixas temperaturas do que a tilápia vermelha. Entretanto, as tilápias vermelhas têm, frequentemente, maior valor no mercado e são mais apropriadas para cultivos em salinidades acima de 10 g/l, além de serem mais fáceis de serem despescadas em viveiros de terra e transportadas vivas (Lovshin, 2000). Marengoni et al., (2010) também cita que a tilápia vermelha quando bem adaptada, possui um crescimento rápido, fácil manejo e boa aceitabilidade ao alimento artificial. Portanto, a escolha pela criação da tilápia vermelha ou tilápia do Nilo é basicamente dependente da preferência do mercado consumidor.

2.2 Proteína na alimentação de peixes

As proteínas são as macromoléculas mais importantes e abundantes no organismo vivo, podendo ocorrer na forma de pequenos peptídeos até grandes polímeros (Nelson e Cox, 2011). Suas unidades formadoras são os aminoácidos, e apenas 20 tipos de aminoácidos (10 essenciais e 10 não essenciais) diferentes constituem todas as proteínas (Lovell, 1998). Segundo esses mesmos autores, essas unidades são unidas entre si por ligações peptídicas e devido às características da cadeia lateral de cada um dos aminoácidos, as células podem formar várias proteínas com funções distintas. Os aminoácidos são constituídos de um carbono ao qual está ligado um grupo carboxila, um grupo amino, diferindo entre si pela cadeia lateral, que varia em estrutura, tamanho e carga elétrica (Nelson e Cox, 2011).

A proteína é o componente mais caro da dieta animal, e a qualidade da proteína de um alimento pode ser comparada com a de outro pela composição dos aminoácidos, especialmente a proporção dos aminoácidos essenciais (Silva et al., 2014). Embora não exerçam função de reserva energética como os carboidratos e os lipídeos, as proteínas, pela oxidação dos aminoácidos, podem liberar energia, mas isso tem alto preço para o produtor e um custo elevado para o organismo e para o ambiente (Silva et al., 2014). A necessidade proteica é um aspecto importante a ser investigado para determinar as exigências nutricionais das espécies de peixes (Gao et al., 2005), uma vez que as proteínas têm papel fundamental não só na deposição muscular, mas também na produção de enzimas, hormônios e anticorpos necessários para muitos processos vitais (Tu et al., 2015). Diversos estudos buscam a concentração proteica ótima da dieta, que pode ser entendida como a quantidade mínima necessária para satisfazer as exigências em aminoácidos, que proporcionarão o máximo crescimento (Sá e Fracalossi, 2002) e, também, a melhor concentração energética (Watanabe et al., 2001). O uso de dietas com quantidades insuficientes de proteína ou composição inadequada de aminoácidos pode interferir em processos metabólicos vitais e reduzir o crescimento dos peixes, enquanto que acima do exigido, o excedente é utilizado como fonte energética aumentando a excreção de resíduos nitrogenados, que são prejudicais ao peixe e ambiente de criação (Hayashi et al., 2002; NRC, 2011).

Os diferentes teores de proteína exigidos nas dietas são geralmente em função do hábito alimentar, idade e tamanho do peixe (Kumar et al., 2013). Abdel-Tawwab et al. (2010) avaliaram três níveis de proteína bruta (25, 35 e 45%) em três categorias de peso: 0,4 – 0,5g; 17 – 22g e 37 – 43g de tilápias, e constataram que o melhor desempenho para as larvas (0,4 – 0,5g) foi com dietas de 45% de PB, enquanto que para juvenis (17 – 22g e 37 – 43g) o melhor

resultado foi com dietas de 35% de PB. Os autores concluíram que, além do prejuízo no desempenho produtivo, a proteína em excesso oferecida aos juvenis foi desaminada e utilizada como energia, aumentando os níveis de amônia ionizada na água.

Em geral, a exigência em proteína para tilápia nilótica tem variado de 26,8 a 45,0% para proteína bruta (Abdel-Tawwab et al., 2010; El-Sayed e Kawanna, 2008; El-Sayed et al., 2003; Furuya et al., 2010; Larumbe-Morán et al., 2010), e de 24,3 a 38,6% em valores digestíveis (Furuya et al., 2005, 2010; Hayashi et al., 2002; Sousa et al., 2013) dependendo da fase de desenvolvimento, ambiente de criação e relação energia/proteína.

As informações relacionadas às exigências em proteína são derivadas de experimentos dose-resposta, utilizando como critério de avaliação o desempenho proporcionado pelas dietas (Furuya et al., 2010). Entretanto, a influência dos nutrientes sobre a fisiologia e metabolismo dos peixes também vem sendo investigada (Abdel-Tawwab et al., 2010; Azaza et al., 2015), pois constituem importante ferramenta ao conhecimento da dinâmica de utilização da proteína. Além disso, a utilização de índices, tais como a taxa de retenção proteica (TRP) e taxa de retenção energética (TRE), calculados através da determinação da composição corporal dos animais, possibilita um melhor entendimento sobre a utilização dos nutrientes da dieta, utilização esta que não é indicada somente com as medidas de ganho em peso e eficiência alimentar. Li et al. (2013) testando níveis de proteína bruta na dieta (20, 25 e 30%) mostraram que a TRP foi máxima quando juvenis de tilápia foram alimentados com a menor concentração proteica, decrescendo à medida que a PB da dieta aumentava. Khan et al. (1993) detectaram um efeito quadrático da proteína da dieta sobre a TRP e a deposição de gordura corporal no bagre da Malásia (*Mystus nemurus*) e propuseram que, a partir de uma determinada concentração proteica da dieta, a proteína não é mais destinada para o incremento do ganho em peso, mas sim desaminada e depositada na forma de gordura. Utilizando outro índice, a TRE, Peres e Oliva-Teles (1999) demonstraram que esta taxa aumentou proporcionalmente ao aumento da concentração proteica da dieta, em juvenis de robalo europeu (*Dicentrarchus labrax)*, evidenciando a importância da utilização de índices na avaliação do desempenho dos peixes frente a diferentes dietas. Também é importante avaliar a influência de fatores que possam atuar no aproveitamento dos nutrientes, pois é provável que as diferenças no desempenho dos peixes sejam, até certo grau, atribuídas às diferenças interespecíficas das características de seus tratos digestivos e à eficiência com que o alimento ingerido é capaz de ser digerido e assimilado.

2.3 Digestão de proteínas: Proteases

A digestão é o processo pelo qual o alimento é transformado em compostos mais simples, capazes de transpor a parede das células intestinais, alcançando a corrente sanguínea para serem transportados aos tecidos (Lundstedt et al., 2004). As proteínas são hidrolisadas em aminoácidos livres ou cadeias peptídicas curtas, os carboidratos são hidrolisados em açúcares simples e as gorduras em ácidos graxos e glicerol. Estes processos são facilitados pela ocorrência das enzimas digestivas que atuam ao longo do trato gastrintestinal (Jobling, 1994).

O processo de digestão proteica nos animais gástricos, como a tilápia, tem início no estômago. Quando o alimento ingerido chega ao estômago, estímulos químicos enviam sinais, que estimulam a secreção de gastrina pelas células G da mucosa estomacal. A gastrina age sobre as células parietais, estimulando a secreção do ácido clorídrico (HCl) (Silva et al., 2014). As células principais da mucosa estomacal secretam pepsinogênio, o qual precisa ser primeiramente ativado pelo HCl a pepsina. O pH ótimo para ativação da pepsina é de 2 a 3,5 (Lovell, 1998). A digestão proteica começa com a ação da pepsina estomacal, uma endopeptidase produzida na glândula pilórica em resposta ao estímulo da gastrina. A pepsina hidrolisa as proteínas nas ligações peptídicas do lado aminoterminal dos resíduos dos aminoácidos aromáticos (tirosina, fenilalanina e triptofano), rompendo as longas cadeias polipeptídicas em uma mistura de peptídeos menores (Bakke et al., 2010).

No intestino, a presença do quimo ácido promove a secreção de secretina, que estimula a secreção de fluidos e bicarbonato, e a presença do alimento promove a secreção de CCK, que estimula a secreção da bile e das enzimas pancreáticas, além de diminuir a motilidade (Silva et al., 2014). O bicarbonato age neutralizando o pH da digesta ácida, que deixa o estômago, proporcionando um ambiente favorável para ativação e ação das enzimas pancreáticas. Juntamente com o bicarbonato, o suco pancreático contém os zimogênios, que necessitam ser ativados. Dessa forma, a mucosa intestinal secreta a enteropeptidase, que, por sua vez, age ativando o tripsinogênio a tripsina (Silva et al., 2014). A tripsina segue se autoativando e também promove a ativação das outras proteases (Silva et al., 2014). O quimotripsinogênio, a procarboxipeptidase e a proelastase são ativados em quimotripsina, carboxipeptidase e elastase, respectivamente (Rust, 2002).

As proteases são classificadas pela região em que elas atuam na molécula proteica. As endopeptidases atuam no interior da cadeia, produzido vários oligopeptídeos; um exemplo é a tripsina, que cliva ligações que envolve lisina e arginina, e a quimotripsina, que hidrolisa ligações que envolve aminoácidos aromáticos (tirosina, triptofano e fenilalanina) (Silva et al.,

2014). As carboxipolipeptidases, são exemplos de exopeptidases, que clivam ligações nas extremidades da cadeia peptídica, reduzindo a proteína a fragmentos ainda menores (Nelson e Cox, 2011). Na borda em escova, aminopeptidases e dipeptidases hidrolisam estes fragmentos tornando-os aptos à absorção (Rust, 2002).

A utilização das proteínas pelos peixes depende da atividade dessas enzimas no trato digestivo, assim como da resposta das mesmas à composição da dieta (Pérez-Jiménez et al., 2009).

2.4 Influência da proteína da dieta na atividade e expressão de enzimas digestivas

Os peixes apresentam a capacidade de modular seu perfil digestivo diante de diferentes fontes, tipos e quantidades de nutrientes da dieta, embora estas habilidades variem entre as espécies (Buddington et al., 1997). Considerando a grande importância da proteína na nutrição de peixes, estudos vêm mostrando as alterações nas enzimas digestivas em resposta à proteína dietética. De acordo com Zambonino Infante e Cahu (2007), a atividade da pepsina parece pouco influenciada pela proteína da dieta, e considerando que algumas espécies de peixes não apresentam estômago, sua presença é colocada como não determinante para a digestão proteica. Porém, outros resultados mostram que para algumas espécies, a atividade proteolítica do estômago é importante para a digestão de proteínas. Em pintado (*Pseudoplatystoma corruscans)*, mesmo não sendo responsiva aos níveis crescentes de proteína bruta da dieta, a protease ácida apresenta alta atividade, o que certamente está associado com o hábito alimentar da espécie (Lundstedt et al., 2004). Por outro lado, o onívoro *Colossoma macropomum* apresenta caráter indutivo desta protease no estômago e os autores argumentam que este traço adaptativo parece estar relacionado à diminuição da proteína da dieta (Almeida et al., 2006). Da mesma forma, Melo et al. (2012) avaliando o efeito de níveis de proteína bruta em dietas (20, 27, 34 e 41 %) sobre enzimas proteolíticas, observaram aumento linear da atividade de protease ácida em relação ao aumento do conteúdo de proteína das dietas para jundiás (*Rhamdia quelen*). Este comportamento também foi observado nas proteases alcalinas, sugerindo um caráter adaptativo destas enzimas frente ao conteúdo proteico da dieta.

Em dietas para juvenis de *Labeo hohita*, o nível de proteína bruta acima de 25% promoveu aumento da atividade de enzimas proteolíticas alcalinas, entretanto, no nível de 40% de PB as concentrações dessas enzimas diminuíram (Debnath et al., 2007). Outras espécies de peixes como *Puntius gonionotus* (Mohanta et al., 2008) e *Carassius auratus* gibelio (Tu et al.,

2015) também apresentaram maior atividade de proteases alcalinas devido ao incremento proteico na dieta até um determinado nível. Segundo Gonçalves et al. (2013) o aumento da inclusão de um nutriente pode estimular a secreção das enzimas responsáveis pela sua digestão até certo ponto, mas quando em excesso, o efeito pode ser contrário ao esperado.

Os efeitos provocados pela proteína dietética na atividade das enzimas proteolíticas incluem, também, mudanças na expressão gênica e tais mudanças são geralmente produzidas pela alteração nos níveis do RNA mensageiro (mRNA) desses genes (Kortner et al., 2011). Péres et al. (1998) mostraram que a proteína dietética modulou os níveis de mRNA da *tripsina* em larva de *Dicentrarchus labrax*. Wang et al. (2006) avaliaram níveis de proteína bruta na dieta (42,8; 47,3 e 52,8%) em larvas de *Pelteobagrus fulvidraco*, e observaram que a proteína afetou significativamente a atividade e o nível de mRNA da *pepsina* e *tripsina*. Os autores também observaram que a expressão da *pepsina* seguiu o padrão da sua atividade enzimática, enquanto que a tripsina não seguiu o mesmo padrão. Alguns aminoácidos também parecem modular a expressão de enzimas proteolíticas. Zhao et al. (2012) avaliando níveis de isoleucina na dieta, demonstraram que o gene da *quimotripsina* no hepatopâncreas de juvenis de *Cyprinus carpio* foi modulado e seguiu um padrão similar ao da sua atividade enzimática. No entanto a expressão da *tripsina* no hepatopâncreas dos juvenis apresentou um padrão oposto ao da sua atividade enzimática. Habte-Tsion et al. (2015) também observaram que dieta suplementada com treonina regulou a expressão de *tripsina* e *quimotripsina* no hepatopâncreas de juvenil de *Megalobrama amblycephala*.

