UNIVERSIDADE FEDERAL RURAL DE PERNAMBUCO
DEPARTAMENTO DE PESCA E AQUICULTURA
PROGRAMA DE PÓS-GRADUAÇÃO EM RECURSOS PESQUEIROS
E AQUICULTURA

EFEITO DO HIDROLISADO PROTÉICO DE CAMARÃO SOBRE AS ENZIMAS DIGESTIVAS DA TILÁPIA DO NILO (*Oreochromis niloticus*, L.)

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RECIFE
2008

Livros Grátis

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Dissertação apresentada ao **Programa de Pós-Graduação em Recursos Pesqueiros e Aquicultura**, como parte dos requisitos necessários para a obtenção do título de **Mestre em Recursos Pesqueiros e Aquicultura.**

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Recife, PE
Fevereiro/2008

UNIVERSIDADE FEDERAL RURAL DE PERNAMBUCO
PROGRAMA DE PÓS-GRADUAÇÃO EM RECURSOS PESQUEIROS E AQUICULTURA

Parecer da comissão examinadora da defesa de dissertação de mestrado de

JULIANA FERREIRA DOS SANTOS

EFEITO DO HIDROLISADO PROTÉICO DE CAMARÃO SOBRE AS ENZIMAS DIGESTIVAS DA TILÁPIA DO NILO (*Oreochromis niloticus*, L.)

Área de concentração: **Aquicultura**

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AGRADECIMENTOS

Agradeço primeiramente a Deus, a Jesus e aos meus protetores do céu e da terra, que sempre estiveram comigo, confortando, harmonizando e trazendo a paz para continuação da jornada.

Aos meus pais José Santos e Rosa Santos, por servirem de referência em minha vida. A Eduardo Martim pela paciência e companheirismo nesses anos de convivência. Aos meus irmãos Paulo Henrique e Ana Paula, e minha cunhada e amiga Selma Espíndola. Aos sobrinhos Gabriela Negreiros, Thainá Araújo, Camila Espíndola, Thaiane Araújo, Pedro Henrique, Vitória Florêncio pela companhia, força, e por ter tornado momentos difíceis em felizes, pelo simples fato de estarmos juntos. As minhas tias Maria José e Darci Gonçalves por tantas contribuições e incentivos dados. Ao meu padrinho e amigo Fernando Guimarães, pelo carinho. Aos primos Rogério Sávio, André Luiz, Cristina Maria, Hector Gonçalves por serem verdadeiros irmãos.

A todos os amigos do LABENZ, em especial a Ranilson de Souza Bezerra, pela orientação e confiança depositada nestes anos. Por toda força dada, não só na parte técnica, mas também nos momentos de diversão agradeço a Augusto Freitas Júnior, Talita Espósito, Thiago Cahú, Fábio Marcel, Renata França, Caio Assis, Suzan Diniz, Helane Costa, Karina Ribeiro, Robson Liberal, Werlayne Mendes, Elba Maciel, Amanda Guedes, Karollina Lopes, Robson Coelho, Mirela Assunção, Felipe Cesar, Diego Buarque, Marina Marcuschi, Patrícia Castro, Ian Amaral, Janilson Felix. Um agradecimento todo especial a Caio Rodrigo, Marina Marcuschi, Elba Maciel, Renata França, Patrícia Castro e Thiago Cahú pelas contribuições dadas nos momentos mais difíceis.

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RESUMO

Foi avaliada a influência de diferentes concentrações de hidrolisado protéico de camarão nas atividades enzimáticas dos intestinos de juvenis de tilápia do Nilo, e correlacionadas com parâmetros de crescimento (peso final, ganho de peso, ganho de peso diário, fator de crescimento específico, fator de conversão alimentar, fator de eficiência protéica) e composição do corpo (conteúdo de proteína e lipídio). Hidrolisado protéico de camarão foi incluído nas dietas em concentrações de 0 (SPH 0), 1,5 (SPH 1,5), 3 (SPH 3) e 6% (SPH 6), uma dieta comercial foi usada como referência. O ensaio enzimático foi realizado com os substratos azocaseína, BApNA, SApNA, AA-β naphytilamide e amido. Apesar de algumas diferenças estatísticas serem observadas, não houve uma correlação lógica entre atividade enzimática e as diferentes concentrações de hidrolisado protéico de camarão nas dietas. Análises de zimogramas também demonstraram as mudanças causadas pela inclusão de proteína hidrolisada no perfil das proteases digestivas da tilápia do Nilo. Zimogramas revelaram doze bandas proteolíticas, sendo que sete delas responderam a inclusão de hidrolisado protéico de camarão. Três tripsinas diminuíram suas atividades, e o inverso foi observado para uma aminopeptidase. Além disso, três proteases não identificadas (baixo peso molecular) aumentaram suas atividades. Bestatina inibiu significativamente nove enzimas do intestino de tilápia do Nilo, sendo que TPCK, PMSF, Benzamidina e TLCK apresentaram menor efeito. Houve uma correlação entre tripsina e aminopeptidase com alguns parâmetros de crescimento, conteúdo de proteína e lipídio. Também foi observado perfis de proteases distintos para cada tratamento, sugerindo a alta adaptabilidade da tilápia do Nilo a diferentes dietas.

Palavras chave: Tilápia do Nilo, hidrolisado protéico de camarão, parâmetros de crescimento, enzimas digestivas, zimograma.

ABSTRACT

The influence of different dietary concentrations of shrimp protein hydrolysate on the enzymatic activity of intestine of Nile tilapia juveniles were evaluated and correlated with growth parameters (final weight, weight gain, average daily gain, specific growth rate, feed conversion ratio and protein efficiency ratio) and body composition (protein and lipid content). Shrimp protein hydrolysate was included in the diets at concentrations of 0 (SPH 0), 1.5 (SPH 1.5), 3 (SPH 3) and 6% (SPH 6) and a commercial diet was used as reference. The enzymatic activity was carried out using azocasein, BApNA, SApNA, AA-β naphytilamide and starch as substrate. Despite some statistical differences observed, there was no logical correlation between enzyme activity and different concentrations of shrimp protein hydrolysate in the diets. Zymogram was also performed to analyze the changes caused by the inclusion of protein hydrolysate on profile of Nile tilapia digestive proteases. Zymograms revealed 12 proteolytic bands, of which 7 have responded to incorporation of shrimp protein hydrolysate. Three trypsins decreased their activity and the inverse was observed for one aminopeptidase. Moreover, three non-identified proteases (lower molecular weight) increased their activity. Bestatin inhibited significantly nine enzymes Nile tilapia intestine, while TPCK, PMSF, Benzamidine and TLCK were less effective. There was a correlation between activity of trypsin and aminopeptidase with some growth parameters, protein and lipid content. It was also found a distinct protease profiles for each treatment, suggesting the known high adaptability of Nile tilapia to the different diets.

Keywords: Nile tilapia, shrimp protein hydrolysate, growth parameters, digestive enzymes, zymograms.

SUMÁRIO

1. INTRODUÇÃO

A produção mundial de tilápia aumentou de 402.064 toneladas em 1991 para 2.096.187 toneladas em 2005, promovendo um rendimento de US$ 565,6 milhões para mais de US$2,5 bilhões. (FAO 2005; FITZSIMONS e GONZALEZ, 2006)

No Brasil, a produção de tilápia duplicou entre os anos de 2000 e 2004, passando de 32.459 para 69.078 toneladas, com crescimento médio de 22,4% ao ano. De toda a produção da aquicultura continental brasileira em 2004, a tilápia representou 38,4% do volume produzido, sendo a espécie de maior representatividade. Deste volume só a Região Nordeste contribuiu com 73% da produção, totalizando 28,5 mil toneladas (IBAMA, 2000; 2004).

O aumento da produção aquícola na última década gerou uma expectativa de crescimento na procura por novos insumos alimentares, que possam ser utilizados na elaboração de rações (SCHULZ et al., 2007). A farinha de peixe continua a ser um importante componente na dieta para organismos aquáticos, devido ao seu perfil de aminoácidos balanceados, ácidos graxos essenciais, energia digestível, vitaminas e minerais (ABDELGHANY, 2003; BORGESON et al., 2006). No entanto, há a preocupação quanto à sustentabilidade a longo prazo deste recurso (BORGESON et al., 2006).

