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JANILSON FELIX DA SILVA

DESEMPENHO ZOOTÉCNICO E FISIOLOGIA DIGESTIVA DO *Litopenaeus vannamei* SUBMETIDOS A CULTIVOS DE BIOFLOCOS E ÁGUAS CLARAS

Recife
2013

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal de Pernambuco como pré-requisito para a obtenção do grau de doutor em Ciências Biológicas.

Orientador: Prof. Dr. Ranilson de Souza Bezerra
Co-orientadora: Prof. Dra. Karina Ribeiro

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JANILSON FELIX DA SILVA

DESEMPENHO ZOOTÉCNICO E FISIOLOGIA DIGESTIVA DO *Litopenaeus vannamei* SUBMETIDOS A CULTIVOS HETEROTRÓFICOS E AUTOTRÓFICOS

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal de Pernambuco como pré-requisito para a obtenção do grau de doutor em Ciências Biológicas.

Aprovado em 30 / 07 / 2013

BANCA EXAMINADORA

_____

Prof. Dr. Ranilson de Souza Bezerra/UFPE

_____

Prof. Dra. Maria Tereza dos Santos Correia/UFPE

_____

Prof. Dr. Eduardo Isidoro Beltrão/UFPE

_____

Dra. Helane Maria da Costa Silva/UFPE

_____

Prof. Dr. Eudes de Souza Correia/UFRPE

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“O homem progride, estranhamente, somente em um ambiente desafiador”

L. Ron.Hubbard

RESUMO

No presente estudo foram avaliados os desempenhos zootécnicos e as atividades das enzimas digestórias do hepatopâncreas do *Litopenaeus vannamei* em diferentes condições de cultivo. Três experimentos foram desenvolvidos. No primeiro (E1), adotaram-se três unidades experimentais, sendo um sistema água clara com aplicação de ração e dois sistemas com bioflocos, um com adição de ração e outro sem alimentação. O segundo experimento (E2) consistiu num delineamento inteiramente casualizado, com quatro tratamentos envolvendo diferentes concentrações de proteínas na ração comercial (20%, 25%, 30% e 35%). Por fim, no terceiro experimento (E3), os camarões foram submetidos às dietas com diferentes níveis de substituição da farinha de peixe (0% (C), 30% (S_{30}), 60% (S_{60}) e 100% (S_{100})) por concentrado protéico de soja (SPC). Para a análise das enzimas digestivas presentes nos extratos enzimáticos realizou-se ensaios *in vitro* na presença dos substratos de cadeia longa (azocaseína 1% e amido 2%), p-nitroanilide (BApNA, SApNA e Leu-p-Nan) e β-naphthylamide (alanina, arginina, leucina, tirosina, serina, glicina, isoleucina e histidina). Além disso, foram realizados SDS-PAGE e zimogramas de atividade proteolítica e amilolítica. Ao final dos experimentos, observou-se no E1 que os sistemas de bioflocos aumentaram as atividades das proteases digestórias quando comparadas às apresentadas pelo sistema autotrófico. No E2 não foram evidenciadas diferenças estatísticas ($P<0,05$) entre os animais, tanto no ganho de peso e biomassa final quanto na taxa de sobrevivência. Maior atividade proteolítica total (1,54 ± 0,11U·mg^{-1}) e tríptica (15,00 ± 0,75 mU·mg^{-1}) foram observadas para o tratamento 20%. Para quimotripsina, a atividade no tratamento 35% (0,16 ± 0,03 mU·mg^{-1}) foi consideravelmente maior que as dos demais grupos. Em relação às leucineaminopeptidases foi observada maior atividade no grupo 30% (0,26 ± 0,03 mU·mg^{-1}). Em relação às atividades enzimáticas do E3, ocorreram maiores atividades de enzimas quimotripsina (13,78±1,61 U.mg^{-1}) e leucine aminopeptidase (0,45±0,03 U.mg^{-1}) foram observadas para o grupo controle (C). Enquanto que a mais elevada atividade tríptica (13,13±0,53 U.mg^{-1}) foi constatada para o tratamento S_{30}. Entre os substratos β-naphthylamide analisados, verificou-se valores mais altos de atividade aminopeptídica para arginina e alanina em todos os tratamentos, principalmente no S_{30} que também obteve maior atividade na presença da glicina (1,05±0,08 U.mg^{-1}). Notou-se que para a serina, a atividade das aminopeptidases sofreu uma redução gradativa à medida que aumentou o nível de SPC na dieta dos camarões. O tratamento S_{60} apresentou maior atividade aminopeptídica para isoleucina (0,69±0,02 U.mg^{-1}) e histidina (0,85±0,04 U.mg^{-1}). Em relação à leucina e tirosina, a atuação das aminopeptidases mostrou-se indiferente estatisticamente às variações dietárias.

De acordo com o perfil eletroforético através de SDS-PAGE, observaram-se 17 bandas nas dietas 35%, 30% e 20% e 16 bandas para 25% no tratamento E2, enquanto que no E3 foram observadas vinte e seis bandas protéicas, compreendidas entre 6,9 e 198,8 KDa, para todos os tratamentos. Os zimogramas revelaram seis bandas com alta intensidade para os sistemas de bioflocos e três nos autototrófico no E1. E2 apresentou 12 bandas com atividades proteolíticas para 35% e 30% e 9 bandas para 25% e 20%. Em E3, o zimograma de protease exibiu dois perfis semelhantes, um com 18 (C e S_{30}) e outro com 12 bandas proteolíticas (S_{60} e S_{100}). Enquanto que o zimograma de amilase revelou cinco bandas com atividade amilolítica para todos os tratamentos. Com os resultados expostos, observou-se que os sistemas de bioflocos, proporcionou um efeito positivo na performance dos animais, mesmo reduzindo o teor de proteína nas dietas, bem como na substituição da farinha de peixe por SPC em 30, 60 e 100%.

Palavras chaves: bioflocos, proteases digestórias, proteína de soja, *Litopenaeus vannamei*