Assim como as enzimas proteolíticas, as atividades da α-amilase e lipase também parecem ser responsivas a proteína da dieta. Melo et al. (2012) avaliando o efeito de níveis de proteína bruta na dieta (20, 27, 34 e 41%) sobre as atividades da α-amilase e lipase no trato digestório de Jundiá (*Rhamdia quelen*), observaram que as mesmas foram inversas ao teor de proteína da dieta. E foi explicado pela redução relativa dos teores de carboidratos e lipídios nas dietas experimentais. O caráter adaptativo da α-amilase frente a proteína da dieta também foi descrito em outras espécies de peixes (Tu et al., 2015; Liu et al., 2009; Mohanta et al., 2008; Giri et al., 2003). No entanto, Debnath et al. (2007) não encontraram diferença significativa para a atividade de α-amilase em *Labeo rohita* alimentados com diferentes níveis de proteína. Isso sugere que outros fatores além da proteína (como o hábito alimentar, o tipo de carboidrato, a temperatura da água e a estação do ano) podem influenciar a atividade da α-amilase em peixes (Debnath et al., 2007). Esses outros fatores também parecem influenciar na atividade de lipase,

uma vez que para algumas espécies a proteína da dieta altera a atividade desta enzima (Melo et al., 2012) e outras não (Tu et al., 2015; Liu et al., 2009; Debnath et al., 2007).

De acordo com esses trabalhos é possível observar que, quando a proteína da dieta muda, a atividade e a expressão das enzimas digestivas podem ser moduladas, e isso pode estar relacionado à maior taxa de crescimento do peixe (Thongprajukaew et al., 2011). Portanto, as enzimas digestivas podem ser consideradas ótimas ferramentas para formulação adequada de dietas para algumas espécies de peixes.

2.5 Metabolismo proteico

Após a hidrólise das proteínas, os aminoácidos livres resultantes deste processo são absorvidos para o sistema porta. Segundo Rotta (2003), a absorção dos aminoácidos livres ocorre na membrana apical do enterócito e é realizada através de transportadores específicos dependentes de Na+, de transportadores não-dependentes de Na+ e por difusão. Posteriormente, os aminoácidos são levados para a circulação geral por meio do fígado e utilizados para a síntese de novas proteínas (anabolismo) ou para energia (catabolismo) (De Silva e Anderson, 1995). As proporções entre proteínas anabolizadas e proteínas catabolizadas dependem da exigência do peixe, do conteúdo de proteína da dieta, da composição em aminoácidos, da exigência energética, da quantidade de energia disponível a partir de outras fontes (lipídios e carboidratos) (Lundstedt et al., 2004) e do hábito alimentar do peixe (peixe carnívoro possui alta exigência, pois obrigatoriamente usa parte da proteína para produção de energia).

Elevados níveis de proteína na dieta do peixe exige um aumento do nível de energia necessário para metabolizá-la (De Silva e Anderson, 1995). Todos os animais têm um limite para utilizar a proteína ingerida, pois os aminoácidos não são estocados em grandes quantidades no corpo (Ballantyne, 2001). Além deste limite, o excesso de aminoácidos circulantes é degradado para a produção de energia ou sofre conversão em glicogênio (gliconeogênese) ou lipídios (cetogênese) para armazenamento (De Silva e Anderson, 1995).

O grupamento amino é removido dos aminoácidos principalmente por transaminação ou por desaminação oxidativa (Lovell, 1988). A transaminação parece ser a principal rota inicial para desaminação em peixes, envolvendo a transferência da amônia do grupo amino para um α-cetoácido, usualmente α- cetoglutarato (Lovell, 1988). As enzimas envolvidas neste processo são a alanina aminotransferase (ALT) e aspartato aminotransferase (AST). Através da determinação dessas enzimas é possível verificar as possíveis situações metabólicas

indesejáveis, tais como a utilização de proteína para obtenção de energia (Melo, 2012). Abdel-Tawwab et al. (2010) estudaram o efeito do nível de proteína na dieta e peso corporal em tilápias nilóticas, observando efeito significativo nas atividades das enzimas ALT e AST no soro, fígado e músculo, e concluíram que a proteína em excesso na dieta pode ser desaminada e usada como fonte de energia.

2.6 Hormônios envolvidos na ingestão alimentar e saciedade

O grupo mais diversificado e abundante dos vertebrados é o dos peixes, sendo que apenas poucas espécies foram examinadas até o momento, no que diz respeito a sua fisiologia, em particular a ingestão alimentar (Volkoff, 2016). No entanto, as pesquisas sobre a regulação endócrina da alimentação de peixes vêm progredindo nos últimos anos. As técnicas tradicionais, como lesões cerebrais e injeções de peptídeos, embora ainda úteis e utilizadas, estão sendo complementadas por novas abordagens, como o estudo da expressão gênica (Volkoff, 2016). No entanto, a literatura sobre modulação da expressão dos hormônios envolvidos na ingestão alimentar pela composição da dieta ainda é escassa.

Em peixe, como em outros vertebrados, a alimentação é regulada por uma infinidade de hormônios produzidos tanto pelo cérebro quanto por órgãos periféricos, incluindo estimulantes (fatores orexigênicos) e inibidores (fatores anorexigênicos) do apetite que interagem para regular o equilíbrio energético. Peptídeos orexigênicos incluem o NPY e a grelina e sinais anorexigênicos incluem a CCK, o PYY a leptina e a insulina (Volkoff, 2016).

O NPY pertence à família dos polipeptídeos pancreáticos, que também inclui o PYY (Holzer et al., 2012). É um dos neuropeptídeos mais abundantes no cérebro e tem um importante papel regulador na homeostase energética e na ingestão de alimentos (Volkoff et al., 2009). O aumento do apetite causado por injeções centrais de NPY tem sido demonstrado em peixes (Kiris et al., 2007; Yokobori et al., 2012), contudo, a influência da composição da dieta sobre esse peptídeo ainda não está clara. Narnaware e Peter (2002) observaram que dietas com alto teor de carboidratos ou lipídeos alteram a expressão de *npy* em *Carassius auratus*, enquanto as dietas com altos níveis proteicos não influenciam a expressão desse gene. Em contraste, dietas com alto teor de proteína e dietas suplementadas com amido diminuíram a expressão do *npy* no hipotálamo de *Oncorhynchus mykiss* (Figueiredo-Silva et al., 2012). Jin et al. (2015) também mostraram que níveis de proteína na dieta modulam a expressão de *npy* no hipotálamo de juvenil de *Ctenopharyngodon idella*.

A grelina é um hormônio polipeptídico produzido pelo estômago que desempenha um papel importante no metabolismo energético e homeostase da glicose, além de ser um hormônio orexígeno e estimular a liberação do hormônio do crescimento (GH) (Volkoff, 2016). Unniappan et al. (2002) forneceram a primeira sequência de cDNA da grelina e a primeira evidência de seu papel orexigênico em peixes, uma vez que injeções centrais de grelina estimularam a ingestão alimentar. Estudos posteriores em várias espécies de peixes confirmaram seu papel como estimuladores do apetite (Jönsson, 2013). Por outro lado, em algumas espécies há evidências contraditórias, no salmão do Atlântico (*Salmo salar*), por exemplo, os níveis plasmáticos de grelina são mais baixos em peixes em jejum (Hevrøy et al., 2011). Em relação ao efeito da composição da dieta sobre esse hormônio, Blanco et al., (2016) observaram que dietas com alto teor de lipídeos a curto prazo (7 dias) aumentou significativamente os níveis de mRNA da *preprogrelina* (percursor da grelina) no intestino de *Carassius auratus,* enquanto as dietas com alto teor de proteína exibiram uma redução significativa desse gene no fígado. Após alimentação prolongada (28 dias), dietas com alto teor de proteínas e lipídeos não produziram nenhum efeito significativo na expressão intestinal da *preprogrelina*. Babaei et al. (2017) mostraram que dietas com alto teor de proteína / baixo carboidrato estimularam a expressão de *grelina* no intestino de *Sparus aurata*. Portanto, a regulação da grelina pela dieta em peixes permanece pouco compreendida.

Em mamíferos, a CCK inibe a ingestão de alimentos e induz a liberação de enzimas digestivas do intestino, pâncreas e secreção da vesícula biliar (Boguszewski et al., 2010). Para as espécies de peixes estudadas, no geral, a CCK parece ter papéis semelhantes aos processos digestivos e alimentares dos mamíferos, isto é, atua como um fator inibidor do apetite e induz a liberação de enzimas digestivas do trato gastrointestinal (Volkoff, 2016). Babaei et al. (2017) mostraram que dietas com alto teor de proteína / baixo carboidrato estimularam a expressão de *cck* no intestino de *Sparus aurata*. Em humanos, a liberação da CCK é estimulada por uma refeição rica em proteína (Morell e Fiszman, 2017).

O PYY é um peptídeo do cérebro-intestino que atua como um sinal anorexigênico em mamíferos (Zhang et al., 2012). A diminuição da ingestão alimentar causados por injeções centrais de PYY, tem sido demonstrada em peixes (Chen et al., 2015; Chen et al., 2013). No geral, parece que na maioria dos peixes estudados até hoje, o PYY pode atuar como um peptídeo anorexígeno / saciante, embora isso não pareça ser verdadeiro para todas as espécies de peixes, por exemplo, no salmão do Atlântico (Kousoulaki et al., 2013). Em relação ao efeito da composição da dieta sobre esse peptídeo, é desconhecida em peixes. Em humanos, assim como

a CCK, a liberação de PYY é estimulada por uma refeição rica em proteína (Morell e Fiszman, 2017).

A leptina é secretada principalmente pelo tecido adiposo em mamíferos (Zhang et al., 1994), mas em peixes é expressa em vários tecidos, incluindo fígado e intestino (Birsoy et al., 2013). É um hormônio peptídico multifuncional em mamíferos (Park e Ahima, 2015) e em peixes (Gorissen e Flik, 2014) está envolvido não apenas na regulação da ingestão alimentar e peso corporal, mas também na reprodução e respostas ao estresse (Volkoff, 2016). De acordo com os estudos da leptina em peixes, existe uma grande variabilidade das respostas desse peptídeo entre as espécies, talvez devido ao metabolismo lipídico e áreas de armazenamento lipídico diferentes (Volkoff, 2016). Pouco se sabe sobre o efeito da proteína sobre a leptina, o estudo de Babaei et al. (2017) mostraram que dietas com alto teor de proteína / baixo carboidrato estimularam a expressão de *leptina* no tecido adiposo de *Sparus aurata*.

Outro importante sinalizador e regulador da homeostase energética é a insulina. A composição dos macronutrientes da dieta também pode influenciar a secreção da insulina (Torres et al., 2009), mas pouco se conhece sobre essa influência em peixes. A insulina é um hormônio secretado pelas células β pancreáticas e exerce um papel central na regulação da homeostase da glicose (Hrytsenko et al., 2008), e também parece atuar, pelo menos em parte, na regulação da ingestão alimentar. A insulina aumenta a captação de glicose, e a queda da glicemia é um estímulo para o aumento do apetite (Woods et al., 1998). Por outro lado, estudos experimentais demonstraram que a insulina tem uma função essencial no sistema nervoso central para incitar a saciedade, aumentar o gasto energético e regular a ação da leptina (Schwartz, 2000).

O efeito da composição da dieta e do estado nutricional na expressão de peptídeos orexígenos e anorexígenos em peixes não é conclusivo e parece ser dependente da espécie.

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3 MANUSCRITO I

Gene expression and enzymatic activity of proteases in juvenile Nile tilapia fed levels of crude protein

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Abstract

The objective of this study was to evaluate the performance, protease activity and expression of genes related to protein intake and digestion in juvenile tilapia fed diets containing different levels of crude protein (CP). Four different experimental treatments were designed containing different CP levels: 25.14, 31.12, 36.60 and 42.05% (analyzed values). Diets were provided until apparent satiation, three times a day for 65 days. The increment of CP resulted in a linear decrease in animal performance and protein and energy retention rates, and a linear increased in hepatic alanine aminotransferase activity. A positive quadratic effect of CP on *pepsinogen* was observed, although acid protease activity was not affected. *Trypsinogen* and trypsin activity in the intestine presented similar patterns, showing a positive quadratic response to dietary protein levels. A linear increase in intestinal *chymotrypsinogen* expression was observed, but the activity of chymotrypsin showed a positive quadratic response. In addition, the expression of *cholecystokinin* (cck) and *peptide yy* (pyy) increased linearly with increasing CP. It is concluded that variation in dietary protein promoted changes in the metabolism of the Nile tilapia, which was reflected in proteolytic activity and expression of genes.

Keywords: crude protein, digestion, proteolytic enzyme, mRNA, food intake

3.1 Introduction

Nile tilapia is an omnivorous fish considered one of the most important species for freshwater aquaculture due its fast growth, good meat quality and robustness for enduring intensive culture conditions (Ng and Romano, 2013). Despite all these favorable characteristics for intensive production systems, without adequate food and nutrition its productive potential cannot be achieved.