Desta forma, a determinação de fontes protéicas de menor custo e que promovam bom crescimento é vantajoso tanto para a indústria de rações, como também para os aqüicultores (COYLE et al., 2004). Assim, vários estudos têm sido realizados para avaliar a substituição parcial ou total desta farinha por outras fontes de proteína (GABER, 1996; OLVERA-NOVOA et al., 1997; PLASCÊNCIA-JATOMEA et al., 2002; EL - SAIDY e GABER, 2003; GABER, 2006). Tais recursos devem garantir os

mesmos ou melhores resultados de produção, sanidade do animal, crescimento e qualidade do produto, como obtido na utilização de ingredientes de origem marinha.

O crescimento da indústria de pescado tem gerado uma grande quantidade de resíduos e subprodutos que representam um desafio para os empresários do setor e para a comunidade científica especializada, em buscar estratégias para que essa atividade seja sustentável e ecologicamente viável (BEZERRA et al., 2001a). Segundo Arruda (2004), cerca de 50 % do pescado mundial produzido em 2000 transformou-se em resíduo. Supondo-se que esse percentual tenha-se mantido, dos 132 milhões de toneladas de pescado produzidas em 2003 (FAO, 2004), 66 milhões teriam sido descartadas, constituindo-se em uma fonte significativa de desperdício de recursos e de contaminação ambiental.

Ao longo dos anos, diversos têm sido os esforços empreendidos por pesquisadores em todo o mundo para desenvolver métodos que possibilitem a transformação desses resíduos em produtos passíveis de utilização tanto na nutrição humana quanto na animal (FAGBENRO, 1996; KRISTINSSON e RASCO, 2000; MARTONE, et al., 2005).

A produção de farinha ou hidrolisado protéico a partir de subprodutos das indústrias pesqueiras representa uma excelente alternativa para o incremento da oferta de proteína animal (MACKIE, 1982; HAARD, 1992; KENT, 1997), já que estes subprodutos são usualmente descartados.

Devido ao grande volume de camarões cultivado e capturado, a farinha de resíduos tem sido identificada como uma fonte de proteína animal de grande potencial (FANIMO et al., 2000), podendo contribuir ainda para a redução de problemas ambientais pela inadequada destinação destes resíduos no processamento (HEU et al.,

2003). Uma das possíveis soluções é transformá-lo em material para uso na formulação de rações animais, inclusive peixes (CAVALHEIRO et al., 2007).

No processamento do camarão, geralmente são removidas a cabeça, o exoesqueleto e a porção posterior. Estes subprodutos correspondem a, aproximadamente 52% do seu peso total, o que torna importante seu aproveitamento, do ponto de vista econômico, industrial e ambiental (HEU et al., 2003).

Toma e James (1975) demonstraram que a proteína desperdiçada do camarão tem um valor nutritivo comparável ao da caseína. Os autores ainda enfatizam a potencial aplicação deste hidrolisado em ração ou como suplemento alimentar para animais.

Vários são os métodos empregados para obtenção do hidrolisado protéico dos produtos e subprodutos pesqueiros (GILBERG, 1993). A presença de enzimas proteolíticas no trato digestório de animais aquáticos tem uma influência significante na produção de hidrolisados, que pode ser obtida empregando o processo de autólise ou um método de hidrólise, através da adição de enzimas (SHAHIDI et al., 1995). Gilberg e Stenberg (2001) demonstraram que a proteína dos subprodutos do camarão pode ser hidrolisada por proteases comerciais e recuperada como hidrolisado protéico com alto conteúdo de aminoácidos essenciais.

Bezerra (2000) descreveu para tambaqui, *Colossoma macropomum,* um processo de produção de hidrolisado protéico por autólise enzimática, com a vantagem de diminuir custos, já que utiliza enzimas do próprio animal. Normalmente usam-se enzimas comerciais (alcalase) para este procedimento, porém, pode-se inviabilizar economicamente o processo.

Silva (2006) elaborou um hidrolisado protéico a partir de cabeças de camarão marinho *Litopenaeus vannamei*, por autólise enzimática, obtendo-se um concentrado

protéico, o qual foi considerando uma excelente fonte alimentar, sobretudo de aminoácidos, sendo os mais abundantes, ácido glutâmico, ácido aspártico, leucina, lisina, tirosina e arginina.

Entretanto, mudanças na origem e quantidade de nutrientes utilizados na elaboração de rações, podem modificar a atividade, concentração e o perfil enzimático no trato digestório dos animais (LUNDSTEDT et al., 2004), gerando ou não adaptações para uma melhor assimilação destes nutrientes. (MORAES e BIDINOTO, 2000).

Sendo assim, pretende-se investigar os níveis de protease e amilase no extrato bruto do intestino da tilápia do Nilo submetidas a diferentes dietas, como forma de avaliar mudanças no perfil da digestão com a adição do hidrolisado protéico de camarão, e sua possível adaptação a este processo. Como também relacionar estes dados aos parâmetros de crescimento (peso final, ganho de peso, ganho de peso diário, crescimento específico, fator de conversão alimentar e fator de eficiência protéica) e a composição corporal da tilápia do Nilo.

2. REVISÃO BIBLIOGRÁFICA

Saldaña e Lopez (1988) mencionam que o valor nutricional de uma dieta não se baseia simplesmente na sua composição química, mas também na capacidade fisiológica do peixe em digerir e absorver, de acordo com seus hábitos alimentares.

Durante a digestão as proteínas, carboidratos e lipídios são degradados em compostos mais simples para então serem absorvidas e utilizadas pelo corpo. Esta degradação é executada no trato digestório com o auxílio de enzimas (NIELSEN-SCHIMIDT, 1996).

Estas enzimas também refletem o hábito alimentar do peixe (herbívoro, detritívoro, onívoro e carnívoro) e sua capacidade de digestão (SMITH, 1980). As espécies de peixes diferem bastante nesta capacidade. Esta variação reflete diferenças anatômicas e funcionais do trato gastrointestinal e dos órgãos associados. Funções digestivas capazes de hidrolisar uma variedade maior de alimentos tornam os peixes onívoros com capacidade de digestão variada, comparada com os carnívoros (ALMEIDA et al., 2006).

Dentre as espécies onívoras, a tilápia tem se destacado pela elevada capacidade de utilizar nutrientes de origem vegetal e animal, o que possibilita a elaboração de rações de baixo custo e alto valor nutritivo (SILVA et al., 2006). Isto sugere que este animal apresenta um sistema enzimático adaptável a mudanças na alimentação.

As enzimas digestivas foram investigadas por muitos anos como uma maneira de compreender as exigências nutricionais e o efeito dos constituintes da dieta na atividade enzimática. Em teleósteos, as enzimas proteolíticas são amplamente encontradas nas vísceras. Bezerra et al. (2000) estudaram as proteases no extrato bruto do estômago, cecos pilóricos, intestino e fígado do tambaqui, demonstrando uma alta atividade ácida e

alcalina no estômago e cecos pilóricos, respectivamente. Um protocolo de purificação parcial para a enzima tripsina presente nos cecos pilóricos do tambaqui foi elaborado por Bezerra et al. (2001b). Esta mesma metodologia foi aplicada para tilápia do Nilo, onde Bezerra et al. (2005), purificaram e caracterizaram uma tripsina encontrada no intestino deste peixe.

Enzimas como as amilases são menos estudadas quando comparadas às proteases, contudo alguns autores relatam a presença de uma atividade visível destas enzimas no trato digestório de peixes (NAGASE, 1964; CHIU e BENITEZ 1981; UGWUMBA, 1993; PELLETIER et al., 1994; HIDALGO et al., 1999; FERNÁNDEZ et al., 2001; BLIER et al., 2002; ALMEIDA et al., 2006; DEBNATH et al., 2007).

Em vertebrados superiores, as enzimas podem se apresentar distintamente, demonstrando claramente suas áreas funcionais. Para peixes, isto não é mostrado de forma clara, uma vez que as mesmas podem estar distribuídas ao longo do trato digestório (LUNDSTEDT et al., 2004). Elas também podem ser influenciadas pela idade e a espécie do animal, bem como pela quantidade e composição da dieta ministrada (PEREZ et al., 1998).