ABSTRACT

In the present study were evaluated the zootechnical performances and activities of digestive enzymes from the hepatopancreas of *Litopenaeus vannamei* in different culture conditions. Three experiments were conducted. In first experiment (E1), three experimental units were created, one autotrophic system with application of feed and two bioflocs systems, one with the addition of feed and one without feed. The second experiment (E2) consisted of a completely randomized design with four treatments with different concentrations of proteins in commercial feed (20%, 25%, 30% and 35%). Finally, the third experiment (E3), the shrimp were exposed to diets with different levels of replacement of fish meal (0% (C), 30% (S_{30}), 60% (S_{60}) and 100% (S_{100})) by soy protein concentrate (SPC). To analyse of the digestive enzymes present in extracts, *in vitro* enzymatic assays were performed in the presence of long-chain substrates (azocasein 1% starch and 2%), p-nitroanilide (BApNA, Sapna and Leu-p-Nan) and β-naphthylamide (alanine, arginine, leucine, tyrosine, serine, glycine, isoleucine and histidine). enzymatic activities were also analyzed by SDS-PAGE and zymograms amylolytic and proteolytic activity. At the end of the experiments, it was observed in E1 that bioflocs systems increased activity of digestive proteases when compared to those for the autotrophic system. In E2 showed no statistical difference (P <0.05) in animals gain weight, final biomass and survival rate. Higher total proteolytic activity overall (1.54 ± 0.11 U•mg^{-1}) and tryptic (15.00 ± 0.75 mU•mg^{-1}) were observed for the 20%. For chymotrypsin activity in the 35% treatment (0.16 ± 0.03 mU•mg^{-1}) was considerably higher than those of other groups. Higher activity for leucineaminopeptidases was observed in the 30% (0.26 ± 0.03 mU•mg^{-1}). Regarding the E3 enzyme activities, higher activities of enzymes chymotrypsin (13.78 ± 1.61 mU•mg^{-1}) and leucine aminopeptidase (0.45 ± 0.03 mU•mg^{-1}) were observed for the group control (C), while higher tryptic activity (13.13 ± 0.53 mU•mg^{-1}) was found for the treatment S_{30}. Among the β-naphthylamide substrates analyzed, it was found higher values of aminopeptidase activity to alanine and arginine in all treatments, especially in S30 that also showed increased activity in the presence of glycine (1.05 ± 0.08 mU•mg^{-1}). It was noted that for serine, the activity of aminopeptidase decreased gradually with the increased level of SPC in the diet of shrimp. S_{60} presented higher aminopeptidase activities for isoleucine (0.69 ± 0.02 mU•mg^{-1}) and histidine (0.85 ± 0.04 mU•mg^{-1}). Regarding leucine and tyrosine, the activity of aminopeptidases showed statistically indifferent to dietary variations. According to the electrophoretic profile in the E2 treatment showed 17 bands in the diets 35%, 30%, 20% and 16 bands to 25%. In the E3 were observed twenty-six protein bands, between 6.9 and

198.8 kDa for all treatments. The zymograms revealed 6 bands with high intensity for the biofloc systems and 3 in autotrophic in E1 experiment. E2 showed 12 bands with proteolytic activity to 35% and 30% and 9 bands to 25% and 20%. In E3, the proteolytic zymogram showed two similar profiles, one with 18 (C and S_{30}) and the other with 12 proteolytic bands (S_{60} and S_{100}), while amylase zymogram revealed 5 bands with amylolytic activity for all treatments. With these results, it was observed that bioflocs systems provided a positive effect on animal performance, whether reducing the protein content of the diets as well as in the replacement of fishmeal by SPC.

Keywords: bioflocs, digestive proteases, soy protein, *Litopenaeus vannamei*

LISTA DE ABREVIATURAS

AA-NA - aminoacil-β-naftilamida

AA-Nan - aminoacil-p-nitroanilida

BAPNA - benzoil arginina ρ-nitroanilida

DFP - diisopropil-fluorfosfato

IUBMB - União Internacional de Bioquímica e Biologia Molecular

KDa - quilo Daltons

LEUPNAN - aminoacil de β naftilamida

pH - potencial hidrogeniônico

PMSF - fluoreto fenil-metil-sulfonil

SAPNA - N-succinil-Ala-Ala-Pro-Phe-p-nitroanilida

SBO - óleo de soja

SBTI - inibidor de tripsina de soja

SDS-PAGE - eletroforese em gel de poliacrilamida utilizando SDS

SPC - concentrado protéico de soja

TAME - tosil-arginina-metil-éster

TCA - Ácido Tricloroacético

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SUMÁRIO

1. INTRODUÇÃO

A carcinicultura é um dos setores mais lucrativos entre os diversos segmentos da aquicultura, apresentando crescimento acelerado desde a década passada. Esta atividade surgiu no sudoeste da Ásia no século XV com a captura de larvas marinhas e o seu progresso só foi possível, graças ao domínio de técnicas de larvicultura do *Marsupenaeus japonicus* desenvolvidas por cientistas japoneses na década de 30 (ROCHA e MAIA, 1998; ARANA, 1999). Em meados de 1980, a disponibilidade de larvas produzidas em laboratórios e de rações comerciais permitiu a intensificação da atividade, levando países asiáticos como Taiwan, Filipinas e China a produções significativas (PRIMAVERA, 1998).

Ao longo dos anos, a carcinicultura tem progredido quanto à intensificação dos sistemas e das técnicas de produção. No entanto, essa rápida expansão não foi devidamente acompanhada por manejos que reduzissem os impactos ambientais gerados por sistemas autotróficos tradicionais (SAMOCHA et al., 2007). Além disso, a alimentação empregada nos viveiros, sempre consistiu num dos fatores mais importantes do cultivo promissor de camarão. Muito embora, sejam necessários estudos que evitem o fornecimento de alimentos que possam apresentar fatores antinutricionais e deficiência de aminoácidos essenciais (LONGAS, 1996). Desta forma, a formulação de uma ração é baseada nos requerimentos nutricionais dos organismos cultivados.

Para camarões, a proteína é o maior e mais caro componente da ração alcançando mais de 50% do custo total de produção (AKIYAMA et al., 1992; SHIAU, 1998; HERTRAMPF e PIEDAD-PASCUAL, 2000; LEMOS, 2003) e sua principal fonte é a farinha de peixe que também apresenta um balanço de aminoácidos e ácidos graxos adequado para o rápido crescimento desses organismos aquáticos (CRUZ-SUÁREZ et al., 2000; HERTRAMPF e PIEDAD-PASCUAL, 2000). Entretanto, o emprego da farinha de peixe é afetado por fatores econômicos, ecológicos e de mercado, os quais elevam seu custo e restringem a sua utilização (GUZMAN, 1996). Com isso, a substituição da farinha de peixe por fontes protéicas alternativas tem sido cada vez mais estudada e utilizada em formulações de rações comerciais (EAPA, 2006; SWICK, 2007). Dentre as fontes alternativas atualmente utilizadas podemos citar os subprodutos da pesca e da pecuária e ingredientes de origem vegetal.

A manipulação de bactérias heterotróficas naturalmente presentes nos ambientes aquáticos desponta como promissora tecnologia aplicada à sustentabilidade em cultivo de camarões, bem como na redução dos custos com a alimentação. Sistemas heterotróficos de cultivo vêm sendo desenvolvidos a nível mundial, juntamente com a aplicação de agentes

probióticos que favorecem a digestibilidade e, consequentemente, absorção dos nutrientes, proporcionando melhor resposta imunológica dos camarões (LIN et al., 2004; WANG, 2007). Desta forma, técnicas de cultivo heterotrófico vêm sendo empregadas como alternativa biotecnológica para elevar as produções de camarões marinhos.

No entanto, nem sempre a aplicação de uma ração nutricionalmente balanceada e de um novo sistema de cultivo irá produzir o crescimento esperado, o que pode consequentemente, comprometer o retorno do investimento empregado (LEE & LAWRENCE, 1997). Tal fato pode ser referido ao emprego de técnicas de manejo inadequado e a falta de conhecimentos referentes à fisiologia digestória dos camarões de cultivo. Sobretudo de suas enzimas digestórias, que podem auxiliar no conhecimento da capacidade destes animais em explorar várias dietas, com o intuito de suprir suas exigências nutricionais (JOHNSTON e FREEMAN, 2005).

2. REVISÃO DA LITERATURA

2.1 *Litopenaeus vannamei*

O camarão *L. vannamei* (Figura 1) uma espécie marinha que ocorre naturalmente no Oceano Pacífico, mais precisamente na região leste, estando distribuída numa faixa que se estende desde Sonora no México, até Thumbes no norte do Peru (Figura 2). Este animal tem preferência por fundos lamosos e pode ser encontrada no infralitoral, bem como em profundidades de até 72 metros (Barbieri Júnior & Ostrensky Neto, 2002).