Among aquaculture production costs, feeding is the most expensive and protein is one of the most expensive nutrients that constitute the animal diet (Li et al., 2013). In addition to its cost, protein is also one of most important nutrients, because it participates in essential physiological processes, from muscle deposition to the formation and maintenance of vital systems, involving enzymatic, endocrine and immunological factors (Tu et al., 2015). Thus, for maximum fish growth, in addition to the correct energy/protein ratio, the amount of protein to be used in the diet should be sufficient for meeting the requirements for essential amino acids

(Ahmed, 2012; Haidar et al., 2018). The use of protein-deficient diets, or an inadequate amino acid balance, impairs the growth, survival and health of fish (Giri et al., 2003; Qiang et al., 2013), whereas protein excess can be converted into energy, increasing nitrogen excretions in the water (Guo et al., 2012). Therefore, to develop formulations that properly meet fish protein requirements, it is necessary to carry out research that takes into consideration not only performance but also relevant physiological and metabolic aspects.

To efficiently formulate diets, some previous knowledge of the digestive process of the animals is required, which may be developed by prospecting digestive enzyme activity (Canada et al., 2017). Proteases are enzymes that specifically break peptide bonds between amino acids in proteins (Rust, 2002), with trypsin and chymotrypsin being the most relevant in the gastrointestinal tract of fish (Santigosa et al., 2008). Secretion of these enzymes, as well as food intake, is regulated by neural and hormonal factors (Konturek et al., 2003; Ji et al., 2015). One of these factors is cholecystokinin (cck), which is secreted in the intestine in response to food intake and acts on gallbladder control, secretion of pancreatic enzymes, reduction of gastric emptying and satiety (Micale et al., 2014). It is also worth mentioning the members of the pancreatic polypeptide family, neuropeptide y (npy) and peptide yy (pyy), that are known to stimulate and inhibit food intake, respectively (Yan et al., 2017).

Fish can modulate proteolytic enzyme secretion patterns, as well as the secretion of hormones involved in food intake, in adjustment to different dietary protein levels (Mohanta et al., 2008; Tu et al., 2015). In addition, dietary protein may also modulate the expression of the genes of these enzymes and hormones (Wang et al., 2006; Jin et al., 2015), thus making animals more (or less) efficient at converting food into body tissue.

The objective of this study was to evaluate how different protein levels affect the performance, protease activity and expression of genes related to food intake and protein digestion of juvenile Nile tilapia.

3.2 Materials and Methods

This study has been approved by the Committee for Ethics in Animals Use of the Universidade Federal de Minas Gerais (CEUA / UFMG - protocol number 159/2016).

Animals and experimental conditions

Male juvenile Nile tilapia (*Oreochromis niloticus*) were purchased from a commercial fish farm and acclimated to laboratory conditions for one week. Then, 240 fish (initial mean weight of 16.56±1.21g) were randomly distributed among 20 fiberglass tanks (12 fish/tank), with 100 l of useful volume each. All tanks were installed in a water recirculation system, including mechanical and biological filter, supplementary aeration and temperature control by means of electric heaters. Water quality parameters were monitored continuously with a YSI 6920 V2 multiparameter probe (Yellow Springs Incorporated – YSI®, OH USA), and all remained within acceptable ranges for the specie (dissolved oxygen of 6.17±0.77 mg/l, temperature of 28.34±0.43ºC and pH of 6.59±0.15). The experiment was conducted in a closed room. Fish were reared under a 10 h of light and 14 h of darkness (10L:14D) regime. The maximum light intensity was 301 lux measured at the water surface during daytime.

Experimental diets and food management

Four isoenergetic experimental diets were formulated to have 24, 30, 36 or 42% of crude protein (CP). The corresponding analyzed values of dietary CP were 25.14, 31.12, 36.60 and 42.05%. Formulations and approximate compositions are presented in Table 1. The ingredients of each diet were mill-milled to reach 0.5 mm, homogenized in a mixer and extruded a single screw extruder (Labor PQ30, Inbramaq®, Ribeirão Preto, Brazil), obtaining pellets approximately 4 mm in diameter. After that, the diets were dried in a forced air oven and stored in a refrigerated room (0°C) until use.

Fish were fed the experimental diets until apparent satiety three times a day (at 08.00, 12.00 and 16.00 hours) for 65 days of experimental period. Each diet was fed to five different tanks, using a total of twenty tanks. After each feeding, leftover diet was collected, stored and frozen (-20ºC). Subsequently, the material was dried in a forced ventilation oven at 55°C for 96 h and weighed to obtain food consumption by the fish. Cleaning of the tanks was done daily after the last feeding time.

Table 1. Formulation, approximate composition and main amino acids of the experimental diets (as-fed basis)

Ingredient	Dietary protein level (%)			
	24	30	36	42
Fishmeal	15.50	21.00	24.00	29.00
Soybean meal	24.00	31.00	33.00	37.00
Corn	40.00	31.50	22.90	13.80
Broken rice	5.00	5.00	5.00	5.00
Albumin	1.00	1.60	6.50	9.10
Soybean oil	8.80	6.60	5.30	3.70
Cellulose	3.00	1.00	1.00	0.00
Bicalcic phosphate	0.50	0.50	0.50	0.50
Vitamin / Mineral Premix[a]	0.50	0.50	0.50	0.50
BHT[b]	0.02	0.02	0.02	0.02
Salt	0.50	0.50	0.50	0.50
Inert	1.18	0.78	0.78	0.88
Analyzed composition[c]				
Crude protein (%)	25.14	31.12	36.60	42.05
Crude energy (MJ kg^{-1})	17.28	17.86	17.93	17.60
Ether extract (%)	9.98	7.77	7.86	6.35
Ash (%)	6.66	7.02	7.49	10.08
Essential amino acids (%)[d]				
Thr	1.02	1.38	1.53	1.80
Val	1.26	1.62	1.93	2.17
Met	0.49	0.65	0.73	0.95
Ile	1.15	1.49	1.76	2.03
Leu	2.13	2.63	2.98	3.29
Phe	1.13	1.45	1.75	1.98
Lys	1.65	2.28	2.61	3.00
His	0.63	0.84	0.99	1.11
Arg	1.70	2.41	2.77	3.13
Non-essential amino acids (%)[d]				
Asp	2.62	3.69	4.12	4.65
Glu	3.95	5.03	5.61	6.45
Gly	1.52	2.23	2.44	2.97
Ala	1.55	1.99	2.24	2.60
Pro	1.45	1.80	1.99	2.26
Tyr	0.88	1.23	1.33	1.56
Cys	0.28	0.39	0.51	0.66
Ser	1.27	1.71	1.98	2.37

[a]Composition of vitamin and mineral supplement: Vit. A, 1 200 000 IU; Vit. D_3, 200 000 IU; Vit. E, 12 000 mg; Vit. K, 2 400 mg; Vit. B_1, 4 800 mg; Vit. B_2, 4 800 mg; Vit. B_6, 4 000 mg; Vit. B_{12}, 4 800 mg; folic acid, 1 200 mg; pantothenic acid, 3 750 mg; Vit. C, 48 000 mg; Biotin, 48 mg; Choline, 65 000 mg; Niacin, 24 000 mg; Fe, 10 000 mg; Cu, 6 000 mg; Mn, 4 000 mg; Zn, 6 000 mg; I, 20 mg; Co, 2 mg; Se, 20 mg.
[b]Butylated hydroxytoluene (antioxidant).
[c]Values presented are means from triplicate analyses.
[d]Values analyzed in the laboratory (HPLC and enzymatic).

Sampling procedures, chemical analysis and calculations

At the end of the experiment, after 12 h of fasting, 60 fish (n = 15 per treatment, 3 fish from each replicate tank) were anesthetized with a benzocaine solution (100 mg/l). These animals were then dissected and samples of hepatopancreas, stomach and intestine (anterior, middle and posterior) were collected for enzymatic assays. Another set of samples of hepatopancreas, stomach, intestine and hypothalamus were collected and cleaned in saline solution for gene expression assay. All samples were immediately frozen in liquid nitrogen and stored at -80°C for later analysis.

All fish were weighed at the beginning and at the end of the experiment. The parameters used to evaluate fish performance and nutrient retention were calculated using the following equations: Weight gain = (final weight − initial weight); Apparent feed conversion = (feed intake/weight gain); Specific growth rate (SGR) = {[(ln final weight – ln initial weight)/days] × 100}; Survival rate = [(number of animals at the end of experiment/number of animals at the beginning of experiment) × 100]; Protein retention rate (PRR) = {[(final weight × body protein) - (initial weight × initial body protein) / total ingested protein] × 100} and Energy retention rate (ERR) = {[(final weight × body energy) - (initial weight × initial body energy) / total ingested energy] × 100}.

For calculating PRR and ERR, two sampling points were collected: one at the beginning of the experiment consisting of a pool of 10 fish, and a final sample of two fish from each tank replicate (n = 10 per treatment) collected at the end of the feeding trial. The animals were euthanized by an overdose of benzocaine (250 mg/l) and frozen at -20°C until processing. Samples were lyophilized and grounded in a knife mill.

Chemical composition of ingredients, diets, and carcass were analyzed according to AOAC methods (AOAC, 2012). Briefly, moisture was determined after drying in an oven at 105 °C for 24 h; ash by incineration in a muffle furnace at 550 °C for 24 h; crude lipid by petroleum ether extraction in a Soxtec System; crude protein (N6.25) by the Kjeldahl method after acid digestion using a Kjeldahl System. Gross energy was determined by direct combustion in an adiabatic bomb calorimeter (PARR 6200, Parr Instrument Company, Illinois, USA).

Enzyme activity assay

Acid protease activity was determined in the stomach, the activity of trypsin and chymotrypsin were determined in the three portions of the intestine (anterior, middle and posterior) and the activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined in hepatopancreas. Tissue extracts from stomach samples were prepared in Tris-HCl 20 mM pH 7.0 and from intestine and hepatopancreas samples in Tris-HCl 100 mM pH 7.8 buffer with 0.1 % Triton X-100 and 0.1 mM Ethylenediamine tetraacetic acid (EDTA).

The activity of acid proteases was determined using bovine haemoglobin (Sigma) as the substrate. Aliquots of 20 µL of enzyme extract were incubated with 250 µL of haemoglobin (0.5 % in 0.1 M glycine buffer pH 2.0) for 20 minutes at 37 °C. This reaction was then stopped by the addition of 250 µL of 20 % TCA (trichloroacetic acid). The activity was measured at 280 nm and for each sample a "blank" containing 20 µL of enzyme extract, 250 µL of 0.5 % (w/v) haemoglobin, and 250 µL of 20 % (w/v) TCA was made. The specific activity was defined as µg of tyrosine released per minute per mg of total soluble proteins at 37 °C, using 0.008 mL/µg per cm as the molar extinction coefficient.

Trypsin activity was determined using Nα-benzoyl-L-arginine-4-nitroanilide (BApNA) as a substrate. Aliquots of 100 µL BApNA (1 mM in 50 mM Tris-HCl pH 8.2 and 20 mM CaCl2) were incubated with 20 µL of enzyme extract at 30 °C. The activity was measured at 410 nm every 30 s for 30 min. The specific activity was defined as nmol of 4-nitroaniline produced per minute (U) per mg of protein (mU^{-1} mg protein). The molar extinction coefficient was 8800 M/cm.

Chymotrypsin activity was determined using N-benzoyl-L-tyrosine ethyl ester (BTEE) as the substrate. Aliquots of 140 µL BTEE (1.13 mM in 100 mM Tris-HCl pH 7.8 and 25 mM $CaCl_2$) were incubated with 20 µL of enzyme extract at 30 °C, and the activity measured at 256 nm every 20 s for 20 min. The specific activity was defined as nmol of Benzoyl-L-tyrosine produced per minute per mg of protein. The molar extinction coefficient was 964 M/cm.

The activities of ALT and AST were determined using commercial kits (BioTécnica®). The total protein concentrations were determined using Bradford's (1976) method, with bovine serum albumin (BSA) as a standard. All readings were performed in triplicate using a microplate reader (Multiskan GO, Thermo Scientific, Waltham, Massachusetts, USA).

Real time PCR analysis

The expression of the gene *npy* was determined in the hypothalamus, the expression of the gene *pepsinogen* was determined in the stomach, the expression of the genes *cck* and *pyy* was determined in the intestine, and in the hepatopancreas, the expression of the gene *trypsinogen* was determined. The expression of the *chymotrypsinogen* and *procarboxypeptidase* genes were evaluated both in the intestine and in the hepatopancreas.

The hypothalamus, stomach, intestine and hepatopancreas samples were transferred into sterile tubes containing 0.5 mL of Trizol (Invitrogen, Carlsbad, Califórnia, USA). The tissue samples were mechanically homogenised and total RNA extraction was performed according to the manufacturer's instructions (Invitrogen). The RNA pellet was dissolved in sterile DEPC water (Invitrogen) and the RNA concentration was determined by spectrometry (Nanodrop ND-1000, Thermo Fisher Scientific, Waltham, MA, USA). Total RNA (1 µg) was retrotranscribed using a commercial kit (QuantiTect Reverse Transcription Kit, Qiagen, Hilden, Germany), which included a genomic DNA elimination step. cDNA was subjected to quantitative PCR analyses in a light thermocycler (7500 Real-Time PCR system, Applied Biosystems, Foster City, Califórnia, USA) following this protocol: 95 ºC for 15 min, followed by 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min. Quantitative PCR reactions were performed using the SYBR Green PCR Master Mix (Applied Biosystems). All samples were run in duplicate and the qPCR reaction was performed in a final volume of 20 µL.

The relative amplification efficiencies of all genes were analysed by cDNA dilution curves, and it was verified that they were similar for all the genes. Melting curves were run after amplification to ensure that only one DNA species was being amplified. The primer concentrations were determined by means of a primer dilution curve. The primers of *pepsinogen, trypsinogen, chymotrypsinogen, npy, cck, pyy*, *elongation factor 1 alpha (ef1α)* and *18s ribosomal RNA (18s)* were added at a final concentration in the reaction of 200 nM. The primers of *procarboxypeptidase* were added at a final concentration of 400 nM. The gene expression values were calculated as relative expression using the ΔΔCT method using ef1α and 18s as the housekeeping references. The primers were designed using the Primer3 software (Untergasser et al., 2012) and their sequences are shown in Table 2. All procedures were adapted from Costa et al. (2016).