Os efeitos de diferentes dietas nas atividades enzimáticas já foram descritas e estudadas em *Oreochromis mossambicus* (NAGASE, 1964), onde a tripsina e α-amilase demonstraram uma correlação positiva com a dieta. Na carpa comum, *Cyprinus carpio* (KAWAI e IKEDA, 1972) e na truta arco-íris, *Salmo gairdneri* (KAWAI e IKEDA, 1973) houve um aumento nos níveis de enzimas proteolíticas de acordo com as taxas alimentares.

Kohla et al. (1992) demonstraram que em *Colossoma macropomum* os níveis de tripsina e α-amilase diminuíram no suco digestivo durante o regime de alimentação, porém, na realimentação essas atividades apresentaram um grande aumento. Eles

demonstraram evidências de que o sistema digestório do tambaqui se adapta a mudanças na qualidade e quantidade de dietas.

O bacalhau do Atlântico apresentou uma correlação positiva em relação à taxa de crescimento e a carboidrase (PELLETIER et al., 1994). Resultados similares também foram encontrados para o salmão prateado (BLIER et al., 2002).

Tripsina, quimotripsina e fosfatase alcalina demonstraram uma correlação positiva com a taxa de crescimento do Bacalhau do Atlântico (LEMIEUX et al., 1999). Para o pintado *(Pseudoplatystoma corruscans),* a atividade proteolítica ácida no estômago não apresentou correlação com a porcentagem de proteína total administrada na dieta, contudo, demonstrou valores expressivos de atividade de pepsina com 30% de proteína bruta ofertada (LUNDSTEDT et al., 2004). Pequenas alterações nas atividades de tripsina e quimotripsina foram observadas com as mudanças nos níveis protéicos das dietas. Na carpa (*Labeo rohita)* alimentada com diferentes níveis de proteína bruta, as atividades de amilase, fosfatase alcalina e protease alcalina não demonstraram correlação positiva com as dietas ministradas. A fosfatase ácida demonstrou um aumento com relação à quantidade de proteína (DEBNATH et al., 2007).

Fountoulaki et al. (2005), demonstraram que a capacidade digestiva depende simultaneamente do nível enzimático, bem como do tempo em que os nutrientes estão sujeitos a ação das enzimas. Assim, as concentrações de enzimas estão relacionadas com o nutriente administrado. Os autores demonstraram uma pequena redução na atividade proteolítica alcalina total, com o aumento nos níveis de gordura na dieta, contudo essa variação não foi significativa.

Para o peixe lobo malhado (*Anarhichas minor)* a atividade alcalina total mostrou-se expressiva no intestino anterior, baixa no posterior e inexpressiva no estômago. Comparado com outros carnívoros, a atividade da pepsina no estômago

também se mostrou baixa e sem correlação com a concentração de proteína e lipídio das dietas ministradas. As atividades de tripsina e quimotripsina apresentaram um aumento com concentrações baixas de proteína e altas de lipídios. Isto pode estar relacionado a uma adaptação do animal para aproveitar melhor as baixas concentrações de proteína da dieta. Os autores sugerem que estas mesmas conclusões sirvam para os níveis de aminopeptidases, carboxipeptidases, elastases e colagenases. (PAPOUTSOGLOU e LYNDON, 2006).

Almeida et al. (2006) investigaram a atividade enzimática do tambaqui submetido a rações com diferentes níveis de proteína e de lipídios. As atividades ácidas do estômago apresentaram correlação positiva com os níveis de proteína administrados. No intestino, a atividade proteolítica alcalina se mostrou baixa. Amilase se apresentou em todo o trato gastrintestinal, tendo correlação positiva com os níveis de proteína nos cecos pilóricos.

Vários autores avaliaram o efeito da adição de hidrolisado protéico em dietas visando a atractabilidade, aumento no crescimento e melhora na sobrevivência (OLIVA-TELES et al., 1999; KOLKOVSKI et al., 2000; PLASCÊNCIA-JATOMEA et al., 2002, REFSTIE et al., 2004, HEVROY et al., 2005; SAVOIE et al., 2006, AKSNES et al., 2006; KOTZAMANIS et al., 2007; LEAL, 2007). Contudo, poucos estudos foram feitos sobre a influência da inclusão destes hidrolisados nas enzimas digestivas.

Kotzamanis et al. (2007) avaliaram a influência do hidrolisado protéico na sobrevivência, taxa de crescimento, níveis enzimáticos (amilase, tripsina, fosfatase alcalina e leucino aminopeptidases) em larvas de *Dicentrarchus labrax.* Os autores observaram que não houve diferença significativa na taxa de sobrevivência, porém, os animais alimentados com menores taxas de hidrolisados protéicos (10%) demonstraram crescimento significativo. Os níveis de tripsina e amilase não apresentaram diferença

estatística, no entanto a atividade tríptica apresentou uma tendência à diminuição com valores mais altos de proteína hidrolisada (19%). Leucino aminopeptidases e fosfatase alcalina apresentaram valores mais expressivos com uma dieta contendo menor valor de hidrolisado protéico. Zambonino-Infante e Cahú (2007) reportam que a secreção de tripsina em larvas de peixes alimentados com proteína nativa ou com níveis moderados de proteína hidrolisada (cerca de 14%) é alta, sendo reduzida quando a incorporação de hidrolisado está em torno de 46%.

Os estudos sobre atividades enzimáticas também podem contribuir para definição de uma concentração ótima de hidrolisado protéico, refletindo em um processo normal de maturação intestinal ou atraso no desenvolvimento das larvas quando alimentadas com uma dieta com excesso de dipeptídeos e tripeptídeos, já que há um número reduzido de transportadores de peptídeos intestinais (BAKKE-MCKELLEP et al., 2000).

3. ARTIGO CIENTÍFICO

Profile of digestive enzymes from Nile tilapia (*Oreochromis niloticus*) submitted to diets with different concentrations of shrimp protein hydrolysate and its correlation with growth parameters and body composition

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3.1 Abstract

The influence of different dietary concentrations of shrimp hydrolysate on the protease and amylase activity of Nile tilapia juveniles were evaluated and correlated with growth parameters and body composition. Shrimp protein hydrolysate (SPH) was included in the diets at concentrations of 0, 1.5, 3 and 6%. A commercial diet was used as reference. Azocasein, BApNA, SApNA, AA-β naphytilamide and starch were used as substrates. Despite some differences observed, there was no logical correlation between enzyme activity and different concentrations of SPH in the diets. Zymogram was also performed to analyze the changes caused by the inclusion of protein hydrolysate on profile of Nile tilapia digestive proteases. Zymograms revealed 12 proteolytic bands, of which 7 have responded to incorporation of SPH. Three trypsins decreased their activity and the inverse was observed for one aminopeptidase. Moreover, three non-identified proteases (lower molecular weight) increased their activity. Bestatin inhibited significantly nine enzymes of intestine, while TPCK, PMSF, Benzamidine and TLCK were less effective. There was a correlation between activity of trypsin and aminopeptidase with some growth parameters, protein and lipid content. It was also found a distinct protease profiles for each treatment, suggesting the known high adaptability of Nile tilapia to the different diets.

Keywords: Digestive enzymes, growth parameters, Nile tilapia, shrimp protein hydrolysate, zymograms.

3.2 Introduction

Tilapia world culture production has been increasing significantly in the last years. This development was followed by increase of feed consumption and has stimulated the search for new ingredients which can be used in diets formulation (SCHULZ et al., 2007). Shrimp processing waste, for instance, has been discharged into environment and represents an important source of water and land pollution. One of the possible solutions is to transform this material into suitable ingredients for use as a component in animal feeds (CAVALHEIRO et al., 2007). Fanimo et al. (2000) have already identified this byproduct as an animal protein source of great potential.

Recently in our laboratory, it was elaborated a protocol to produce protein hydrolysate through autolysis of shrimp processing waste (from Litopenaeus vannamei). Through this method it was obtained a protein concentrate, which is considered to be an excellent nutrient source, mainly of amino acids, among them the most abundant are glutamate, aspartate, leucine, lysine, tyrosine and arginine (SILVA, 2006). In fact, shrimp protein hydrolysate has been used in fish feeds both as a new protein source (PLASCÊNCIA-JATOMEA et al., 2002) as, in low quantity, a flavor to amend the attractiveness of diet (KOLKOVSKI et al., 2000).

Variations in the quality and quantity of nutrients used in diet formulations can modify the enzymatic profile and activity in the digestive tract of the animals (LUNDSTEDT et al, 2004). Thus, these different feeds could induce some biological adaptations, increasing the absorption of nutrients (MORAES and BIDINOTO, 2000).