Figura 1. Exemplar do camarão branco *L. vannamei* (Boone, 1931).

Figura 2. Distribuição geográfica (mancha vermelha) do camarão marinho *L. vannamei* (Boone, 1931). **Adaptado da FAO (2012).**

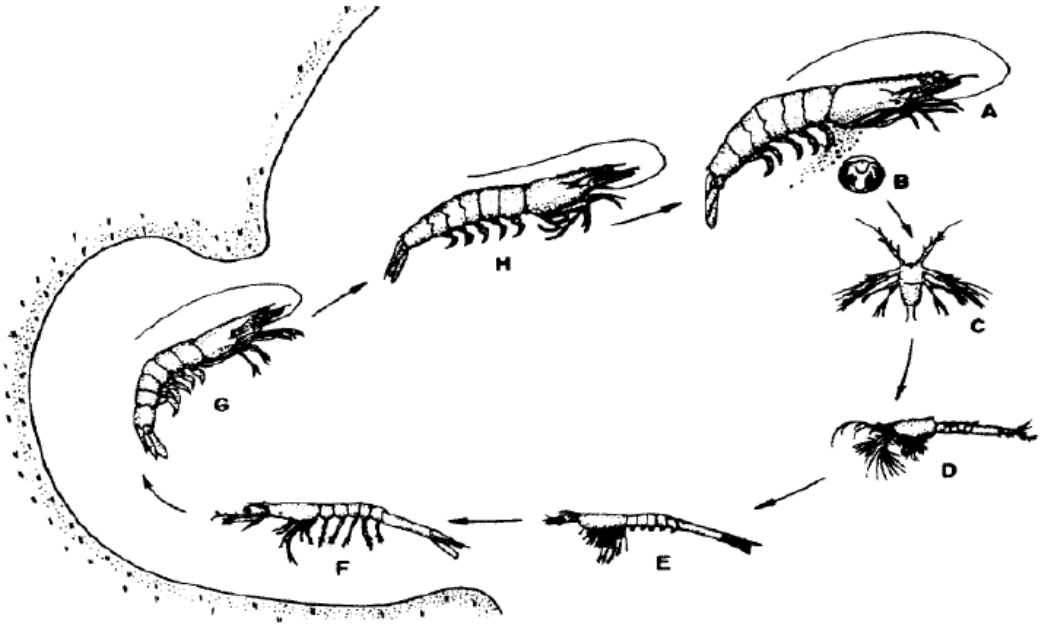
2.1.1 Ciclo de vida

O *L. vannamei* possui três estágios larvais (náuplios, protozea e misis) e estágios pós-larvais durante o seu desenvolvimento (Figura 3). A fase larval desta espécie é planctônica oceânica, enquanto que as pós-larvais se dividem em juvenil estuarina e adulta. Nesta última fase o animal retorna ao ambiente marinho para maturação e desova (VALLES-JIMENEZ et al., 2005). Segundo Nunes (2001), essa mudança de habitats no processo de desenvolvimento dos camarões em ambiente natural, tem a finalidade de aumentar as chances de sobrevivência da prole.

As fêmeas eliminam cerca de 100.000 a 500.000 ovos durante a sua fase reprodutiva e após 14 horas de fecundados nascem os náuplios (PRIMAVERA, 1984). Esta fase apresenta vários subestágios e de acordo com Kitani (1986), em *L. vannamei* observam-se seis fases como náuplio (N1 a N6). Este estágio sofre uma série de mudanças morfológicas, desenvolvendo oito pares de apêndices, e também no comportamento passando, assim, após 36 horas, ao estágio de protozoea. Este nível de desenvolvimento tem duração de 48 horas e é constituída por três subestágios (Z1 a Z3). Nesta fase o animal começará a capturar seu próprio alimento adquirindo, assim, um hábito carnívoro (ALFONSO; COELHO, 1997). Posteriormente, inicia-se a fase de misis com três subestágios (M1 a M3) com duração de três

dias. Neste momento observa-se a constituição da carapaça recobrindo o seu tórax. A fase do desenvolvimento larval será finalizada após a aquisição de todos os apêndices abdominais. Assim o camarão passa ao nível de pós-larva, que por sua vez, é semelhante ao indivíduo adulto tanto na forma quanto na fisiologia. O estágio de pós-lava é seguido do juvenil, o qual o camarão é exatamente igual ao indivíduo adulto porém faltando atingir a maturação gonadal (DALL et al., 1999; BARBIERI JR; OSTRENSKY NETO, 2001; ANDREATTA; BELTRAME, 2004).

Figura 3. Ciclo de vida do camarão marinho peneídeo. A, reprodutor desovando; B, ovo; C, náuplio; D, zoea; E, misis; F, pós-larva; G, juvenil; H, Adulto.



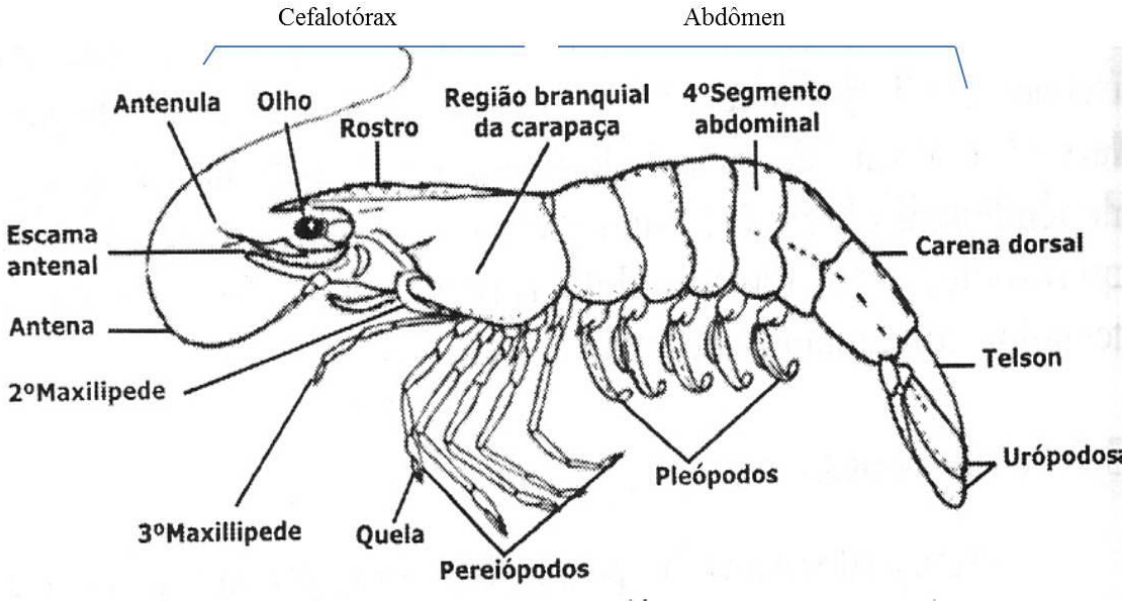
Fonte: (FREITAS, 2003).