Table 2. Primer sequences used in the quantitative PCR (qPCR) assays of tilapia

Gene	Ensembl/GenBank	Fw/Rv	Primer sequence (5´to 3´)
pepsinogen	JQ043215.1	Fw	TGACCAATGACGCTGACTTG
		Rv	GGAGGAACCGGTGTCAAAAATG
trypsinogen	AY510093.1	Fw	AGTGCGCAAAGAACTCTGTG
		Rv	AATGTTGTGCTCACCAAGGC
chymotrypsinogen	ENSONIG00000003237	Fw	TTCTGCCTTCGCTTCTCATC
		Rv	TTCAACGCCATCTGCTACTG
procarboxypeptidase	ENSONIG00000003887	Fw	TGAGGGGCATAAAGTGCTTC
		Rv	GCTCGAACTCCATCATTTCC
npy	ENSONIG00000004499	Fw	ACACCCAACACTGCTTGAAG
		Rv	TGTTGCACAGATGACGACTC
pyy	ENSONIG00000006306	Fw	TCAATCTGATCACCAGGCAGAG
		Rv	TGTTATCGCCACCAAACAGC
cck	ENSONIT00000019439.1	Fw	AGAAACTCCACGGCAAACAG
		Rv	ACTCATACTCCTCTGCACTGC
ef1α	AB075952	Fw	GATTGACCGTCGTTCTGGCAAGAAGCGGCA
		Rv	AAGCGACCAAGGGGAGCAT
18s	JF698683	Fw	GGACACGGAAAGGATTGACAG
		Rv	GTTCGTTATCGGAATTAACCAGAC

Abbreviations: Fw, forward; Rv, reverse; *npy*, neuropeptide y; *pyy*, peptide yy; *cck*, cholecystokinin; *ef1α*, elongation factor 1 alpha; 18 s = 18 s ribosomal RNA

Statistical analysis

Data were submitted to Shapiro-Wilk and Levene's tests to verify a normal data distribution and homogeneity of variances, respectively. All gene expression and digestive enzyme activities data were square root transformed. The variables were subjected to an analysis of variance (ANOVA) and to first and second degree polynomial regression analysis. The model was selected based on the behaviour of the data, the level of significance of the mean square of the residues and the coefficient of determination. Results were considered statistically significant when $p < 0.05$. Statistical analyses were performed using R software. The results are presented as mean values with their standard errors.

3.3 Results

There was no significant effect of dietary protein levels on the activity of acid protease in the stomach (13.49 ± 0.75 U/mg protein; $p > 0.05$). However, alkaline proteases (trypsin and Chymotrypsin) were significantly affected. A positive quadratic effect was observed for trypsin activity in the three portions of the intestine (anterior, middle and posterior) ($p < 0.05$), with greater activity for the estimated levels of 31.07, 32.14 and 31.28% CP, respectively (Figure 1a). Chymotrypsin activity had a similar profile to that of trypsin in the three portions of the intestine ($p < 0.05$), with the greatest activity at the estimated levels of 31.06, 31.43 and 30.22% CP, respectively (Figure 1b). The greatest activities of these enzymes were observed in the anterior intestine followed by the middle intestine.

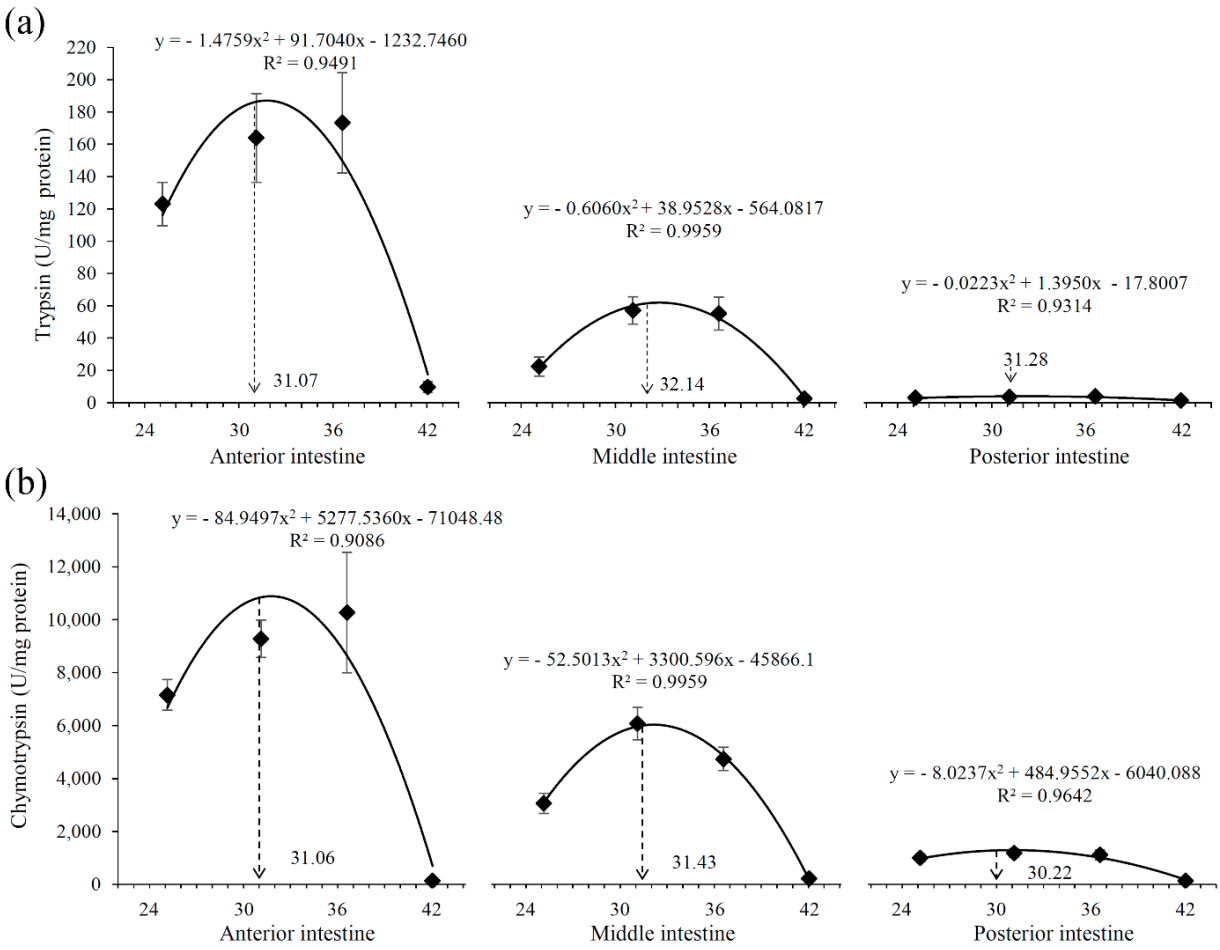

Figure 1. Quadratic regression analysis of trypsin activity (a) and chymotrypsin activity (b) measured at three different segments of the intestine (anterior, middle and posterior) for the juvenile Nile tilapia fed diets containing different levels of crude protein for 65 days. Values are means (n = 15) with their standard errors represented by vertical bars.

The treatments influenced in a quadratic manner the expression of *pepsinogen* ($p < 0.05$) of juvenile tilapia, which increased until the crude protein estimate of 35.76% (Figure 2).

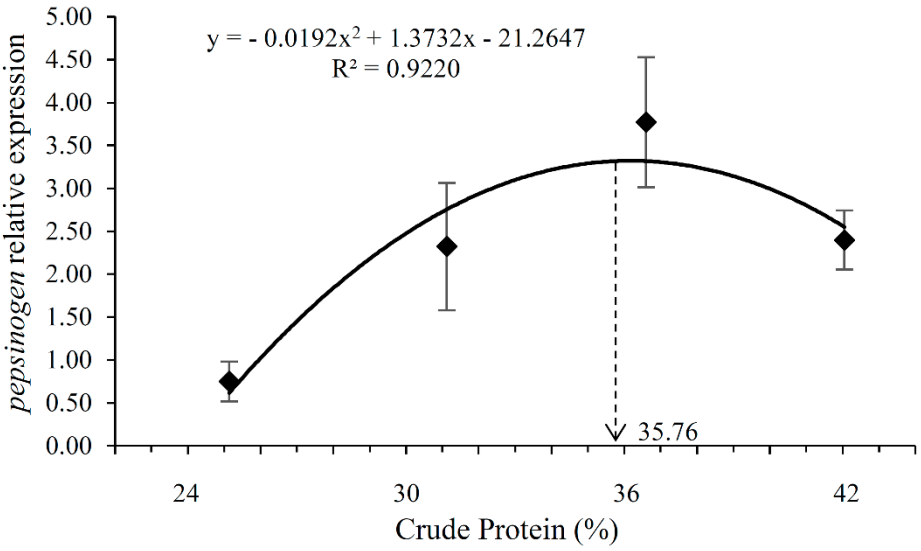

Figure 2. Quadratic regression analysis of relative expression of gastric *pepsinogen* for the juvenile Nile tilapia fed diets containing different levels of crude protein for 65 days. Values are means (n = 15) with their standard errors represented by vertical bars.

The relative expression of *trypsinogen* in the hepatopancreas presented a quadratic effect ($p < 0.05$), increasing to the estimated level of 30.65% CP and then decreasing from that level (Figure 3a). On the other hand, in the intestine the expression of *chymotrypsinogen* increased linearly ($p < 0.05$) with increasing dietary protein concentration (Figure 3b). The relative expression of *chymotrypsinogen* in the hepatopancreas did not presented statistical differences between treatments (1.50 ± 1.93; $p > 0.05$), as well as for the expression of *procarboxypeptidase* in the hepatopancreas (1.5 ± 1.33; $p > 0.05$) and intestine (1.19 ± 0.85; $p > 0.05$).

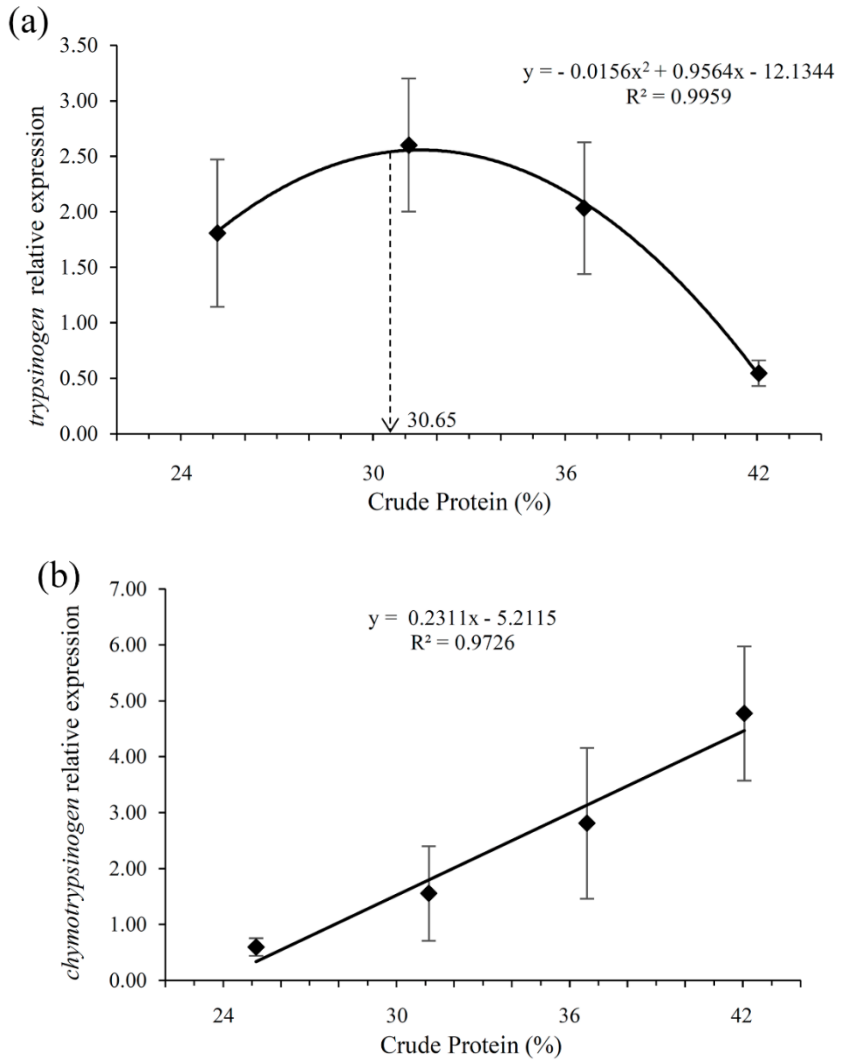

Figure 3. Quadratic regression analysis of relative expression of *trypsinogen* in the hepatopancreas (a) and linear regression analysis of relative expression of *chymotrypsinogen* in the intestine (b) for the juvenile Nile tilapia fed diets containing different levels of crude protein for 65 days. Values are means (n = 15) with their standard errors represented by vertical bars.

For endocrine factors, the relative expression of *npy* (2.05 ± 0.43; $p > 0.05$) showed no significant difference between the protein levels tested. Meanwhile, there was a linear increase in the expression of *cck* and *pyy* ($p < 0.05$) with increasing protein levels in the diet (Figure 4).