Digestive enzymes have been investigated for many years as a way to understand the nutritional requirements and the effects of diet composition on the enzymatic activity, contributing to reduce feeding costs in fish farms (CARUSO et al., 1996). Most of researches evaluate the effect of feeds with different concentrations of

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protein, carbohydrate and lipids, correlating these results to the enzymatic activity. However, such results, combined with growth parameters, may contribute to the establishment of the appropriate quantities of nutrients to be inserted in the newly developed feed. Fountoulaki et al. (2005) also demonstrated that the differences in enzymatic profile are related to the nutrients present in the diet.

Thus, the present work has focused on the following hypothesis: a) the inclusion of shrimp protein hydrolysate at different concentrations in tilapia's feeds could promote detectible changes in the main digestive enzymes activity; b) the use of substrate-SDS-PAGE zymograms could be an effective tool to improve the analysis of these changes; and c) there is a correlation between the enzymatic activity of the main digestive enzymes and growth parameters and content of protein and lipid in the body.

3.3 Materials and methods

Materials

All reagents were of analytical grade and purchased from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany).

Diet preparation

Four isonitrogenous (37% crude protein - CP) and isocaloric (440 kcal 100 g^{-1}) experimental diets were formulated to feed O. niloticus juveniles. Shrimp protein hydrolysate (SPH) was included in the diets at concentrations of 0 (control – SPH0), 1.5 (SPH1.5), 3 (SPH3) and 6% (SPH6). The 1:2 animal:plant protein ratio was observed. SPH was incorporated to soybean meal and the dough was dried at 65°C for 24 h. The ingredients were then mixed and the diets prepared under industrial conditions to obtain 1mm diameter pellets. A commercial diet (36% CP) was adopted as reference. Centesimal composition and total aminoacid content of shrimp hydrolysate are

presented in Tables 1 and 2, respectively. The centesimal composition of diets is displayed in Table 3.

Animals and culture conditions

Juvenile sex-reversed Nile tilapias were obtained from Universidade Federal Rural de Pernambuco Aquaculture Station. Fish were stocked in fifteen 40-L glass aquaria (8 per aquarium), supplied with biological filter and continuous aeration, and were submitted to a 7-days acclimatization period, both for diets and environmental conditions, in a completely randomized design with five treatments and three replicates. Before beginning the feeding trial, fish were weighed (1.7±0.4g) and measured (4.7±0.4cm). Fish were fed four times a day at rates ranging from 15% to 6% of biomass, adjusted every nine days during 45 days.

Aquaria were siphoned twice daily, with 66% of water exchange. Temperature, dissolved oxygen, pH, ammonia and nitrite were monitored and averaged (mean± SD) 28.7±0.59ºC, 3.5±0.92 mg L^{-1}, 8.1±0.19, 0.14±0.22 mg L^{-1} and 0.08±0.02 mg L^{-1}, respectively.

Enzyme extraction

At the end of trial, six fish from each aquarium, after 24 hours fasting, were removed and sacrificed in an ice bath for biometric measurements and tissue removal. Intestines were immediately collected and homogenized (40 mg tissue/mL) in 0.01M Tris-HCl pH 8.0 buffer, containing 0.15M NaCl, using a tissue homogenizer. The resulting preparations were centrifuged at 10,000 x g for 10 min at 4° C to remove cell debris and nuclei. The supernatants (crude extracts) were frozen at -20° C and used in further assays (BEZERRA et al., 2005).

Enzymatic assay

Alkaline proteolytic activity (APA)

In a microcentrifuge tube (triplicates), 1% azocasein prepared in 0.1M Tris-HCl buffer pH 8.0 was incubated with intestine crude extract (30 µL) for 60 min at 25 °C. Then, 240 µL of 10% trichloroacetic acid (TCA) were added to stop the reaction. After 15 min, centrifugation was carried out at 8,000 x g for 5 min. The supernatant (70 µL) was added to 1 M NaOH (130 µL) in a 96-well microtiter plate and the absorbance of this mixture was measured in a microtiter plate reader (Bio-rad 550) at 450 nm against a blank similarly prepared except that 0.01M Tris-HCl, pH 8.0 replaced the crude extract sample. Previous experiment demonstrated that, under the conditions described above, for the first 60 min, the reaction carried out followed first order kinetics. One unit (U) of enzymatic activity was defined as the amount of enzyme capable of hydrolyzing azocasein to produce a 0.001 change in absorbance per minute per milligram of protein (BEZERRA et al., 2005).

Trypsin and chymotrypsin activity

Trypsin and chymotrypsin activity was determined using 8mM BApNA (Nα-benzoyl-DL-arginine-p-nitroanilide) and 8mM SApNA (Suc-Ala-Ala-Pro-Phe p-nitroanilide) in DMSO (Dimethyl sulfoxide), respectively. Intestine crude extract (30 µL) was incubated with 0.1M Tris-HCl buffer pH 8.0 (140 µL) and respective substrates (30 µL) in a microtiter plate reader (Bio-rad 550). The absorbance was measured at 405 nm against a blank similarly prepared except that 0.1M Tris-HCl, pH 8.0 replaced the crude extract sample. Enzymatic activity was performed in triplicates. Trypsin and chymotrypsin units of activity were expressed as change in absorbance per minute per milligram of protein (BEZERRA et al., 2005).

Aminopeptidase activity (AP)

Aminopeptidase activity was evaluated using aminoacyl of β-naphthylamide (AA of arginine) as substrate. The procedure adapted from Oliveira et al. (1999) was carried out by incubating 4.2mM substrate (50 µL), 50mM sodium phosphate buffer pH 7.0 (600 µL) and H_2O (50 µL) at 37 ºC. After temperature equilibration, the intestine crude extract (50 µL) was added. After 30 minutes, the reaction was stopped by adding fresh Garnet reagent (250 µL) in 0.2M sodium acetate buffer, pH 4.2, containing 10% Tween 20 (v/v). Absorbance was measured at 525 nm and the amount of β-naphthylamine was determined using a standard curve of β-naphthylamine. The activity was expressed as protease millunits per milligram of protein. One unit of enzymatic activity was defined as the amount of enzyme required to hydrolyze one µmol of p-nitroaniline per minute.

Amylase activity

Amylase activity was evaluated according to Bernfeld (1955) using starch 2% as substrate: 60 µL intestine crude extract were incubated with 375 µL starch solution and 375 µL 10mM phosphate buffer pH 8.0 containing 15mM NaCl at 25ºC. After 20 min 3.5-dinitro salicylic acid (DNSA) was added and the solution was submitted to 100º C for 10 min. After temperature equilibration the absorbance was measured at 570 nm against a blank similarly prepared except that 10mM phosphate buffer replaced the crude extract sample. Enzymatic activity was perceived in triplicates. One unit of enzymatic activity was defined as the amount of enzyme required to hydrolyze 1mg of maltose per milligram of protein per min.

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<u>Total soluble protein determination</u>

Protein concentration was determined according to Bradford (1976), using bovine serum albumin (BSA) as standard and reported as mg protein equivalent to BSA.

Hydrolysis profile of SPH

The hydrolysis profile of shrimp hydrolysate was evaluated in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), using stacking gel at 4% (w/v) and separation gel at 17% (LAEMMLI, 1970). SPH was produced as described by Bezerra (2000) and enzymatic hydrolysis was followed at sampling times of 0, 30, 60, 90, 120, 150 and 180 minutes.

Enzyme characterization in substrate-SDS-PAGE

Proteases from intestine crude extract of <u>O. niloticus</u> were studied in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using stacking gel at 4% (w/v) and separation gel at 12.5% (LAEMMLI, 1970). Zimograms were also carried out according to Garcia-Carreño et al. (1993). After electrophoresis, gels were immersed in 2.5% Triton X-100 dissolved in 0.1M Tris-HCl buffer pH 8.0 to remove SDS and incubated with 3% casein (w/v) in 0.1M Tris-HCl buffer pH 8.0, for 30 min at $4^{o}C$. The temperature was raised to $37^{o}C$ and kept for 90 min to allow the digestion of casein by the active fractions. Finally, gel was stained overnight for protein in 0.18% (w/v) Coomassie Brilliant Blue R250, prepared in acid acetic and methanol (10:25% v/v) and the background of the gel was destained in acetic acid and methanol (10:25% v/v). Clear bands in blue background denoted protease bands by digestion of casein substrate.