2.1.2 Aspectos morfológicos

2.1.2.1 Características externas

O camarão branco *L. vannamei*, assim como os outros artrópodes, possui um envoltório externo denominado exoesqueleto. Esta estrutura é composta por proteínas, quitina e substâncias calcárias como carbonato de cálcio que a torna rígida (RUPPERT e BARNES, 1996). Além dessa estrutura, o camarão branco também apresenta várias características morfológicas que são semelhantes aos dos outros peneídeos. Isto permite a apresentação de um plano corporal geral que represente todos os integrantes desta família (Figura 4).

Figura 4. Vista lateral de um camarão marinho peneídeo Adaptado de Barbieri Junior e Ostrensky Neto, 2002.



De acordo Barbieri Junior e Ostrensky Neto (2001), e como pode ser observado na figura 3, o corpo dos peneídeos é divido em duas regiões bem características, sendo uma localizada na região anterior, o cefalotórax, e a outra na porção posterior, o abdômen. O cefalotórax é formado pela fusão entre cabeça e tórax e dispõe de estruturas consideradas de grande importância funcional para os camarões. Dentre elas, está a carapaça que tem a função de recobrir e proteger as brânquias e os órgãos vitais, os olhos pedunculados, responsáveis pela visão e o rostro que é estrutura pontiaguda com função de proteger o animal contra os predadores. Esta última estrutura também é importante na identificação das espécies de camarões. No *L. vannamei* o rostro apresenta-se moderadamente longo com 7-10 dentes dorsais e 2-4 ventrais (FAO, 2012). Ainda no cefalotórax, encontram-se apêndices com modificações evidentes. Os dois primeiros pares, situados pré-oralmente, são antenas e são responsáveis basicamente pela função sensorial. Os três últimos pares localizam-se atrás da boca, sendo um par de mandíbulas possuindo bordas capazes de moer e cortar os alimentos e dois pares de maxilas que ajudam as mandíbulas na manipulação do alimento. São encontrados também no cefalotórax cinco pares de patas denominados pereiopodes (apêndices ambulatórios) que desempenham funções como captura de alimento, locomoção, cópula nos indivíduos machos e transporte de óvulos nas fêmeas. Na região abdominal encontram-se os pleiopodos responsáveis pela natação do animal. Na extremidade desta região está o telson,

uma estrutura pontiaguda que juntamente com os uropodes formam o último segmento abdominal. Ambos são responsáveis por direcionar o animal durante o deslocamento natatório.

2.1.2.2 Características internas

Assim como ocorre com as estruturas externas, a anatomia interna do *L. vannamei* também se assemelha aos representantes do grande grupo dos artrópodes e, consequentemente, aos peneídeos. No interior do cefalotórax encontram-se vísceras importantes como o cérebro, coração, hepatopâncreas, estômagos e as gônadas, enquanto que no abdômen está o intestino e a maior parte da musculatura dos peneídeos (BARBIERI JR e OSTRENKY NETO, 2001; ANDEATTA e BELTRAME, 2004). Os órgãos excretores são pareados e compostos de um saco terminal, um canal excretor e um duto de saída, todos localizados na cabeça sendo chamados de glândulas antenais ou maxilares, uma vez que os poros excretores encontram-se na base das antenas ou das maxilas. As brânquias excretam amônia e são responsáveis pelo equilíbrio salino. O estômago possui muitos músculos permitindo que só seja repassado ao hepatopâncreas o que está totalmente liquefeito. O hepatopâncreas é uma glândula de suma importância assumindo um papel no metabolismo destes organismos interagindo com os processos fisiológicos de muda, produzindo respostas rápidas a alterações induzidas por fatores endógenos e ambientais. É também responsável pelo armazenamento de substâncias de reservas e produção de enzimas digestivas. O sistema circulatório é aberto, possuindo hemolinfa (sangue) onde circulam os hemócitos. Os hemócitos são produzidos pelo tecido hematopoiético localizado próximo ao estômago. A hemolinfa passa por todo o corpo retornando sempre para o coração, principal órgão propulsor, constituído por três partes de óstio. O órgão linfóide é o órgão responsável pela defesa se tornando hipertrofiado em algumas enfermidades. O sistema nervoso dos camarões marinhos e bem rudimentar e apresenta um cordão nervoso direcionado para todos os segmentos (RUPPERT e BARNES, 1996).

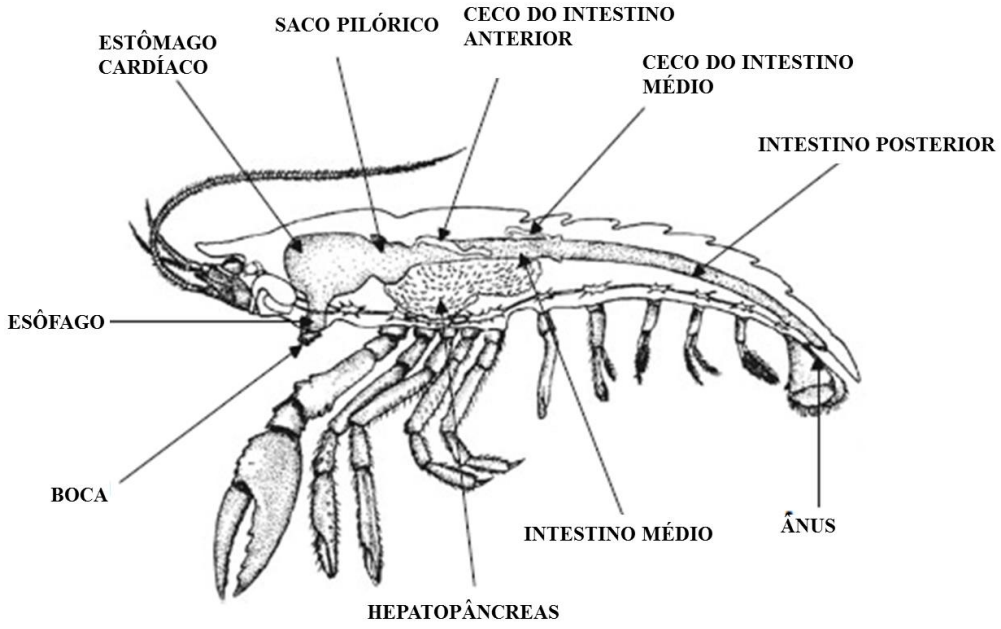
2.1.2.3 Sistema digestório

2.1.2.3.1 Visão geral

O aparelho digestório do *L. vannamei*, de uma maneira geral, segue os mesmos padrões morfológico e fisiológico dos camarões decápodas. Essencialmente é um tubo interno

que está dividido em três partes funcionais: os intestinos anterior, médio e posterior (Figura 5). A primeira região é uma estrutura altamente especializada que funciona tanto na digestão mecânica quanto na extracelular. A porção mediana regula o movimento dentro do hepatopâncreas onde ocorre a digestão intracelular, enquanto movimentos peristálticos rítmicos do intestino posterior expele uma membrana peritrófica contendo fezes (McGAW e CURTIS, 2013).