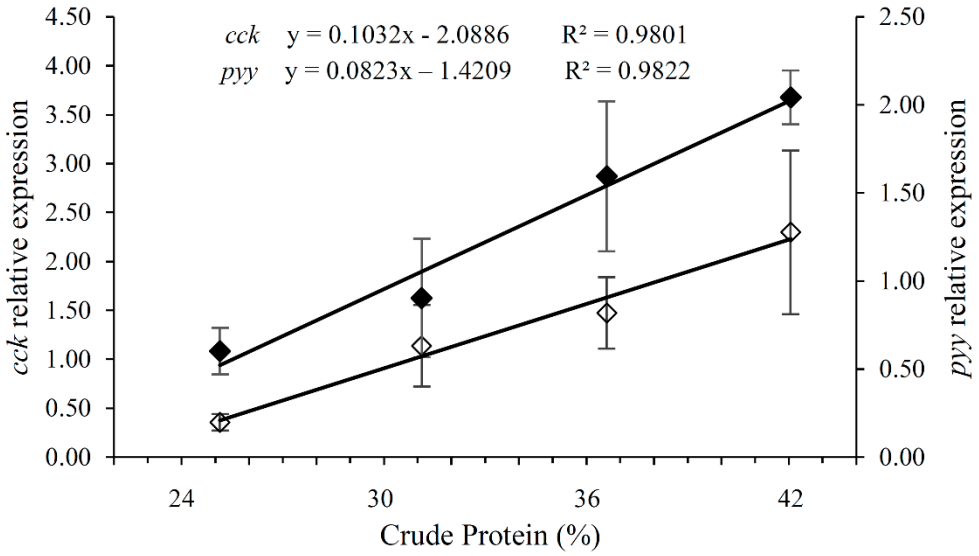

Figure 4. Linear regression analysis of relative expression of *cck*, cholecystokinin (◊) and *pyy*, peptide yy (♦) in the intestine for the juvenile Nile tilapia fed diets containing different levels of crude protein for 65 days. Values are means (n = 15) with their standard errors represented by vertical bars.

The results for performance parameters, nutrients retention rates and hepatic transaminases of juvenile tilapia are presented in Table 3. There was no significant effect of dietary CP levels on apparent food conversion, survival and AST ($p > 0.05$). However, increasing CP in diets resulted in decreased linearly final weight, weight gain, food intake, SGR, PRR and ERR ($p < 0.05$). In contrast, ALT activity showed a positive linear effect ($p < 0.05$) with increasing diet protein levels.

Table 3. Performance parameters, nutrients retention rates and hepatic transaminases of juvenile tilapia fed with varying levels of dietary crude protein

Variables	Levels of crude protein analyzed (%)				*p*-value		
	25.14	31.12	36.60	42.05	ANOVA	Linear model	Quadratic model
Performance[a]							
Initial weight (g)	16.35±0.20	16.46±0.49	16.55±0.25	16.87±1.00	0.9288	-	-
Final weight (g)	145.63±4.92	142.26±8.34	120.17±8.87	113.84±5.83	0.0269	0.0047	0.8495
Food intake (g)	191.47±3.88	180.66±9.16	153.80±7.07	153.56±8.59	0.0049	0.0009	0.4827
Weight gain (g)	134.52±1.30	127.25±7.35	114.39±4.69	110.03±5.80	0.0243	0.0034	0.7888
SGR (%/day)	3.36±0.06	3.31±0.07	2.94±0.02	2.95±0.04	0.0000	0.0000	0.56909
Apparent feed conversion	1.42±0.03	1.42±0.01	1.31±0.02	1.38±0.10	0.3214	-	-
Survival (%)	91.67±3.73	95.00±3.33	90.00±3.12	88.33±4.25	0.6160	-	-
Nutrients retention rates[b]							
PRR (%)	34.46±2.65	24.61±2.85	19.37±1,47	17.18±1.89	0.0003	0.0000	0.1126
ERR (%)	33.67±2.98	27.43±3.43	20.64±1.39	19.61±1.81	0.0040	0.0005	0.3199
Transaminases[c]							
ALT (U/mg protein)	83.52±4.58	86.11±6.85	152.19±11.09	156.87±12.33	0.0000	0.0000	0.9051
AST (U/mg protein)	256.33±14.52	237.49±10.16	234.45±20.28	215.47±12.63	0.3273	-	-
Regression equation							R^2
$Y_{Final\ weight} = -2.0001x + 196.5631$							0.9079
$Y_{Food\ intake} = -2.3956x + 248.6352$							0.8816
$Y_{Weight\ gain} = -1.4560x + 169.5161$							0.9643
$Y_{SGR} = -0.0278x + 4.0542$							0.8202
$Y_{PRR} = -0.9512x + 55.2912$							0.9166
$Y_{ERR} = -0.8161x + 52.2702$							0.9318
$Y_{ALT} = 4.7456x - 36.8189$							0.8421

Note: Values are presented as mean ± standard errors.
Number of observations (n) used in the statistical procedure: [a]n = 5 (composed of a set of 12 fishes); [b]n = 10 (2 fish from each replicate tank); [c]n = 15 (3 fish from each replicate tank).
Abbreviations: SGR, Specific growth rate; PRR, protein retention rate; ERR, energy retention rate; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

3.4 Discussion

The capacity of fish to utilize ingested protein depends to a large extent on the proteolytic enzymes present in the digestive tract and on the response of these enzymes to diet composition (Pérez-Jiménez et al., 2009). Protein digestion in tilapia begins in the stomach through the action of pepsin, which initiates protein hydrolysis (Tengjaroenkul et al., 2000). In our study, acid protease activity was not affected by the treatments, indicating that protein levels between 25.14 and 42.05% were not sufficient to modify the activity of this enzyme, as also observed for *Colossoma macropomum* (Kohla et al., 1992) and *Pseudoplatystoma corruscans* (Lundstedt et al., 2004).

Alkaline proteases are synthesized in the exocrine pancreas and secreted into the lumen of the intestine as inactive precursors (Zambonino Infante and Cahu, 2001). In the present study, greater activities of trypsin and chymotrypsin were found in the anterior portion of the intestine. This was to be expected, because the anterior portion of the intestine is the site where the pancreatic enzymes are released (Uscanga et al., 2010). The activity of these enzymes throughout the intestine varied with dietary protein. According to our results, increased dietary protein led to increased trypsin and chymotrypsin activity in the three portions of the intestine (anterior, middle and posterior) reaching maximum values at 31% CP, with enzyme activity levels being reduced again with greater CP levels. The increase of the activity of these proteases demonstrates an adaptive character and, therefore, the possibility of adjustment of nutrients for optimization of the diets. Considering the regression equations obtained for the enzymatic activity of trypsin and chymotrypsin, possibly 31% CP is the maximum level for the greatest protein synthesis in juvenile tilapia, considering the amount of energy supplied. Other species of fish, such as *Labeo rohita* (Debnath et al., 2007), *Puntius gonionotus* (Mohanta et al., 2008) and *Carassius auratus* (Tu et al., 2015), also showed greater alkaline protease activity due to increased protein in the diet to a certain level.

Enzymatic activity can be modified at the level of synthesis (induction or repression of gene expression) (Panserat and Kaushik, 2010). There appears to be a relationship between increased gene expression and increased enzyme activity, depending on dietary protein (Péres et al., 1998; Wang et al., 2006). In our study, the estimated level of 35.76% CP determined the maximum expression of *pepsinogen*. However, this result did not reflect the activity of the acid protease in the stomach of juvenile tilapia. With these results, we observed that increased gene expression does not always have the effect of increased enzymatic activity, because factors other than protein may influence the response. According to Huvet et al. (2003), several factors

may influence the increase of translatable mRNA including the rate of gene transcription, the efficiency of processing of the primary transcript and the stability of the mature cytoplasmic mRNA.

The expression of *trypsinogen* in the hepatopancreas followed a pattern similar to that of trypsin activity in the intestine, because it exhibited a quadratic effect in relation to dietary protein levels, with the greatest estimated values at 30.65 and 31.50% CP for *trypsinogen* and activity of trypsin in portions of the intestine, respectively. This suggests that dietary protein may modulate the expression of *trypsinogen* and, consequently, increase the activity of trypsin in the intestine. This result is interesting for the process of protein digestion because, among the alkaline proteases, trypsin is considered a key enzyme in this process because it activates its own precursor form (trypsinogen) as well as several other proteases, including chymotrypsin (Sunde et al., 2004).

The *chymotrypsinogen* gene was identified in both the hepatopancreas and the intestine, indicating intestine also as expression site of zymogens in tilapia. Similarly, the expression of *trypsinogen* and *chymotrypsinogen* was detected all along the digestive tract, including the posterior intestine in *Sparus aurata* (Psochiou et al., 2007). The relative expression of *chymotrypsinogen* in the hepatopancreas did not change among CP levels, whereas in the intestine it increased linearly as a function of increasing levels of CP in the diet. These results show that the expression of *chymotrypsinogen* in the intestine is more influenced by dietary protein than in the hepatopancreas of tilapia. In addition, the results indicate that 42.05% CP stimulated the greatest expression of *chymotrypsinogen* in the intestine, but the activity of its active form (chymotrypsin) begins to drop with approximately 31% CP. Likewise, the *procarboxypeptidase* gene was identified in both the hepatopancreas and the intestine of the juvenile tilapia. However, the protein levels tested were not able to modulate the relative expression of these genes.

The results of the enzymatic activity and gene expression show that the increase of the protein in the diet can stimulate the secretion of proteolytic enzymes. However, high enzymatic activity is not always associated with higher fish growth. In the present study, the results of productive performance were higher for the diet of 24% CP (decreasing linear response), whereas the alkaline proteases (trypsin and chymotrypsin) reached higher activity for the estimated level of 31% CP (quadratic response positive). It is suggested that juveniles of tilapia fed with up to 31% of CP efficiently digested the ingested protein due to the high activity of the proteolytic enzymes. However, upon reaching the intermediate metabolism, the absorbed

amino acids were deaminated and not used by fish to form body protein, which was supported by nutrient retention results. Juvenile tilapia fed lower levels of protein had greater PRR, as also observed by Li et al. (2013) for the same species, which indicates better use of the protein consumed. On the other hand, animals fed greater protein levels showed low PRR, indicating that excess protein in the diet did not imply increased protein synthesis, but amino acid deamination instead. The greater ALT activity measured in the hepatopancreas of juvenile tilapia receiving the 36.60 and 42.05% CP treatments confirms greater deamination rates, given the role of this enzyme in amino acid catabolism (Abdel-Tawwab et al., 2010). According to Haidar et al. (2018), when using high levels of protein in the diet, part of the protein is catabolized to meet energy demands, thereby reducing protein efficiency. In addition, considering that diets were isoenergetic, treatments containing lower CP levels would also have a greater energy/protein ratio when compared to diets with greater CP levels. It is possible that the energy available in the diet containing 42.05% CP was not enough to ensure an efficient metabolization of that protein amount in the animal's organism, resulting in lower PRR. The ERR, which indicates how much of the raw energy of the diet is converted into gross body energy, exhibited a similar effect to that of PRR.

Along with the proteolytic enzymes activities, the endocrine mechanisms that control feed intake are also important regulators of digestive function in fish. Feeding is controlled by a number of hormones produced by both the brain and the peripheral organs. These hormones include appetite stimulants (npy) and inhibitors (pyy and cck) that interact to regulate energy balance in fish (Volkoff, 2016). The stimulant effects of appetite, caused by central injections of npy, have been demonstrated in fish (Kiris et al., 2007; Yokobori et al., 2012; Zhou et al., 2013), but the influence of diet composition on this hormone is not yet clear. Narnaware and Peter (2002) observed that *npy* expression in *Carassius auratus* was modulated by high carbohydrate or lipid diets but not by diets with high protein levels. In our study, the expression of *npy* in the hypothalamus of the juvenile tilapia was also not influenced by the protein levels tested. In contrast, Jin et al. (2015) showed that dietary protein levels modulate *npy* expression in *Ctenopharyngodon idella*. Hence *npy* regulation by protein levels seems to be species dependent.

It has been shown that pyy and cck function as satiety hormones in fish (Volkoff et al., 2003; Chen et al., 2015) and, according to our results, these hormones appear to be influenced by protein. We observed that the expression of *pyy* and *cck* in the intestine of tilapia is greater with increasing CP in the diet. It is suggested that the high concentration of free amino acids in

the bloodstream generated after the digestion of the diets with 42.05% CP stimulates the release of pyy and cck, favoring the reduction of the dietary intake of fish, as observed in humans (Morell and Fiszman, 2017). This also justifies the lower consumption of diets with 36.60 and 42.05% CP in our study. Proteins have greater satiety power than carbohydrates and lipids (Caron et al., 2016), due to the greater energy expenditure to digests diets with high protein (Veldhorst et al., 2008).