Inhibition assays

The following inhibitors prepared in DMSO at 2mM final concentration were used: Tosyl phenylalanine chlorometyl ketone (TPCK - chymotrypsin inhibitor),

Phenyl-methyl-sulfonil-fluoride (PMSF - serine proteases inhibitor), benzamidine and tosyl-lysine chloromethyl ketone (TLCK), both trypsin inhibitors and bestatin (leucine aminopeptidase inhibitor) (BEZERRA et al., 2005). Samples of enzyme extract and inhibitors were incubated at 25ºC for 30 min and zymogram was performed as described above. The 100% values (control) were established using DMSO without inhibitors.

Growth parameters

Fish performance was evaluated through average daily gain (ADG), specific growth rate (SGR), feed conversion ratio (FCR) and protein efficiency ratio (PER), according to the following formulae: WG = 100 (final weight - initial weight) / initial weight; ADG = weight gain (g) / time (days); SGR = $100 (\text{Ln } W_f - \text{Ln } W_i)$ / time (days); FCR = dry feed offered (g) / wet weight gain (g); PER = wet weight gain (g) / protein fed (g).

Centesimal composition of body

Initial and final body analyses were performed with a pooled sample of fish which were frozen before and after the feeding trial (two fish from each aquarium). Lipid and protein contents were determined using the standard methods (AOAC, 1980).

Statistical analysis

Data of enzymatic activity were analyzed by one-way ANOVA (One Way Analysis of Variance with Tukey´s test). Data that not passed normality test (chymotrypsin) were transformed (ln) and submitted to parametric ANOVA. Interaction/ Relation between variables were estimated by Pearson Product Moment Correlation and regression analysis. Differences were reported as statistically significant when $p < 0.05$ using the Jandel Scientific SigmaStat software version 2.0.

3.4 Results

The hydrolysis profile of SPH is displayed in Figure 1. It can be observed that the enzymatic autolysis visibly promoted digestion of the proteins of highest molecular weights over time. At time 0 (lane 2) most of proteins presented molecular weight smaller than 100 kDa, but also proteins higher than 220 kDa. At 150 min (lane 7) most proteins were smaller than 20 kDa.

All enzyme activity performed is displayed in Table 4. Statistical differences ($P<0.05$) between animals fed formulated diets (SPH0, SPH1.5, SPH3 and SPH6) were observed for chymotrypsin, trypsin and total alkaline proteases but the major differences were always observed between formulated and commercial diets. The lowest values for enzymes were always related to commercial diets.

Zymograms revealed caseinolytic activity in crude extracts of fish from all treatments (Figure 2). Twelve caseinolytic bands were found in animals fed SPH0: P1, P2, P3, P4, P5, P6, P7, P8, P9, P10, P11 and P12. In all treatments, highest molecular weight proteins (P1 to P5) were not significantly affected by protein hydrolysate inclusion, although the lowest ones (P6 to P12) underwent alterations. As hydrolysate concentration increases it is observed a slowdown in the activity of proteases P6, P7 and P8. The inverse situation occurred for P9, P10, P11 and P12, in which the caseinolytic bands showed to be more intense.

Inhibition of alkaline proteases performed with SPH0 crude extract is displayed in Figure 3. Lane 1 (control) revealed 12 caseinolytic bands, as also demonstrated in Fig 2. TPCK (lane 2) did not affect significantly caseinolytic activity of O. niloticus (P11 was the only enzyme slightly inhibited). PMSF, a serine protease inhibitor, (lane 3) showed high (P9), medium (P1, P2, P3, P4, P5 and P11) and low (P6, P7 and P8) inhibition of caseinolytic bands. Benzamidine (lane 4), which is a trypsin inhibitor,

acted totally inhibited P6 and P8 proteins. It was observed one band between P5 and P6 enzymes that demonstrated activity in presence of benzamidine but did not appear in the control. Benzamidine slightly activated P5, P7, P9 and P10. TLCK, another trypsin inhibitor, (lane 5) strongly inhibited P3, P4, P6, P7 and P8 enzymes, while P11 and P12 proteins were partially inhibited and P9 and P10, on the other hand, presented an increase in its activity. The inhibitor of aminopeptidase, bestatin (lane 6) affected significantly nine enzymes of O. niloticus: P3, P4, P5, P6, P7, P8 and P9 were totally inhibited by this compound while P1 and P2 were slightly inhibited and, in contrast, P10 and P12 showed to be more intense.

There was no correlation between growth parameters (FW, WG, ADG, SGR, FCR, PER), body composition (protein, and lipid contents), chymotrypsin, amylase and total alkaline proteases.

Positive correlations were found between growth parameters (FW, WG, ADG and SGR), body protein content and trypsin activity (Figure 4). Aminopeptidase activity showed positive correlations with all parameters above listed, except with WG (Figure 5). Negative correlations were observed between FCR and body lipid content and both trypsin and aminopeptidase (Figures 4 and 5)

3.5 Discussion

The nutritional quality of protein hydrolysate is related to high concentration of small peptides and essential amino acids as long as to compounds that stimulates immune response in fish, promoting growth and resistance to diseases (GILDBERG and STENBERG, 2001). According Silvestre et al (1994), is preferable to use hydrolysate in diets than amino acids mixtures, since di and tri-peptides are absorbed by the intestine faster than free amino acids. These peptides are also accepted as attractive food due to

the chemical stimulation in larvae and juveniles contributing to consumption of artificial diets in fish hatchery.

Several authors related digestive enzyme activity of aquatic organisms and different diets and the observed results were distinct. Nagase (1964) and Kohla et al. (1992) observed an enhancement in trypsin activity, corresponding to an increase in feeding rates for the species O. mossambicus and C. macropomum, respectively. Papoutsoglou and Lyndon (2006) found for the species Anarhichas minor an increase in the chymotrypsin activity when the protein concentration was reduced, indicating an adaptation of this animal to low protein concentrations as a way to better absorb the nutrients from the diet.

Regarding to the specific effects of the hydrolysate protein inclusion in diets on digestive enzymes, growth parameters and body composition of fishes, there are little information. In the present work, there was not a logical correlation between different concentrations of hydrolysate in the diets and the activity of all enzymes studied. Kotzamanis et al. (2007), evaluating the amylase and trypsin activity in Dicentrarchus labrax larvae fed diets with 10 and 19% of protein hydrolysate, did not observe statistical differences between treatments. However, aminopeptidase activity was greater at 10% protein hydrolysate concentration.

In the present work, although it has been observed differences between enzyme activity (total alkaline proteases, trypsin and chymotrypsin) of fish fed formulated diets, the major differences were found between commercial and formulated diets, which should be related rather to the content of fish meal of formulated diets (about 20%), since its concentration in tilapia Brazilian commercial feeds is, in general, 5%. Quantitative studies of digestive enzymes seemed not be helpful to distinguish the effects of different concentrations of SPH in diets. On the other hand, the use of

substrate SDS-PAGE revealed interesting results. In fact, according Garcia-Carreño et al. (1993), this technique is a biochemical tool several times more sensitive than others methods for detecting proteinase composition of tissues crude extracts, which also allows the observation of enzyme activity zones cause by proteinase inhibitors.

Through analyses of inhibition zymograms (Figure 3) it was possible to identify: two classical trypsins (P6 and P8) that were inhibited by PMSF, TLCK and benzamidine; four aminopeptidases (P1, P2, P5 and P9); P3, P4, P7 and P11 seemed to be proteases of low specificity, with aminopeptidase/trypsin activity or chymotrypsin/trypsin activity (P3, P4 and P7, inhibited by Bestatin and TLCK, and P11, inhibited be PMSF, TPCK and TLCK). It was not possible to identify P10 and P12 since none of the inhibitors have inhibitory effect on them.

Comparing inhibition zymogram to Figure 2, it was observed that as shrimp protein hydrolysate concentration increases there was a slowdown of proteases with trypsin activity (P6, P7 and P8). On the other hand, aminopeptidase activity identified in P9 was higher when SPH increased. Moreover, proteases P10 and P12 (non identified) and P11 (with chymotrypsin/trypsin activity) also showed an increase on activity with SPH inclusion. Similar results were reported by Cahu et al. (2004) who related that trypsin secretion was high in larvae of sea bass Dicentrarchus labrax fed diets with 14% of protein hydrolysate and reduced with elevate concentrations (46%).