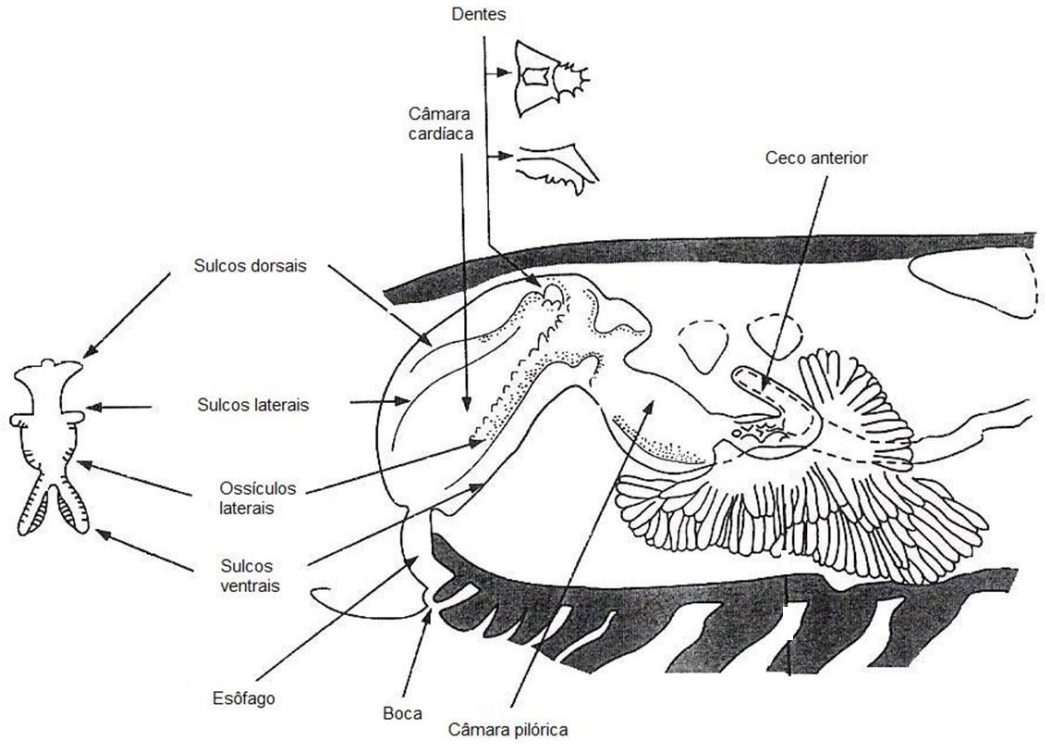
Figura 5. Anatomia do aparelho digestório de um decápoda. Adaptado de McGAW e CURTIS (2013).



2.1.2.3.2 Intestino anterior

O intestino anterior engloba o esôfago e o estômago ou proventrículo (Figura 6). Ele tem início na boca formada por um lábio rígido e circundada por vários pares de apêndices (maxilas, maxílulas, mandíbulas e maxilípedes) especializados na quimiorecepção e apreensão dos alimentos (GUILLAUME E CECCALDI, 1999).

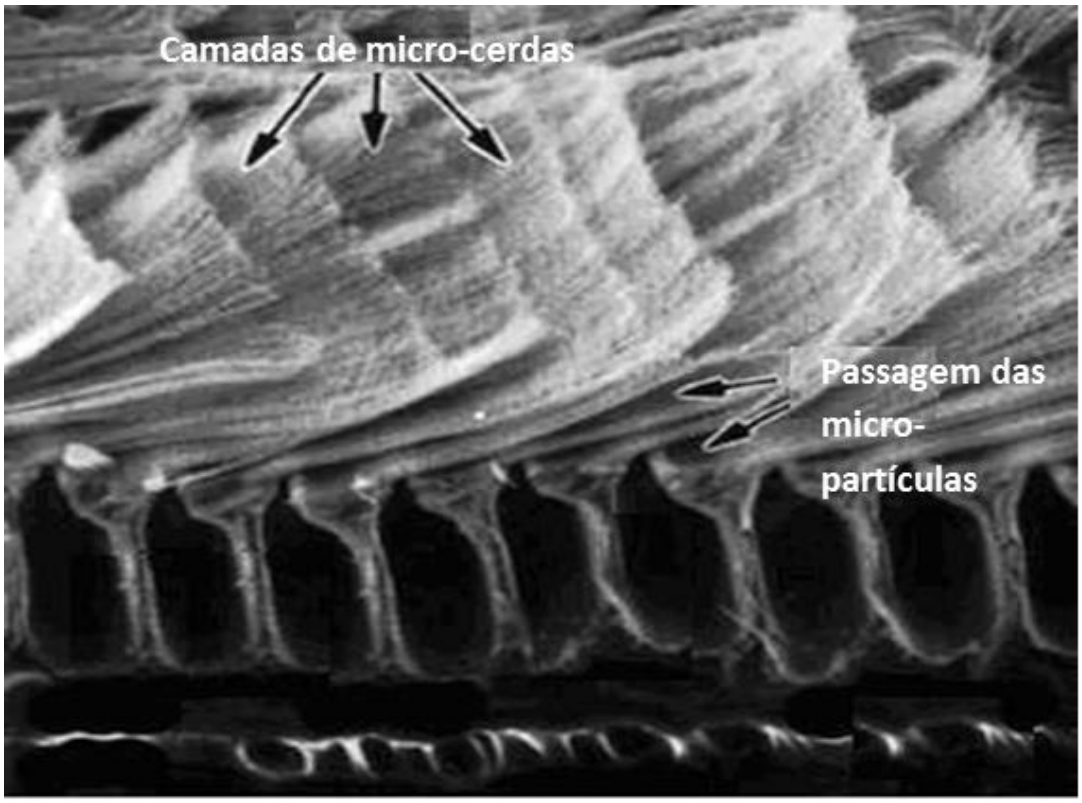
Figura 6. Estruturas do intestino anterior de camarões. Adaptado de Ceccaldi (1997).



O esôfago constitui-se em um tubo curto, reto e contrátil, revestido por uma camada quitino-protéica, cuja função básica é conduzir o alimento ao estômago. A parede desse órgão ainda possui glândulas tegumentares que secretam muco e com objetivo de lubrificar o alimento ingerido (McGAW e CURTIS, 2013). O estômago ou proventrículo é uma estrutura mais complexa e se apresenta dividido em uma porção anterior (câmara cardíaca) e uma posterior (câmara pilórica), separadas por uma válvula cárdio-pilórica. As duas câmaras são providas por peças calcáreas articuladas movidas por músculos específicos localizados na parede externa. Essas peças possuem funções diversas, segundo sua localização. Algumas peças são mais fortes e mais calcificadas (ossículos, discos e dentes) e localizam-se na câmara cardíaca, formando o moinho gástrico, cuja função é triturar os alimentos. Na câmara pilórica, encontram-se peças menores e menos calcificadas, que participam do processo de filtração. A ação combinada dessas peças possibilita a maceração do alimento e impede a passagem de partículas grandes para a o intestino médio. A câmara pilórica está, por sua vez, dividida em uma porção dorsal, com sulcos laterais, que levam ao intestino médio, e outra ventral, onde se

localiza o filtro-prensa. Essa estrutura é composta por um sistema de inúmeras micro-cerdas que filtram as partículas que passam para a glândula digestiva (Figura 7). Somente partículas menores que 1µm e fluído gástrico passam por essa rede de cerdas.

Figura 7. Filtro-prensa do estômago de *Penaeus monodon* Adaptado de Lin (2000).