In conclusion, juvenile tilapia fed with diets containing lower CP levels (25.14 and 31.12%) showed better performance and protein retention rates than animals receiving higher CP levels (36.60 and 42.05%). The dietary protein modulates the activity of alkaline proteases in the intestine, as well as the expression of the genes of these proteases, although a decrease with levels above 31% CP was observed. In addition, dietary protein also modulated the gene expression of hormones involved in appetite regulation (*cck* and *pyy*). The changes in proteases and appetite regulating hormones observed in response to dietary protein levels may help to expand the understanding of food intake and digestion control, thereby improving feed formulations for aquaculture.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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4 MANUSCRITO II

Dietary protein modulates digestive enzyme activities and gene expression in red tilapia juveniles

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Abstract

It is known that the level of dietary protein changes the enzymatic activity of the digestive tract of fish, however, its effect at the molecular level on these enzymes and the hormones regulating appetite has not been well characterised. The objective of this study was to evaluate the effect of CP on the activity of proteases and the expression of genes related to the ingestion and protein digestion of juveniles of red tilapia. A total of 240 juveniles (29.32±5.19 g) were used, distributed across 20 tanks of 100 L in a closed recirculation system. The fish were fed to apparent satiety for 42 days using four isoenergetic diets with different CP levels (24, 30, 36, and 42 %). The results indicated that the diet with 42 % CP was associated with the highest expression of *pepsinogen* and the lowest activity of acid protease ($P < 0.05$). The expression of hepatopancreatic *trypsinogen* increased as CP levels in the diet increased ($P < 0.05$) up to 36%, whereas trypsin activity showed a significant reduction with 42 % CP ($P < 0.05$). The diet with 42 % CP was associated with the lowest intestinal *chymotrypsinogen* expression and the lowest chymotrypsin activity ($P < 0.05$). *α-amylase* expression decreased with increasing ($P < 0.05$) CP levels up to 36 %. No significant differences were observed in the expression of *procarboxypeptidase, lipase,* or *leptin* among all the groups ($P > 0.05$). In addition, the diet with 42 % CP resulted in a decrease ($P < 0.05$) in the expression of *ghrelin* and *insulin* and an increase ($P < 0.05$) in the expression of *cholecystokinin* and *peptide yy*. It is concluded that the activity and expression of digestion and appetite regulating genes are modulated by dietary protein levels in juvenile red tilapia.

Keywords: crude protein, digestion, enzymology, mRNA, appetite

4.1 Introduction

Protein is the most expensive ingredient in fish diets (Tu *et al.*, 2015) and also one of the most important, since its regular intake is necessary for fish to be able to use amino acids to build new proteins (such as during growth and reproduction) or to replace existing proteins (maintenance) (National Research Council, 2011). In excess, protein can lead to additional energy costs, increase nitrogen excretion, and reduce fish growth (Huang *et al*., 2017), even in species with high productive potential such as tilapia. Red tilapia is becoming increasingly popular in the world aquaculture landscape due to its rapid growth, salinity tolerance, easy

adaptation to different farming systems, and market acceptance (Pradeep *et al*., 2014). Thus, the formulation of diets that more efficiently meet the protein requirements of tilapia is of extreme importance for the success of the aquaculture.

The ingestion and digestion processes are determinants for the use of a diet to optimise fish growth and food utilisation (Volkoff *et al*., 2010; García-Meilán *et al*., 2016). Food intake is regulated by a multitude of hormones produced by both the brain and peripheral organs that stimulate (orexigenic) or inhibit (anorexigenic) appetite (Volkoff, 2016). Ghrelin is one of the orexigenic hormones, and leptin, the peptide yy (pyy), cholecystokinin (cck) and insulin are among the anorexigenic hormones. These hormones are involved in the regulation of several other physiological processes, for example, cck act both in satiety and in the secretion of pancreatic enzymes, gut motility and gallbladder contraction (Micale *et al*., 2014). Nutrient degradation in the digestive tract of fish depends largely on digestive enzymes (Ma *et al*., 2019), and their activity depends on the nutrients present in the ingested food (Pérez-Jiménez *et al*., 2009). The most important proteolytic digestive enzymes are the acid protease (pepsin), secreted by the gastric mucosa, and the serine proteases trypsin and chymotrypsin, secreted by the pancreas and present in the intestine (García-Meilán *et al*., 2016).

The level of dietary protein can modulate the expression of digestive enzymes and appetite regulating hormones at the transcriptional level (Jin *et al*., 2015; Wang *et al*., 2006). However, despite reports in the literature regarding the influence of protein on the performance or physiology of tilapia juveniles, there are few studies showing this influence on gene expression. The objective of this study was to evaluate the activity of proteolytic enzymes and the gene expression of digestive enzymes and appetite regulating hormones of red tilapia juveniles submitted to diets containing different crude protein levels.

4.2 Material and methods

Animals and experimental conditions

Male red tilapia juveniles (*Oreochromis* sp.) were purchased from a commercial fish farm and transported to Laboratório de Aquacultura at the Escola de Veterinária of the Universidade Federal de Minas Gerais (UFMG) where the experiment was carried out. After 15 days of acclimatisation, 240 fish (initial mean weight of 29.32±5.19 g) were selected and randomly distributed across 20 glass fibre tanks (100 L; 12 fish per tank), mounted on a

recirculation water system with mechanical and biological filters, air blowers, and central heating. During the test, the temperature was maintained at 28.34±0.43 °C, the dissolved oxygen at 6.17±0.77 mg/L, and the pH at 6.59±0.15.

Experimental diets and food management

Four isoenergetic diets (17.67 MJ/kg) were formulated to contain 24, 30, 36, and 42 % CP. For the preparation of the diets, the ingredients were milled to produce particles less than 0.5 mm in diameter. After weighing and homogenisation, the blends were submitted to the extrusion process (Labor PQ30, Inbramaq®, Ribeirão Preto, São Paulo, Brazil), obtaining pellets approximately 4 mm in diameter. After extrusion, soybean oil was added and the diets were stored in a cold room (0 °C) until use. Analyses of the approximate composition of the diets were performed following Association of Official Analytical Chemists (2012) procedures. Briefly, the crude protein (N × 6.25) was quantified according to the Kjeldahl method, the ethereal extract using an Soxhlet extractor, the crude energy by using a calorimetric pump (PARR 6200, Parr Instrument Company, Milone, Illinois, USA), and mineral matter by burning the samples in a muffle oven (600 °C for 4 h). The formulation and approximate composition of the experimental diets are shown in Table 1. The experiment lasted 42 days and during this period the fish were fed with the experimental diets twice daily (08:00 h and 16:00 h) until apparent satiation. Each diet was fed to five different tanks, using a total of twenty tanks. Tank cleaning was done daily after the last feeding.

Table 1. Formulation and approximate composition diets for tilapia juvenile

Ingredient	Dietary protein level (%)			
	24	30	36	42
Fishmeal	15.50	21.00	24.00	29.00
Soybean meal	24.00	31.00	33.00	37.00
Corn	40.00	31.50	22.90	13.80
Broken rice	5.00	5.00	5.00	5.00
Albumin	1.00	1.60	6.50	9.10
Soybean oil	8.80	6.60	5.30	3.70
Cellulose	3.00	1.00	1.00	0.00
Bicalcic phosphate	0.50	0.50	0.50	0.50
Vitamin / Mineral Premix[1]	0.50	0.50	0.50	0.50
BHT[2]	0.02	0.02	0.02	0.02
Salt	0.50	0.50	0.50	0.50
Inert	1.18	0.78	0.78	0.88
Analyzed composition[3]				
Crude protein (%)	25.14	31.12	36.60	42.05
Crude energy (MJ kg^{-1})	17.28	17.86	17.93	17.60
Ether extract (%)	9.98	7.77	7.86	6.35
Ash (%)	6.66	7.02	7.49	10.08

[1] Composition of vitamin and mineral supplement: Vit. A, 1 200 000 IU; Vit. D_3, 200 000 IU; Vit. E, 12 000 mg; Vit. K, 2 400 mg; Vit. B_1, 4 800 mg; Vit. B_2, 4 800 mg; Vit. B_6, 4 000 mg; Vit. B_{12}, 4 800 mg; folic acid, 1 200 mg; pantothenic acid, 3 750 mg; Vit. C, 48 000 mg; Biotin, 48 mg; Choline, 65 000 mg; Niacin, 24 000 mg; Fe, 10 000 mg; Cu, 6 000 mg; Mn, 4 000 mg; Zn, 6 000 mg; I, 20 mg; Co, 2 mg; Se, 20 mg.

[2] Butylated hydroxytoluene (antioxidant).

[3] Values presented are means from triplicate analyses.

Sample collection

At the end of the experiment, after 12 h of fasting, three fish from each tank (n = 15/treatment) were euthanised with a benzocaine overdose (250 mg/L) and dissected to remove fragments from the stomach, anterior intestine, and hepatopancreas. All samples were cleaned in saline solution and immediately stored at -80 °C for enzymatic and gene expression analyses.

Enzyme activity assay

Acid protease activity was determined in the stomach and the activity of trypsin and chymotrypsin were determined in the anterior intestine. Tissue extracts from stomach samples were prepared in Tris-HCl 20 mM pH 7.0 and from anterior intestine samples in Tris-HCl 100 mM pH 7.8 buffer with 0.1 % Triton X-100 and 0.1 mM Ethylenediamine tetraacetic acid (EDTA). The activity of acid proteases was determined using bovine haemoglobin (Sigma) as the substrate. Aliquots of 20 µL of enzyme extract were incubated with 250 µL of haemoglobin (0.5 % in 0.1 M glycine buffer pH 2.0) for 20 minutes at 37 °C. This reaction was then stopped by the addition of 250 µL of 20 % TCA (trichloroacetic acid). The activity was measured at 280 nm and for each sample a "blank" containing 20 µL of enzyme extract, 250 µL of 0.5 % (w/v) haemoglobin, and 250 µL of 20 % (w/v) TCA was made. The specific activity was defined as µg of tyrosine released per minute per mg of total soluble proteins at 37 °C, using 0.008 mL/µg per cm as the molar extinction coefficient.

Trypsin activity was determined using Nα-benzoyl-L-arginine-4-nitroanilide (BApNA) as a substrate. Aliquots of 100 µL BApNA (1 mM in 50 mM Tris-HCl pH 8.2 and 20 mM $CaCl_2$) were incubated with 20 µL of enzyme extract at 30 °C. The activity was measured at 410 nm every 30 s for 30 min. The specific activity was defined as nmol of 4-nitroaniline produced per minute (U) per mg of protein (mU^{-1} mg protein). The molar extinction coefficient was 8800 M/cm. Chymotrypsin activity was determined using N-benzoyl-L-tyrosine ethyl ester (BTEE) as the substrate. Aliquots of 140 µL BTEE (1.13 mM in 100 mM Tris-HCl pH 7.8 and 25 mM $CaCl_2$) were incubated with 20 µL of enzyme extract at 30 °C, and the activity measured at 256 nm every 20 s for 20 min. The specific activity was defined as nmol of Benzoyl-L-tyrosine produced per minute per mg of protein. The molar extinction coefficient was 964 M/cm. The total protein concentrations were determined using Bradford's (1976) method, with bovine serum albumin (BSA) as a standard. All readings were performed in triplicate using a microplate reader (Multiskan GO, Thermo Scientific, Waltham, Massachusetts, USA).

Real time PCR analysis

The expression of the genes *pepsinogen* and *ghrelin* was determined in the stomach, the expression of the genes *leptin, cck,* and *pyy* was determined in the anterior intestine, and in the hepatopancreas, the expression of the genes *procarboxypeptidase*, *lipase,* and *α-amylase* was determined. The expression of the *trypsinogen* and *chymotrypsinogen* genes were evaluated

both in the anterior intestine and in the hepatopancreas. The stomach, intestine, and hepatopancreas samples were transferred into sterile tubes containing 0.5 mL of Trizol (Invitrogen, Carlsbad, Califórnia, USA). The tissue samples were mechanically homogenised and total RNA extraction was performed according to the manufacturer's instructions (Invitrogen). The RNA pellet was dissolved in sterile DEPC water (Invitrogen) and the RNA concentration was determined by spectrometry (Nanodrop ND-1000, Thermo Fisher Scientific, Waltham, MA, USA). Total RNA (1 µg) was retrotranscribed using a commercial kit (QuantiTect Reverse Transcription Kit, Qiagen, Hilden, Germany), which included a genomic DNA elimination step. cDNA was subjected to quantitative PCR analyses in a light thermocycler (7500 Real-Time PCR system, Applied Biosystems, Foster City, Califórnia, USA) following this protocol: 95 ºC for 15 min, followed by 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min. Quantitative PCR reactions were performed using the SYBR Green PCR Master Mix (Applied Biosystems). All samples were run in duplicate and the qPCR reaction was performed in a final volume of 20 µL.

The relative amplification efficiencies of all genes were analysed by cDNA dilution curves, and it was verified that they were similar for all the genes. Melting curves were run after amplification to ensure that only one DNA species was being amplified. The primer concentrations were determined by means of a primer dilution curve. The primers for *pepsinogen,* hepatopancreatic *trypsinogen, lipase, insulin, cck, pyy, elongation factor 1 alpha (ef1α),* and *18 s ribosomal RNA (18 s)* were added at a final concentration of 200 nM in the reaction mixture. The primers for intestinal *trypsinogen, procarboxypeptidase, α-amylase,* and *ghrelin* were added at a final concentration of 400 nM. The primer for *leptin* was added at a final concentration of 600 nM. The primers for hepatopancreatic and intestinal *chymotrypsinogen* were added at a final concentration of 1000 nM. The gene expression values were calculated as relative expression using the ΔΔCT method using ef1a and 18s as the housekeeping references. The primers were designed using the Primer3 software (Untergasser *et al*., 2012) and their sequences are shown in Table 2. All procedures were adapted from Costa *et al*. (2016).