Dietary protein is one of the most important sources of amino acids for the synthesis of functional proteins and for muscle growth. As protein digestion is the main mechanisms that promote the breakdown of these molecules and fish use energy and monomers obtained from their diets for synthesis of functional proteins and for muscle growth. Ingested food is subjected to enzymes that break it down into compounds, which are absorbed by cells in gastrointestinal tract. Thus, it is very tempting to think

that there is a correlation between digestive enzyme activity and fish growth parameters or body composition. In fact, it was found in this study positive correlation between trypsin and aminopeptidase activity with some growth parameters (final weight, weight gain, average daily gain and specific growth rate) and body protein content. This result indicates the important role of these enzymes on regulation of tilapia growth and incorporation of proteins. Similar results were found by Lemieux et al. (1999) who described positive correlations of the Atlantic cod trypsin and chymotrypsin with growth rates.

Negative correlation was observed between trypsin and aminopeptidase with body lipid content. In fact, the muscle growth (synthesis of protein) increases the energy demand in the cell. Consequently, there is a decrease in concentration of the precursors of endogenous lipid synthesis (NELSON and COX, 2007) which could be related to the low concentration of lipids in body.

3.6 Conclusions

Despite it has been observed some differences between digestive enzyme activity of fish fed experimental diets, there was no logical correlations between the enzyme activity and different concentrations of shrimp protein hydrolysate in these diets.

Substrate-SDS-PAGE zymograms showed to be an efficient tool to detect the changes in enzyme activity of fish submitted to different diets. Through this technique it was possible to observe different protease profiles for each experimental diet. This data reinforce the known ability of tilapia of adapting to different feeding sources.

There were correlations between trypsin and aminopeptidase activity of fish fed the experimental diets with some growth parameters and body protein content, which demonstrate the importance of these enzymes in growth regulation

3.7 Acknowledgements

3.8 References

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3.9 Figure legends

Figure 1. Hydrolysis profile of SPH in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using stacking gel at 4% (w/v) and separation gel at 17%. Lanes: 1 (MW – molecular weight), 2 (0 min), 3 (30 min), 4 (60 min), 5 (90 min), 6 (120 min), 7 (150 min) and 8 (180 min) of enzymatic hydrolyze.

Figure 2. SDS-PAGE zymogram of digestive proteases (3% casein as substrate) of intestine from *O. niloticus* fed different diets. Lanes: 1 (commercial); 2 (SPH 0); 3 (SPH 1.5); 4 (SPH 3); 5 (SPH 6).

Figure 3. Inhibition zymogram of digestive proteases (3% casein as substrate) of intestine from *O. niloticus* fed SPH 0. Lanes: 1 (control without inhibitors); 2 (TPCK); 3 (PMSF); 4 (Benzamidine); 5 (TLCK); 6 (Bestatin).

Figure 4. Relationship between trypsin activity (8mM BAPNA as substrate) and (A) final weight (potential); (B) weight gain (polynomial); (C) average daily gain (potential); (D) specific growth rate (potential); (E) feed conversion ratio (polynomial); (F) [Protein] (potential); (G) [Lipid] (exponential) of *O. niloticus* fed different diets. Error bars represent S.E. of the mean trypsin activity (n=6).

Figure 5. Relationship between aminopeptidase activity (AA of arginine as substrate) and (A) final weight (potential); (B) average daily gain (polynomial); (C) specific growth rate (polynomial); (D) feed conversion ratio (polynomial); (E) [Protein] (polynomial); (F) [Lipid] (polynomial) of *O. niloticus* fed different diets. Error bars represent S.E. of the mean aminopeptidase activity (n=6).

3.10 Tables

Table 1. Centesimal composite and energy of lyophilized SPH (*Litopenaeus vannmei*) according to Silva (2006).

Composition	Value
Moisture (%)	9.68
Protein (%)	43.63
Fat (%)	6.25
Ash (%)	7.32
Carbohydrate (%)	33.12
Energy (Kcal 100 g^{-1})	363.27

Table 2. Total amino acids content of the SPH (*Litopenaeus vannamei*) according to Silva (2006).

Amino acids	SPH	
	mg/100g	%
Essential		
Arginine	3400 ± 0.043	7.4
Histidine	1060 ± 0.005	2.3
Isoleucine	2000 ± 0.021	4.7
Leucine	3490 ± 0.021	7.6
Lysina	3350 ± 0.000	7.3
Methionine	1290 ± 0.005	2.8
Phenylalanine	2370 ± 0.002	5.2
Threonine	2120 ± 0.031	4.7
Thryptophan	670 ± 0.016	1.4
Valine	2250 ± 0.012	4.9
Non-essential		
Tyrosine	3370 ± 0.004	7.4
Aspartic acid	4270 ± 0.031	9.4
Glutamic acid	5780 ± 0.003	12.7
Glycine	2890 ± 0.005	6.3
Serine	2030 ± 0.001	4.4
Alanine	3070 ± 0.017	3.7
Proline	2970 ± 0.024	6.5
Cystine	410 ± 0.015	0,9
TOTAL	**46790**	**100**

Table 3. Composition and proximate analysis of the experimental diets.

Ingredients (%)	Diets				
	Commercial	SPH 0	SPH 5	SPH 10	SPH 20
Fish meal		23.0	22.0	21.0	18.0
SPH		0.0	1.5	3.0	6.0
Soybean meal		47.0	47.5	47.5	47.5
Wheat meal		16.0	13.5	13.5	15.5
Corn starch		10.5	12.0	11.5	9.5
Soybean oil		1.0	1.0	1.0	1.0
Dicalcium phosphate		1.0	1.0	1.0	1.0
Mineral and vitamin mix[1]		1.0	1.0	1.0	1.0
Salt		0.5	0.5	0.5	0.5
Antioxidant BHT		0.02	0.02	0.02	0.02
Proximate analysis (on as-fed basis)					
Dry matter (g kg^{-1})	918.0	944.8	935.9	936.5	946.7
Crude protein (g kg^{-1})	345.6	371.9	374.3	376.2	380.6
Ether extract (g kg^{-1})	65.0	48.1	56.2	52.1	35.9
Crude fibre (g kg^{-1})	34.9	39.7	38.8	41.1	46.6
Ash (g kg^{-1})	67.5	105.7	102.9	101.6	101.9
Nitrogen-free extract (g kg^{-1})	487.0	434.6	427.8	429.0	435.0
Calcium (g kg^{-1})	14.3	22.2	21.7	20.0	17.2
Phosphorus (g kg^{-1})	11.3	12.4	12.5	12.6	12.8
Gross energy (kcal 100 g^{-1})[2]	461.5	438.3	444.5	442.2	431.8

[1] Mineral and vitamin mix (kg^{-1} premix): vitamin A (20,000 UI), vitamin D_3 (5,000UI), vitamin E (250 mg), vitamin K_3 (25 mg), vitamin B_1 (37.5 mg), vitamin B_2 (37.5 mg), vitamin B_6 (25 mg), vitamin B_{12} (0.053 mg), vitamin C (250 mg), niacin (200 mg), pantothenic acid (100 mg), biotin (1,25 mg), choline (1.000 mg), inositol (250 mg), Fe (100 mg), Cu (12 mg), Zn (125 mg), Mn (37,5 mg), Se (0,25 mg), I (1,25 mg), Co (0,25 mg).
[2] Based on 5.65, 4.2 and 9.5 kcal g^{-1} protein, carbohydrate and fat, respectively.

Table 4. Digestive enzymes activity from the crude extracts of *O. niloticus* submitted to diets containing increasing shrimp protein hydrolysate (SPH) levels.