2.1.2.3.3 Intestino médio

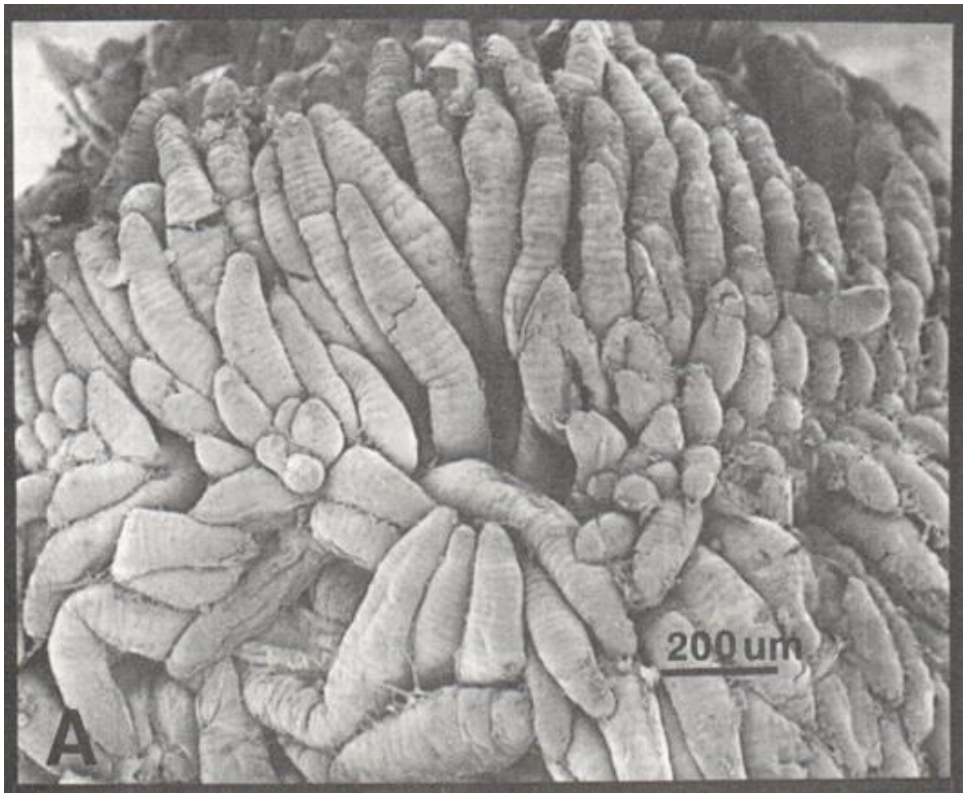
O intestino médio se estende dorsalmente do final do estômago pilórico até o ceco posterior da porção mediana na junção entre a carapaça e o abdômen (McGAW e CURTIS, 2013). Suas paredes apresentam cecos ou divertículos volumosos, onde se distinguem células nervosas, hemócitos e células endócrinas. Nessa região são secretados o muco e a película de quitina que envolve as fezes, mas essa membrana não impede a absorção dos nutrientes residuais presentes no bolo fecal. É no intestino médio que está localizado um dos principais órgãos no processo de digestão do *L. vannamei* e nos decápodas em geral, a glândula digestiva ou hepatopâncreas (Figura 8).

Este órgão exerce várias funções como a síntese e excreção de enzimas digestivas (McGAW e CURTIS, 2013) e absorção e estocagem do material ingerido (VOGT et al., 1989; JOHNSTON et al.,1998). Além disso, é responsável também pelo armazenamento de cálcio e fosfato do exoesqueleto durante o ciclo de muda (BECKER et al., 1974; CHEN et al., 1974; LORENT e DEVOS, 1992) e glicogênio e lipídeos durante períodos de abundância de alimentos (GIBSON e BAKER, 1979). Ele ainda atua na detoxificação de agentes xenobióticos (GIBSON e BAKER, 1979; ICELY e NOTT, 1992; VOGT, 1994).

A glândula digestiva é um grande órgão que ocupa mais da metade da região dorsal do cefalotórax, podendo se estender até o abdômen e consiste de duas metades que se dispõem uma de cada lado da linha horizontal média do corpo do camarão. Cada metade apresenta três lobos que são conectados separadamente ao estômago e intestino médio por um ducto primário que se divide em ductos secundários em cada lóbulo. Os ductos secundários se ramificam amplamente em dúctulos, sendo que cada um termina em um complexo de túbulos com fundo cego (GIBSON e BAKER, 1979; FACTOR, 1995). De acordo com Gibson e Baker (1979), o hepatopâncreas é morfologicamente similar na maioria dos decápodas, embora Icely e Nott (1992) afirmaram que o número de lobos pode variar nas diferentes espécies.

Os túbulos digestórios estão ligados e dispersos em uma extensa rede de tecido conjuntivo constituído por fibras colágenas bem definidas, que apresentam uma variedade de estruturas características incluindo-se sinusóides hemolinfáticos, células circulantes da hemolinfa e fibroblastos (FACTOR e NAAR, 1985; 1990; FRANCESCHINI-VICENTI et al., 2006). Os espaços entre a membrana basal e a lâmina própria do túbulo hepatopancreático são ocupados por células contráteis de miofilamentos. Estas células apresentam processos em disposição circular e longitudinal ao túbulo formando uma malha de tecido contrátil ao redor da lâmina basal do túbulo (FACTOR e NAAR, 1985; ICELY e NOTT, 1992).

Figura 8. Micrografia eletrônica de varredura de baixo poder de resolução mostrando o arranjo e o grande número de túbulos de fundo cego do hepatopâncreas do *L. vannamei*. Adaptado de Caceci et al. (1988).



Nos túbulos digestivos foram reconhecidos quatro tipos celulares que variam de localização ao longo do seu comprimento (ICELY e NOTT et al., 1992; CORRÊA Jr et al., 2002). As células são classificadas de acordo com o esquema proposto por Jacobs (1928) e Hirsch e Jacobs (1930) como células E (embrionária), B (vesicular), F (fibrilar) e R (reabsortiva). As células E estão restritas à região distal em fundo cego. Já as células R ocorrem ao longo de todo o comprimento do tubo. As células F ocorrem principalmente na região distal, enquanto as células B localizam-se na região proximal do túbulo hepatopancreático (ICELY e NOTT, 1992). Em alguns decápodas foi identificado um quinto tipo celular denominado de células M (basal) (AL-MOHANNA et al., 1985b). Este tipo celular está disperso em todo o comprimento do túbulo e apóia-se na membrana basal do epitélio, porém não mantém contato com o lúmen do hepatopâncreas (ICELY e NOTT, 1992).

As células E são indiferenciadas e apresentam núcleo grande que ocupa a maior parte do volume citoplasmático (AL-MOHANNA et al., 1985b; ICELY e NOTT, 1992). Esse tipo celular é responsável pela renovação celular do epitélio dos túbulos hepatopancreáticos (ICELY e NOTT, 1992).

As células F apresentam núcleo localizado próximo à região basal da célula. Estas células são especializadas em sintetizar e secretar enzimas digestivas durante algumas fases do ciclo alimentar (ICELY e NOTT, 1992). Após a ingestão de alimento estas células apresentam numerosos grânulos de zimogênio que estão localizados na região apical do citoplasma. Desta forma, as células F sintetizam e secretam zimogênio para a digestão extracelular. Posteriormente captam material para a digestão intracelular e se diferenciam em células B (AL-MOHANNA et al., 1985a). Estas células presentam ainda um grande vacúolo supranuclear, podendo formar um reservatório de atividade digestiva latente antes da alimentação (AL-MOHANNA et al., 1985a).