Table 2. Primer sequences used in the quantitative PCR (qPCR) assays of tilapia

Gene	Ensembl/GenBank	Fw/Rv	Primer sequence (5´to 3´)
pepsinogen	JQ043215.1	Fw	TGACCAATGACGCTGACTTG
		Rv	GGAGGAACCGGTGTCAAAAATG
trypsinogen	AY510093.1	Fw	AGTGCGCAAAGAACTCTGTG
		Rv	AATGTTGTGCTCACCAAGGC
chymotrypsinogen	ENSONIG00000003237	Fw	TTCTGCCTTCGCTTCTCATC
		Rv	TTCAACGCCATCTGCTACTG
procarboxypeptidase	ENSONIG00000003887	Fw	TGAGGGGCATAAAGTGCTTC
		Rv	GCTCGAACTCCATCATTTCC
lipase	ENSONIG00000005832	Fw	TCGGTGGATGGCATGATGGAGA
		Rv	GCGACTGGATAGTGCTGCTGAG
α-amylase	ENSONIG00000018530	Fw	TGGCGTTGGGCTGACATTGC
		Rv	TTCCTGTTCCACCACCAGATCC
insulin	ENSONIT00000000430.1	Fw	AGGAATGCTGTCACAAACCC
		Rv	AACAAAGTCCAGCAGAGCAG
ghrelin	ENSONIG00000019046	Fw	GCCTGTTGGCTTTTCTCTTG
		Rv	AGTGACGCCAATTTCAAAGG
leptin	KF955989.1	Fw	ATGAACTGCAGCACCCTTTC
		Rv	ACTGCTGGTTTGGGCTTAAG
pyy	ENSONIG00000006306	Fw	TCAATCTGATCACCAGGCAGAG
		Rv	TGTTATCGCCACCAAACAGC
cck	ENSONIT00000019439.1	Fw	AGAAACTCCACGGCAAACAG
		Rv	ACTCATACTCCTCTGCACTGC
ef1α	AB075952	Fw	GATTGACCGTCGTTCTGGCAAGAAGCGGCA
		Rv	AAGCGACCAAGGGGAGCAT
18s	JF698683	Fw	GGACACGGAAAGGATTGACAG
		Rv	GTTCGTTATCGGAATTAACCAGAC

Fw = Forward; Rv = Reverse; *pyy* = peptide yy; *cck* = cholecystokinin; *ef1α* = elongation factor 1 alpha; 18 s = 18 s ribosomal RNA

Statistical analysis

Statistical analyses were carried out using SPSS v16.0 software (SPSS Inc., Chicago, IL, USA). Data were submitted to Shapiro-Wilk and Levene's tests to verify a normal data

distribution and homogeneity of variances, respectively. Data sets were transformed appropriately when the data did not meet the assumptions of ANOVA. Data were analysed using a one-way ANOVA followed by a Tukey's post-hoc test. Significant differences were considered when $P < 0.05$. The results are presented as mean values with their standard errors.

4.3 Results

The acid protease activity was lower for fish fed with 42 % CP (Figure 1a, $P < 0.05$) than 30 and 36 % CP diets. In contrast, *pepsinogen* expression was higher for fish fed with 42 % CP (Figure 1b, $P < 0.05$) than 24 and 30 % CP diets.

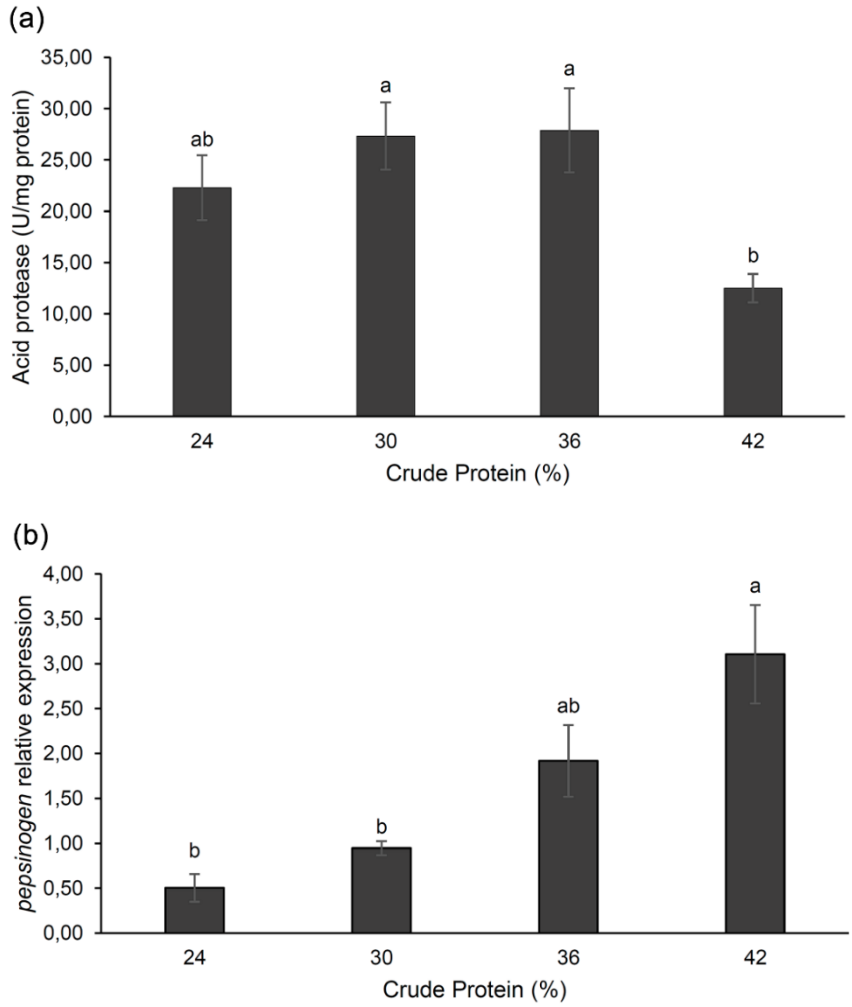

Figure 1. Enzymatic activity of acid protease (a) and relative expression of *pepsinogen* (b) in the stomach of red tilapia juveniles fed diets containing different levels of crude protein for 42 days. Different letters indicate statistically significant differences between groups (Tukey's test, $P < 0.05$). Values are means (n = 15) with their standard errors represented by vertical bars.

The trypsin activity in the anterior intestine was similar for the diets with 24, 30, and 36 % CP, and the lowest level was recorded for the diet with 42 % CP (Figure 2a, $P < 0.05$). In the hepatopancreas, *trypsinogen* expression was lowest with 24 %, intermediate with 30 %, and highest and similar with 36 % and 42 % CP (Figure 2b, $P < 0.05$). No dietary effect was observed among the treatments on the expression of *trypsinogen* in the anterior intestine (Figure 2b, $P > 0.05$).

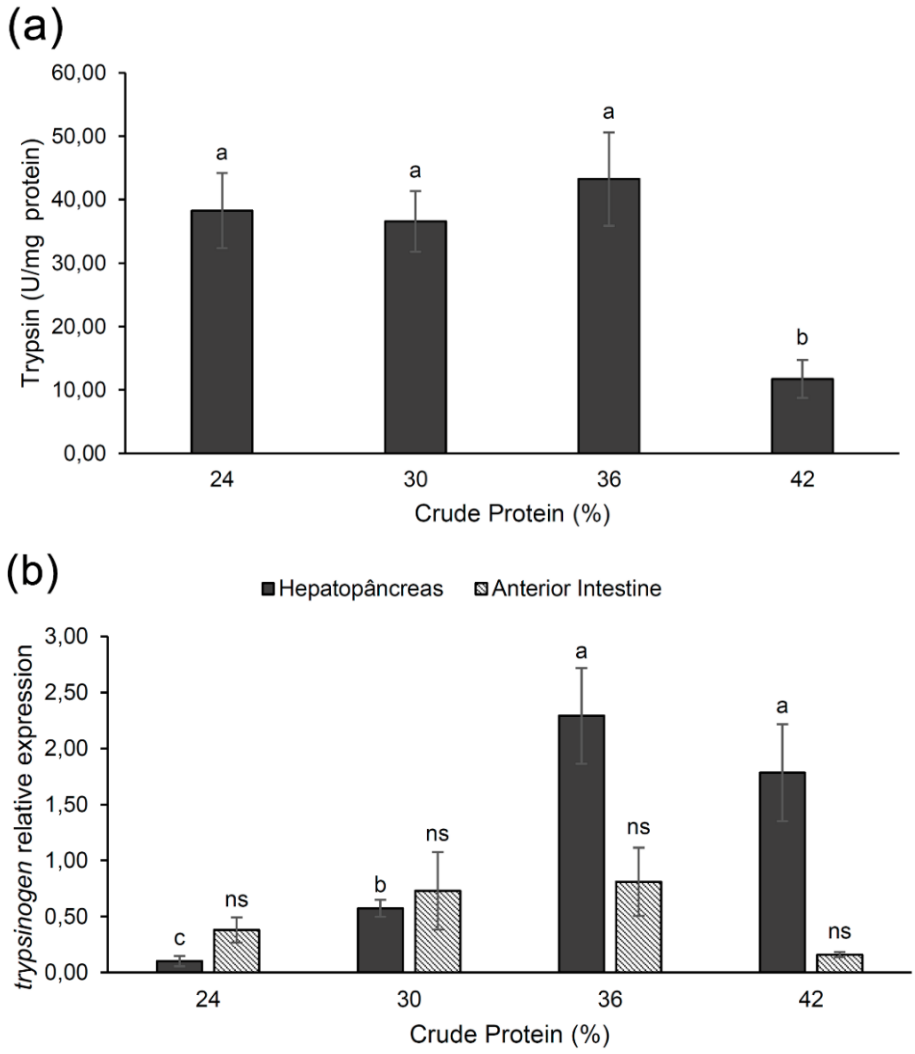

Figure 2. Enzymatic activity of trypsin in the anterior intestine (a) and relative expression of *trypsinogen* in the hepatopancreas and anterior intestine (b) of red tilapia juveniles fed diets containing different levels of crude protein for 42 days. Different letters indicate statistically significant differences between groups (Tukey's test, $P < 0.05$). ns: not significant. Values are means (n = 15) with their standard errors represented by vertical bars.

The chymotrypsin activity in the anterior intestine was higher for diets with 30 and 36 % CP (Figure 3a, $P < 0.05$). *Chymotrypsinogen* expression in the anterior intestine was lower for the diet with 42 % CP and higher for the diet with 36 % CP (Figure 3b, $P < 0.05$). In the hepatopancreas, the expression of the same gene was higher with levels of 36 and 42 % CP (Figure 3b, $P < 0.05$).

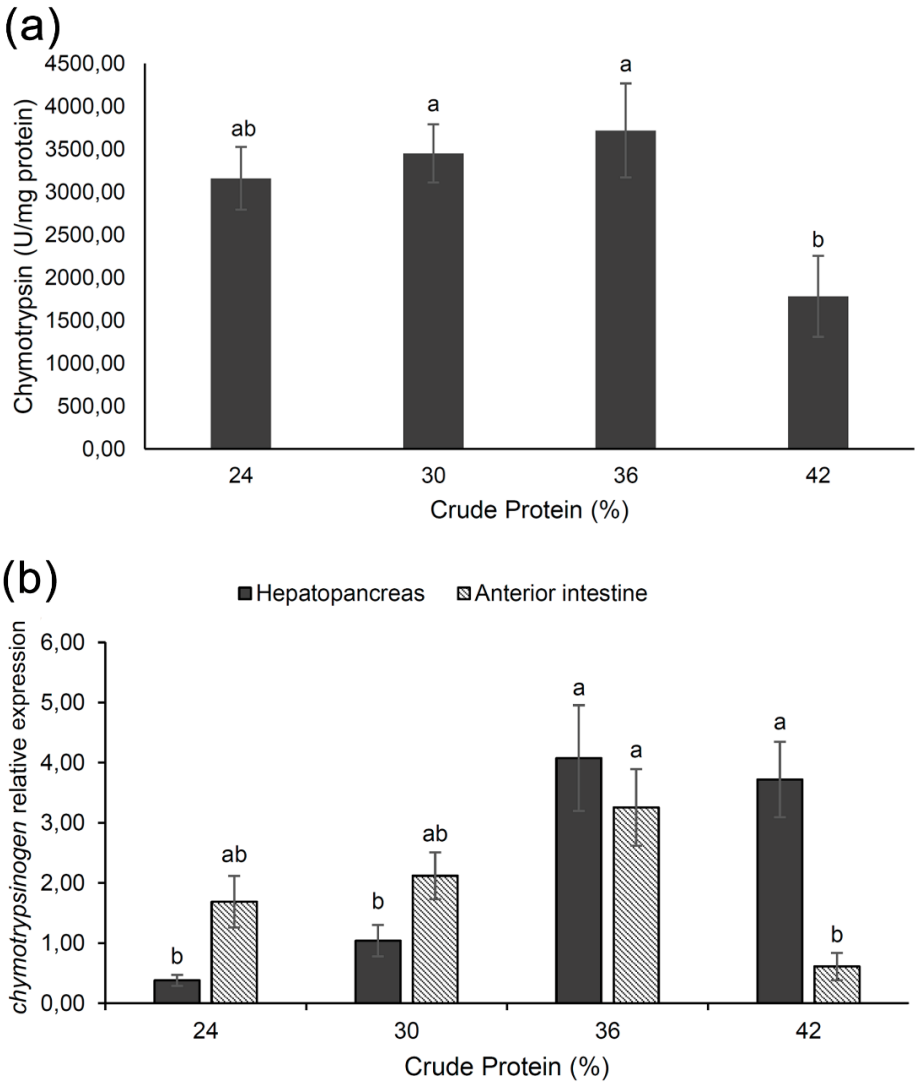

Figure 3. Enzymatic activity of chymotrypsin in the anterior intestine (a) and relative expression of *chymotrypsinogen* in the hepatopancreas and anterior intestine (b) of red tilapia juveniles fed diets containing different levels of crude protein for 42 days. Different letters indicate statistically significant differences between groups (Tukey's test, $P < 0.05$). Values are means (n = 15) with their standard errors represented by vertical bars.