Dietary	Alkaline proteolytic activity	Trypsin	Chymotrypsin	Aminopeptidase	Amylase
Commercial	8.26 ± 1.05^c	1.13 ± 0.14^c	11.96 ± 2.13^c	$20.49\pm3.65^{\ b}$	25.24 ± 3.74^b
SPH 0	11.80 ± 1.23^b	2.58 ± 0.21^b	22.70 ± 2.52^b	$41.68\pm6.65^{\ a}$	45.47 ± 8.78^a
SPH 1.5	15.35 ± 1.70^a	3.38 ± 0.40^a	39.74 ± 7.88^a	$45.34\pm3.66^{\ a}$	53.50 ± 10.41^a
SPH 3	13.60 ± 1.64^{ab}	$2.82\pm0.45^{\ ab}$	30.89 ± 5.41^{ab}	$47.66\pm4.16^{\ a}$	48.79 ± 9.64^a
SPH 6	15.23 ± 1.78^a	$2.62\pm0.30^{\ ab}$	36.66 ± 7.20^a	$39.85\pm5.22^{\ a}$	56.77 ± 8.63^a

Activity are expressed as follows: alkaline proteolytic activity as U/mg of Protein (P); trypsin, chymotrypsin and aminopeptidase as mU/ mg of P; and amylase as mg of maltose per mg of protein released mgM/ mg of P. Different superscripts in the same column signify statistical differences ($p < 0.05$) (mean ± S.D. of six replicates), data were analyzed by ANOVA(One Way Analysis of Variance with Tukey´s test). Data that not passed normality test (chymotrypsin) were transformed (ln) and submitted to parametric ANOVA.
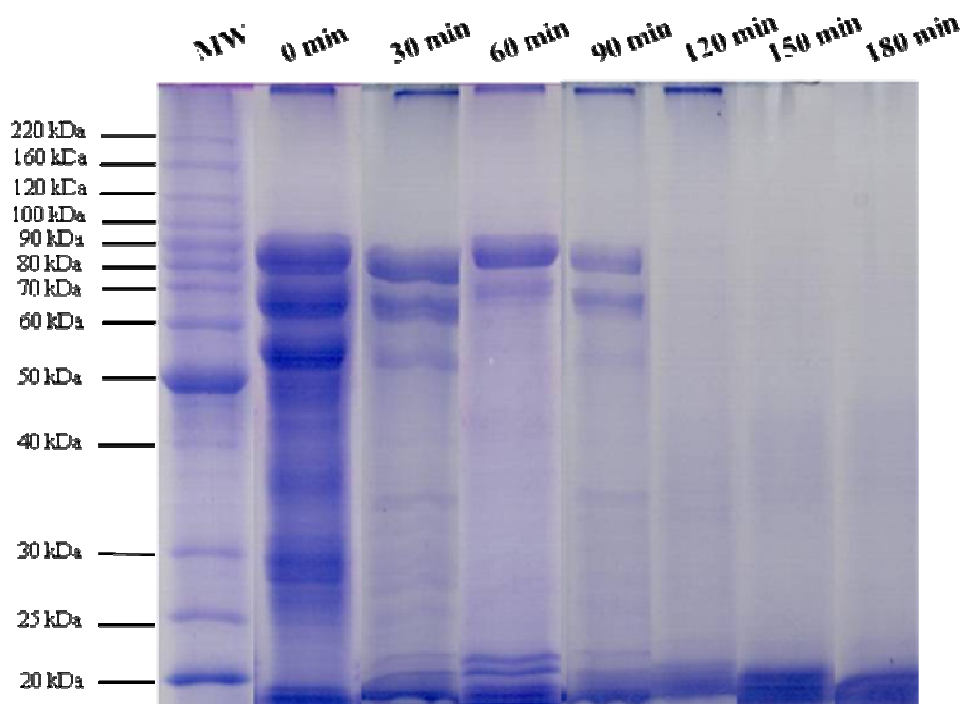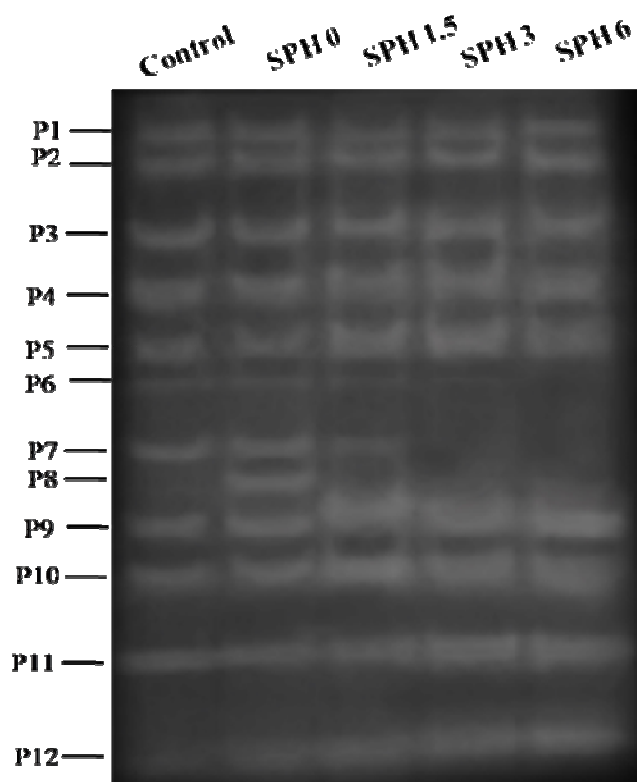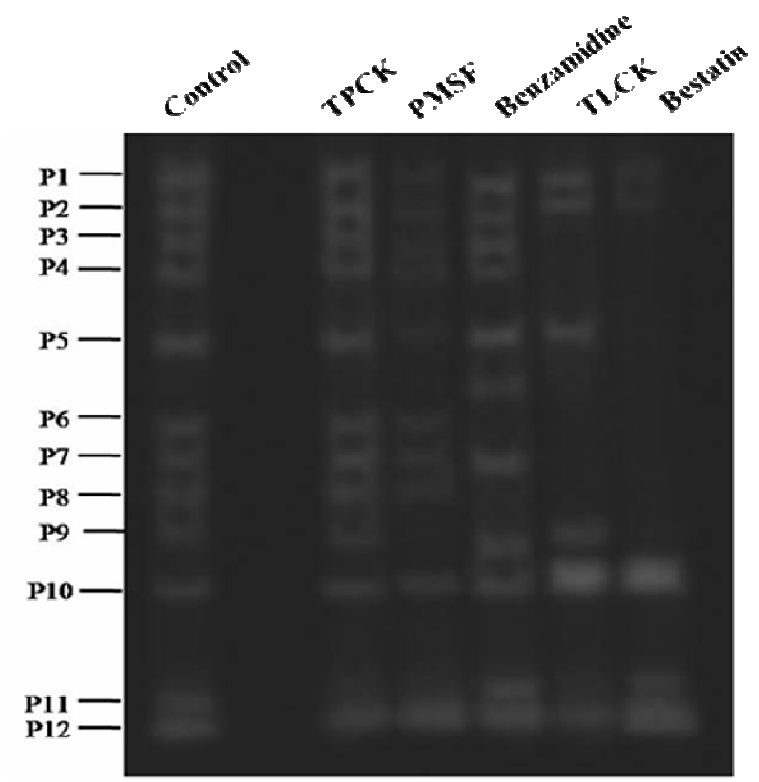
3.11 Figures