As células B são diferenciadas a partir das células F e estão envolvidas com a digestão intracelular (AL-MOHANNA e NOTT, 1986). São os maiores tipos celulares do hepatopâncreas e apresentam um grande vacúolo envolto por uma fina camada de citoplasma e o núcleo está restrito à região basal da célula (ICELY e NOTT, 1992). Logo após a alimentação, a porção apical da membrana das células B desenvolve invaginações as quais se estendem como canais para dentro das células, produzindo as vesículas pinocíticas. Com o avanço do processo de digestão intracelular, pequenas regiões translúcidas aparecem na matriz dos corpos digestivos que fundem-se para formar grandes vacúolos digestivos. No final do processo de digestão intracelular as células B há uma tendência dos vacúolos aumentarem progressivamente de tamanho e moverem-se basalmente e se fusionarem formando um vacúolo único contendo esferas densas. Após a digestão celular, as células B iniciam a fase de extrusão e o vacúolo é expelido pela célula para o interior do lúmem (Al-MOHANNA e NOTT, 1986).

As células R são as mais abundantes nos túbulos hepatopancreáticos dos decápodas. O núcleo dessas células localiza-se na região basal, tendendo a ser pequeno e conter menos cromatina que os núcleos dos demais tipos celulares. As células R absorvem nutrientes solúveis do lúmen do intestino e estocam lipídios e glicogênio (AL-MOHANNA e NOTT, 1989). Desta forma, as células R constituem o principal local de estocagem de lipídios e glicogênio (ICELY e NOTT, 1992).

As células M são conhecidas pela acumulação contínua de material orgânico denso, o qual ocupa todo o volume celular e também por estocar material de origem protéica. Estas células tendem a ser arredondadas, permanecendo em contato com a lâmina basal e, ocasionalmente, podem produzir extensões citoplasmáticas com ramificações entre as células vizinhas (AL-MOHANNA et al., 1985b).

2.1.2.3.4 Intestino posterior

A última porção do tubo digestivo dos camarões surge por trás do ceco do intestino posterior e prolonga-se desde o abdômen até o ânus. O intestino posterior é considerado como um simples tubo com cutículas envolto por camadas externas de músculos estriados circular e longitudinal. A função desses músculos é expelir, por contrações rítmicas ao longo do tubo, a membrana mucoperitrófica e seus conteúdos. Glândulas tegumentares ao longo das paredes do intestino posterior secretam mucos para lubrificar as paredes. Algum processo de digestão ainda pode ser realizado no intestino posterior e o mesmo não é esvaziado até a próxima refeição ser ingerida. Como em outros artrópodes, o intestino posterior pode também está envolvido na absorção e transporte ativo de íons (McGAW e CURTIS, 2013).

2.2 Enzimas

Enzimas são biomoléculas catalisadoras que atuam diminuindo o nível de energia de ativação, implicando no aumento da velocidade das reações bioquímicas (HARVEY et al., 2009). Todas as enzimas conhecidas, com exceção de certos RNAs catalíticos, são proteínas (NELSON e COX, 2004), e estão presente em todos os organismos vivos, sendo essenciais, tanto para a manutenção, como para o crescimento e a diferenciação celular (GUPTA et al., 2002).

As enzimas agem em sequências organizadas e catalisam centenas de reações sucessivas, pelas quais as moléculas de nutrientes são degradadas. Essas biomoléculas catalisadoras não reagem quimicamente com as substâncias sobre as quais atuam, nem alteram o equilíbrio das reações. De uma maneira geral, uma enzima liga-se ao seu substrato formando um complexo Enzima-Substrato (ES), de caráter transitório. Provavelmente, apenas uma fração da molécula denominada sítio ativo é a responsável pela ligação da enzima ao substrato, e essa fração determina a especificidade enzimática (NELSON e COX, 2004).

Uma vez que a reação química catalisada por uma enzima é a propriedade específica que distingue uma enzima de outra, a IUBMB (União Internacional de Bioquímica e Biologia Molecular) dividiu as enzimas em seis grandes divisões (Tabela 1).

Tabela 1. Classificação das enzimas segundo a IUBMB.

CLASSE	REAÇÕES QUE CATALISAM
1. Oxidorredutases	Reações de oxidação-redução
2. Transferases	Reações de grupos contendo C, N ou P -
3. Hidrolases	Clivagem das reações adicionando água
4. Liases	Clivagem de C-C, C-S e certas ligações de C-N
5. Isomerases	Racemização de isômeros ópticos ou geométricos
6. Ligases	Formação de pontes entre C e O, S, N acoplados a hidrólise de fosfatos de alta energia.

C, carbono; N, nitrogênio; P-, íon fosfato; S, enxofre; O, oxigênio. Fonte: (NELSON e COX, 2004).

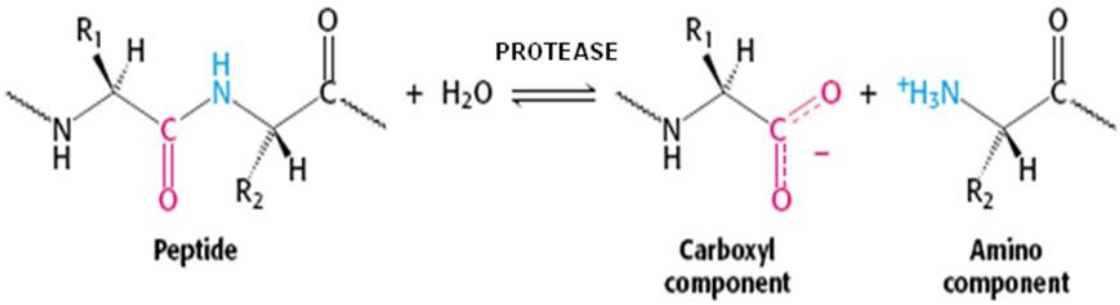
2.2.1. Enzimas digestivas

O estudo do metabolismo das enzimas digestórias é necessário para a escolha de ingredientes a ser introduzido nas dietas de organismos aquáticos, favorecendo o desenvolvimento de uma nutrição adequada.

As proteases estão entre as enzimas de crustáceos que recebem maior atenção (FERNÁNDEZ GIMENEZ et al., 2002), pois são responsáveis pela digestão de proteínas dos alimentos ingeridos, os componentes mais caros da alimentação de camarões (SÁNCHEZ-PAZ et al., 2003)

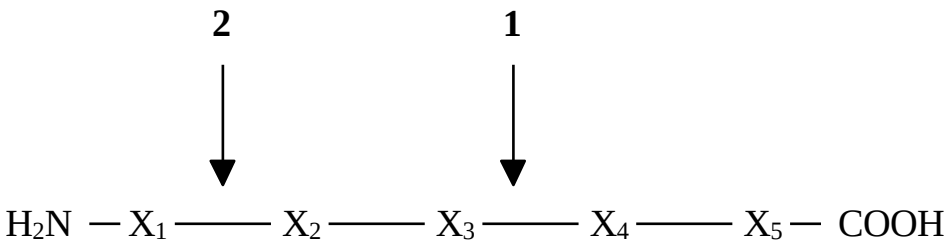
De acordo com a IUBMB as proteases estão inseridas no subgrupo 4 do grupo 3 (Hidrolases), pois por uma reação de hidrólise, elas clivam a proteína adicionando uma molécula de água à ligação peptídica (BERG et al., 2004) (Figura 9).