There was no effect ($P > 0.05$) of dietary protein on *procarboxypeptidase* or *lipase* expression (Figure 4). However, *α-amylase* expression decreased ($P < 0.05$) with increasing dietary CP levels up to 36 %, then remained constant with the inclusion of up to 42 % CP (Figure 4).

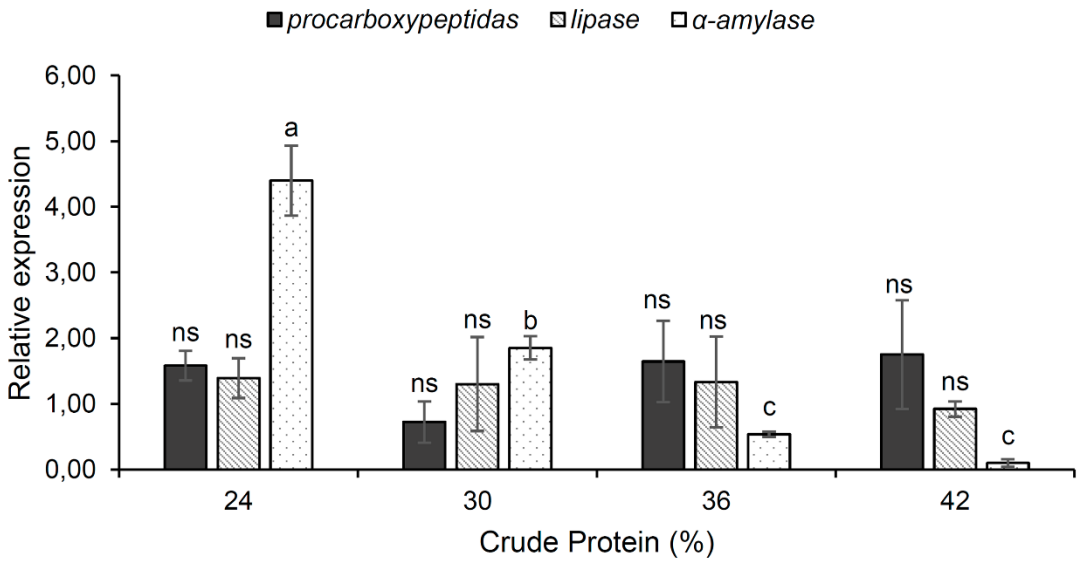

Figure 4. Relative expression of *procarboxypeptidase*, *lipase*, and *α-amylase* in the hepatopancreas of red tilapia juveniles fed diets containing different levels of crude protein for 42 days. Different letters indicate statistically significant differences between groups (Tukey's test, $P < 0.05$). ns: not significant. Values are means (n = 15) with their standard errors represented by vertical bars.

Leptin expression remained constant ($P > 0.05$) among the fish fed different diets (Figure 5). Fish fed the diet containing 42 % CP had a lower ($P < 0.05$) *ghrelin* expression than those receiving 24 and 36 % CP (Figure 5). *Insulin* expression gradually declined ($P < 0.05$) as CP levels in the diet increased up to 36 % and then remained constant (Figure 5). The *pyy* gene exhibited higher ($P < 0.05$) expression in the fish fed the diet containing 42 % CP than in the fish fed the other diets (Figure 5). *cck* expression was higher ($P < 0.05$) in fish fed with 42 % CP than fish fed with 24 % CP (Figure 5).

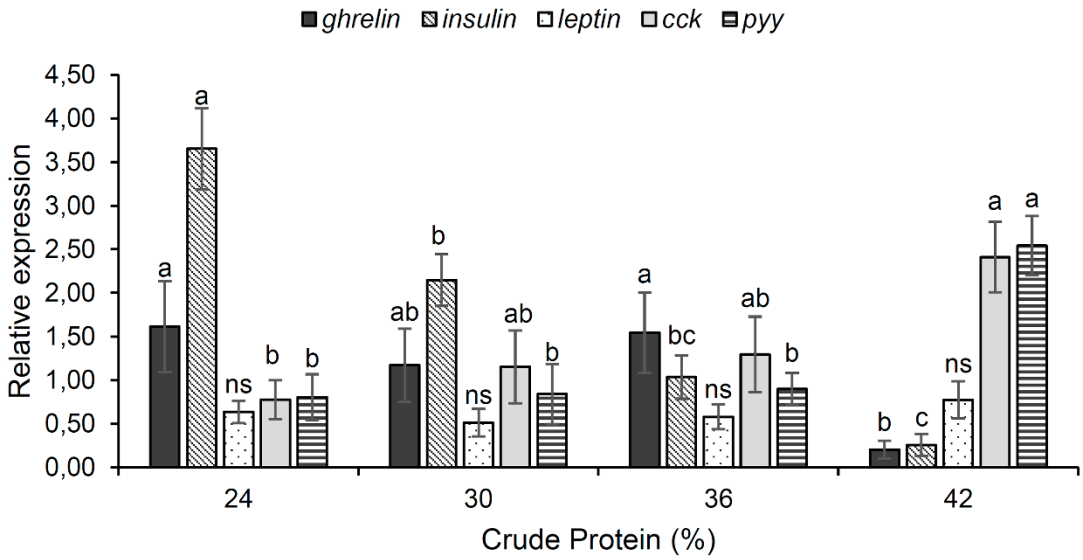

Figure 5. Relative expression of *ghrelin* in the stomach; *insulin* in the hepatopancreas; and *leptin*, cholecystokinin (*cck*), and peptide yy (*pyy*) in the anterior intestine of red tilapia juveniles fed diets containing different levels of crude protein for 42 days. Different letters indicate statistically significant differences between groups (Tukey's test, $P < 0.05$). ns: not significant. Values are means (n = 15) with their standard errors represented by vertical bars.

4.4 Discussion

The activity of digestive enzymes in fish might vary according to diet composition (Pérez-Jiménez *et al*., 2009). This study agrees with the results found in previous studies, since the activity of proteolytic enzymes was affected by dietary protein. There was a significant reduction in acid protease activity with a 42 % CP diet, indicating the important role of this enzyme in the digestion of red tilapia. Likewise, the activity of trypsin and chymotrypsin were influenced by protein and presented similar profiles in each diet. The activity of these enzymes showed little variation up to a CP inclusion level of 36 %. However, at the highest level (42 %), there was a significant reduction. These results suggest that excess protein might lead to decreased protease activity. Therefore, when offered a higher level of substrate (protein, in this case), the glands that synthesise enzymes might increase the production of specific enzymes to digest the substrate, however, there is a limit to this capacity (Giri *et al*., 2003; Tu *et al*., 2015). Similar results were found by Mohanta *et al*. (2008), Tu *et al*. (2015), and Huang *et al*. (2017) who showed a decrease in the activity of proteolytic enzymes in *Puntius gonionotus*, *Carassius auratus*, and *Nibea coibor*, respectively, submitted to higher levels of protein.

The secretion of alkaline proteases in the digestive tract is mediated by neural and hormonal stimuli which, in turn, are associated with the presence of nutrients in the chyme (Tillner *et al*., 2013). Furutani *et al*. (2012) demonstrated that dietary protein stimulates *cck* secretion and, consequently, the release of digestive enzymes. In fact, our results for trypsin and chymotrypsin activity coincided with changes in *cck* gene expression with dietary CP levels of up to 36 %. However, at the highest protein level (42 %), *cck* expression was higher and the alkaline protease activity was lower. These results suggest that *cck* might also be related to the inhibition of proteolytic enzymes when protein levels are above that required by the animal. Tillner *et al*. (2013) suggested that a negative feedback mechanism is present between trypsin and *cck* activity in *Gadus morhua* larvae.

The genes involved in the digestion and appetite processes are mainly regulated at the transcriptional level and appear to vary their expression profiles depending on the composition of the diet (Morais *et al*., 2004; Kortner *et al*., 2011). Our results showed that CP regulated the levels of gene expression of the main proteolytic enzymes, with the exception of *procarboxypeptidase*. For *pepsinogen*, we observed an inconsistency between its expression and enzymatic activity, that is, the expression of *pepsinogen* was higher with 42 % CP, whereas the acid protease activity was lower with the same protein level. These results suggest a post-transcriptional regulation for acid protease. For the alkaline proteases, the results of this work inferred the existence of digestive enzyme synthesis not only in the exocrine pancreas, but also in the anterior intestine of red tilapia juveniles. Psochiou *et al*. (2007) also detected trypsinogen and *chymotrypsinogen* gene expression throughout the digestive tract of *Sparus aurata*. The expression of *trypsinogen* in the intestine was not altered by the protein level in the diet, however, in the hepatopancreas, its expression increased as the level of protein in the diet increased up to 36 % and then remained constant. These results do not match the results for trypsin activity, indicating a post-transcriptional regulation for this protease in tilapia. Perhaps not all *trypsinogen* mRNA was translated into trypsinogen and available for trypsin activation, since the transcripts are subject to degradation in the cytosol (Gawlicka and Horn, 2006). Chymotrypsin also partly presented this non-parallel pattern, since the expression of hepatopancreatic *chymotrypsinogen* was higher with 36 and 42 % CP and its enzymatic activity was lower with 42 % CP. In contrast, most of the regulation of chymotrypsin occurred at the transcriptional level, since variations in the expression profiles of intestinal *chymotrypsinogen* were reflected in the activity of chymotrypsin at the different levels of protein tested.

Other important digestive enzymes are α-amylase and lipase, which are responsible for the digestion of carbohydrates and lipids, respectively. In this study, *lipase* expression did not present a significant difference between the treatments, which can be attributed to the low variation in ethereal extract in the experimental diets. However, *α-amylase* expression was highest in diets with 24 % CP, followed by 30 % CP, which might be attributed to the higher amounts of carbohydrate in those diets at the expense of protein. The results for *insulin* expression were similar. The expression of the *insulin* gene gradually declined as CP levels in the diet increased. It is likely that the higher amount of glucose released by the diets with more carbohydrate (24 and 30 % CP) stimulated the secretion of insulin, since this is responsible for the entry of glucose into the cells. However, there might also be more circulating insulin in fish fed with 24 and 30 % CP to increase glucose uptake, and decreasing glucose levels in the diet might act as a stimulus to increase appetite.

Diet composition plays an important role in the regulation of appetite-regulating hormones (Blanco *et al*., 2016). In mammals, several studies have described a modulation of the secretion and expression of these hormones by the macronutrient composition of diets (Gomez *et al*., 2012; Vester *et al*., 2012, Wang *et al*., 2012), however, this information for fish is scarce. What has been demonstrated in fish is that central injections of hormones, such as ghrelin and leptin, cause the stimulation or inhibition of appetite, respectively (Unniappan *et al*., 2002; Gong *et al*., 2016). In the present study, *ghrelin* expression was affected by dietary protein; it showed little variation up to a CP level of 36 % but a significant reduction with 42 % CP. It is suggested that 42 % CP in the diet might suppress the secretion of ghrelin in tilapia juveniles. Unlike ghrelin, leptin is a peptide that inhibits food intake (Tinoco *et al*., 2012). In this study, the dietary protein levels tested were not sufficient to modify the expression of this peptide in tilapia juveniles. However, other hormones that promote the sensation of satiety seem to be influenced dietary protein levels. The results of this work demonstrate that the diet with 42 % CP exhibited the highest *pyy* expression and *cck* expression increased as the level of crude protein in the diet increased. It is suggested that the higher concentration of free amino acids in the bloodstream generated after the digestion of diets with higher protein levels stimulates the release of pyy and cck. Babaei *et al*. (2017) showed that high protein/low carbohydrate diets stimulated *cck* expression in the intestine of *Sparus aurata*.

4.5 Conclusions

Diets containing 42 % CP reduced the activity of acid protease, trypsin, and chymotrypsin in red tilapia. The expression of genes encoding digestive enzymes showed different expression profiles with varying levels of dietary protein. In addition, dietary protein modulated the expression of appetite regulating hormones in juvenile red tilapia. These responses indicate that diets high in protein probably would not be well utilised by this species, since they can impair both digestion and food intake, thus generating unnecessary production costs.

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Declaration of interest

I declare that there is no conflict of interest related to this article.

Ethics statement

All procedures herein described were previously approved by the Committee for Ethics in Animals Use of the Universidade Federal de Minas Gerais (CEUA/UFMG, protocol number 159/2016).

Software and data repository resources

None of the data were deposited in an official repository

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5 CONSIDERAÇÕES FINAIS

As respostas fisiológicas e metabólicas dos juvenis de tilápia (nilótica e vermelha), em adaptação as variações da proteína bruta da dieta, são semelhantes.

A proteína da dieta modulou a expressão de genes envolvidos na regulação da digestão e do apetite.

Altos níveis de proteína bruta na dieta podem reduzir o crescimento, aumentar a desaminação e diminuir a atividade de enzimas proteolíticas, além de favorecer a diminuição da ingestão alimentar.

Os resultados deste estudo podem ajudar a obter uma visão mais aprofundada sobre as diferenças fisiológicas e moleculares das enzimas digestivas em resposta a proteína da dieta, e assim melhor direcionar a formulação de dietas para tilápia. Além disso, os resultados da expressão dos genes associados à regulação do apetite podem levar ao desenvolvimento de novas técnicas para melhorar o manejo alimentar e reduzir perdas de nutrientes.