Figure 1

Figure 2

Figure 3
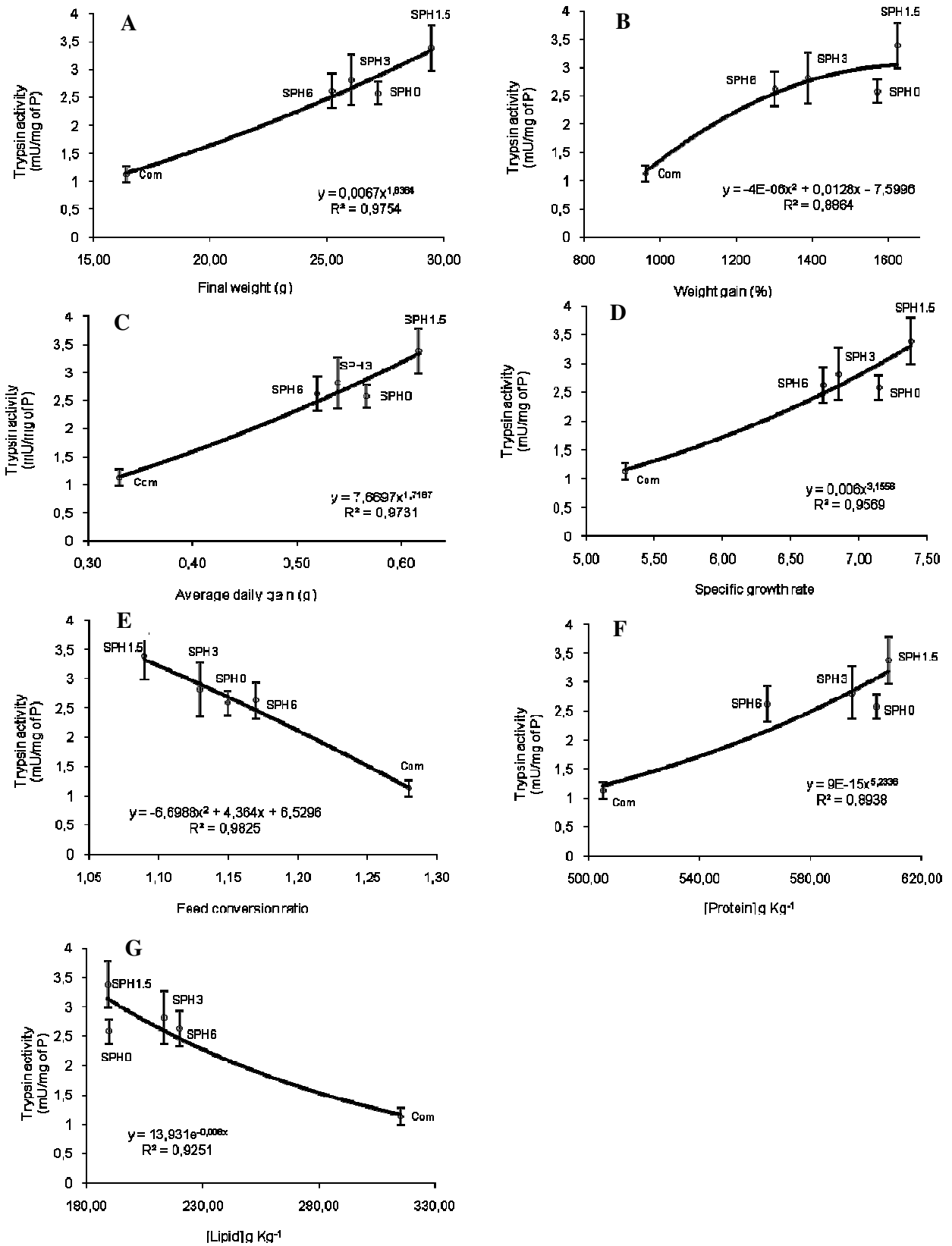
Figure 4
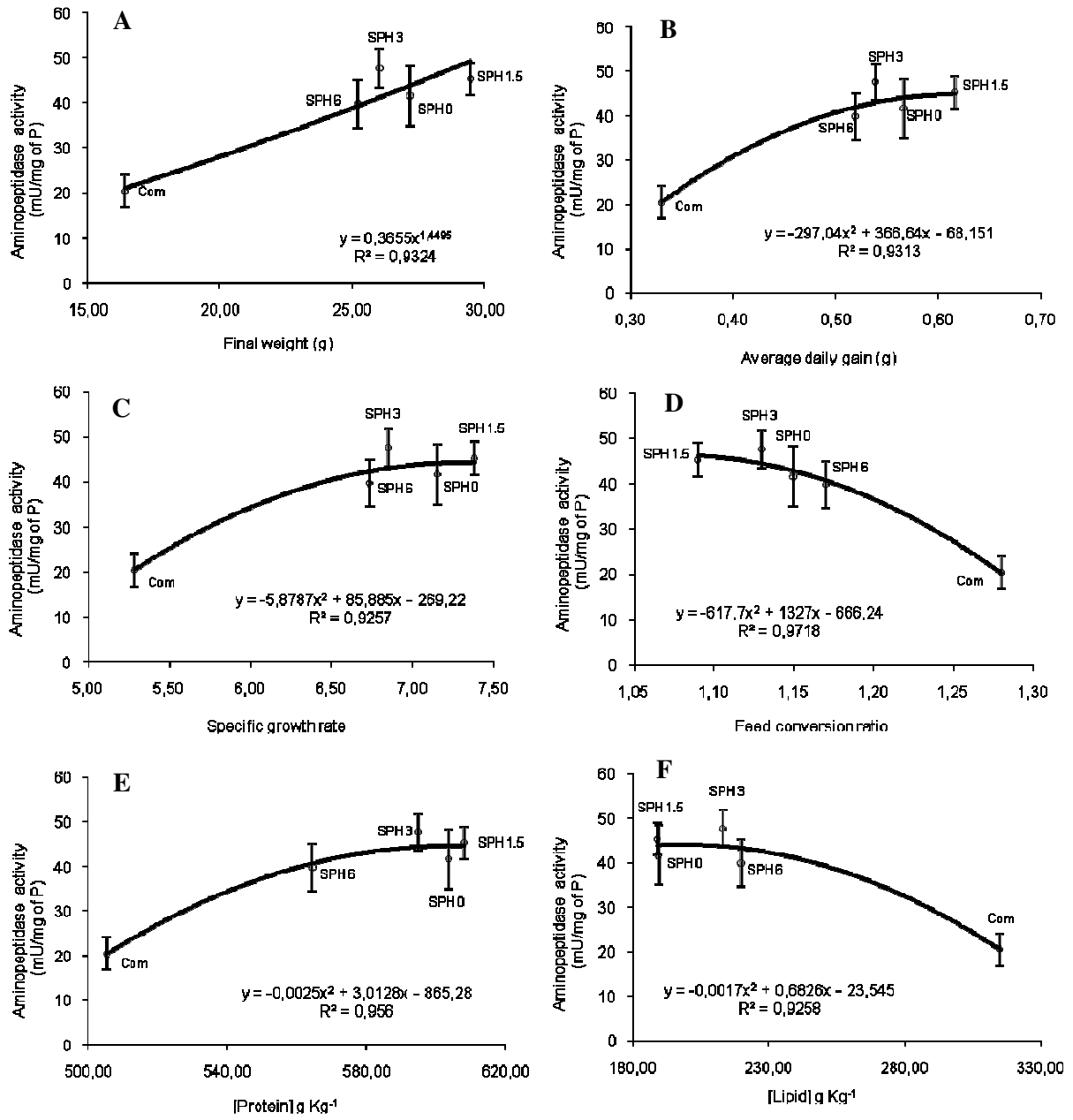
Figure 5

4. CONSIDERAÇÕES FINAIS

Apesar de serem observadas algumas diferenças quantitativas nas atividades enzimáticas dos peixes submetidos a diferentes concentrações de hidrolisado protéico de camarão, não se pôde observar uma correlação lógica entre estes parâmetros.

Estudos feitos a partir de zimogramas demonstraram uma maior eficiência na detecção de mudanças nos perfis enzimáticos da tilápia do Nilo, demonstrando que houve uma adaptação para melhor assimilação dos nutrientes ministrados.

Observou-se também que as atividades tríptica e de aminopeptidase foram as que apresentaram correlação com os parâmetros de crescimento e conteúdo de proteína e lipídio no corpo, demonstrando também perfis de proteases distintos para cada tratamento. Este fato reforça a conhecida habilidade deste peixe para se adaptar a diferentes fontes dietárias.

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6. ANEXOS

6.1 Normas da revista Comparative Biochemistry and Physiology

Guide for Authors

The journal publishes original articles emphasizing comparative and environmental aspects of the physiology, biochemistry, molecular biology, pharmacology, toxicology and endocrinology of animals. Adaptation and evolution as organizing principles are encouraged. Studies on other organisms will be considered if approached in a comparative context.

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Part C. Toxicology and Pharmacology is concerned with chemical and drug action at different levels of organization, biotransformation of xenobiotics, mechanisms of toxicity, including reactive oxygen species and carcinogenesis, endocrine disruptors, natural products chemistry, and signal transduction. A molecular approach to these fields is encouraged.

Part D. Genomics and Proteomics covers the broader comprehensive approaches to comparative biochemistry and physiology that can be generally termed as "-omics", e.g., genomics, functional genomics (transcriptomics), proteomics, metabolomics, and underlying bioinformatics. Papers dealing with fundamental aspects and hypotheses in comparative physiology and biohcemistry are encouraged rather than studies whose main focus is purely technical of methodological.

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Lindsley, J.E., Rutter, J., 2004. Nutrient sensing and metabolic decisions. Comp. Biochem. Physiol. B 139, 543-559.

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