Figura 9. Hidrólise enzimática de uma proteína hipotética. (Fonte: BERG et al., 2004).



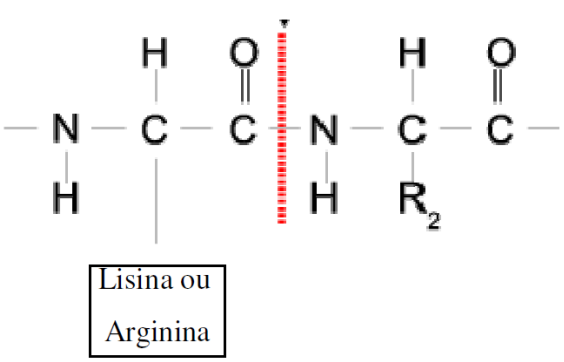
Dentre as proteases de maior importância encontram-se a tripsina, a quimotripsina e as aminopeptidases. A tripsina e a quimotripsina são endoproteases, ou seja, clivam as ligações peptídicas dentro da proteína, enquanto que as aminopeptidases são exoproteases (Figura 10), isto é, clivam resíduos de aminoácidos na posição N-terminal da proteína (GONZALES e ROBERT-BAUDOUY, 1996)

Figura 10. Classificação das proteases: Endoproteases clivam ligações peptídicas dentro da proteína (1). Exoproteases, mais especificamente as aminopeptidases, clivam resíduos localizados na posição N-terminal da proteína (2). Figura modificada de Gonzales e Robert-Baudouy (1996).



A tripsina é a protease mais abundante no sistema digestivo de crustáceos e sua contribuição para a digestão protéica em peneídeos é em torno de 60% (FERNANÉZ GIMENEZ et al., 2003). Ela faz parte da família das serinoproteases, caracterizadas por apresentar um mecanismo comum, envolvendo a presença de uma tríade catalítica composta de resíduos específicos: serina, histidina e ácido aspártico. Esta enzima cliva as ligações peptídicas no lado carboxila de resíduos de aminoácidos carregados positivamente como arginina e lisina (KOMKLAO et al., 2007) (Figura 11).

Figura 11. Sítio de hidrólise específico para tripsina.

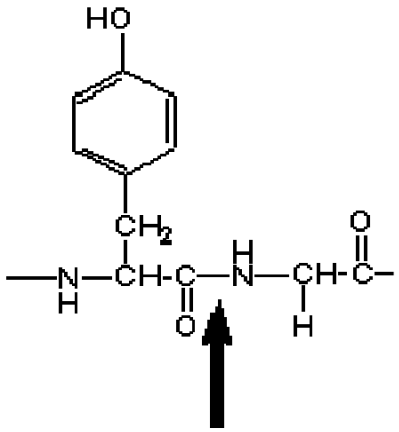


A atuação da tripsina é importante em vários processos biológicos como: digestão protéica propriamente dita, ativação de zimogênios e mediação entre a ingestão do alimento e a assimilação dos nutrientes (SAINZ et al., 2004). Devido à extrema relevância funcional da tripsina, associada a uma ampla aplicabilidade industrial, esta enzima é uma das mais estudadas em organismos aquáticos (KLEIN et al., 1996).

A tripsina se caracteriza por apresentar o maior nível de atividade nos valores de pH entre 8,0 e 11,0 e em temperaturas de 35°- 45°C. Esta enzima pode ainda ter sua atividade alterada em pH abaixo de 5,0 e acima de 11,0 ou pela presença de alguns inibidores como diisopropil-fluorfosfato (DFP), fluoreto fenil-metil-sulfonil (PMSF), inibidor de tripsina de soja (SBTI) e aprotonina. Dentre os substratos sintéticos hidrolizados pela tripsina e usados em pesquisas científicas destacam-se: N-α-benzoil-L-arginina-p-nitoanilida (BApNA) e tosil-arginina-metil-éster (TAME) (WHITAKER, 1994; SIMPSON, 2000).

Conforme a atividade proteolítica, a quimotripsina é considerada a segunda enzima mais abundante no sistema digestório de crustáceos (GARCIA-CARREÑO et al., 1994). Esta endopeptidase, solúvel em água catalisa a hidrólise de ligações peptídicas de proteínas na porção carboxila de aminoácidos aromáticos como: fenilalanina, tirosina e triptofano (Figura 12) e também substratos sintéticos, tais como SAPNA (DE VECCHI e COPPES, 1996; VIPARELLI et al., 2001; ABUIN et al., 2004; CASTILLO-YAÑEZ et al., 2006).

Figura 12. Sítio de hidrólise específica para quimotripsina.



As principais enzimas responsáveis pela liberação dos aminoácidos livres são as aminopeptidases. Além dos aminoácidos, as aminopeptidases liberam também pequenos

peptídeos através da hidrólise das ligações peptídicas na posição N-terminal de proteínas (GONZALES e ROBERT-BAUDOUY, 1996). Essas enzimas, geralmente inespecíficas, estão amplamente distribuídas na natureza, presentes em vários organismos, e apresentam importâncias biológicas e médicas por causa da sua função na degradação de proteínas (OLIVEIRA et al., 1999). As aminopeptidases vêm sendo amplamente investigadas por estudos bioquímicos e a viabilidade potencial de sua dosagem constitui-se em uma medida diagnóstica ou preventiva em algumas patologias relacionadas com seu papel fisiológico. Essas enzimas atuam também catalisando a hidrólise de substratos artificiais tais como aminoacil-β-naftilamida (AA-NA) e aminoacil-p-nitroanilida (AA-Nan).

Para a realização da digestão do amido há a atuação de diversas enzimas. A α-amilase [EC 3.2.1.1] é uma endocarboidrase encontrada na saliva e no trato digestivo de animais vertebrados (SALEH et al., 2005), responsável pela hidrólise de ligações glicosídicas α(1,4), no amido e glicogênio. Nesse processo são produzidos oligossacarídeos, α-dextrinas e maltose (VAN WORMHOUDT e FAVREL, 1988), que são hidrolisados à glicose pela ação complementar da α-glicosidase [EC 3.2.1.20], da sacarase-isomaltase [EC 3.2.1.48] e da α-dextrinase [EC 3.2.1.20]. Dentre essas, a α-glicosidase está diretamente relacionada à exo-hidrólise de ligações glicosídicas α(1,4) da maltose e demais oligossacarídeos formados após a atuação da α-amilase (LE CHEVALIER e VAN WORMHOUDT, 1998; DOUGLAS et al., 2000; ROSAS et al., 2000).

2.3. Nutrição dos camarões peneídeos

Nos estágios iniciais de desenvolvimento, os peneídeos são classificados como onívoros, alimentando-se de fitoplâncton e mudando para zooplâncton ao atingir o estágio pós-larval. Os juvenis são descritos como onívoros e os adultos como onívoros, detritívoros, carnívoros ou predadores. De acordo com Nunes (2000), a detecção do alimento pelos camarões é estimulada por baixas concentrações de compostos orgânicos liberados na água, como aminoácidos (argina e lisina), bem como compostos ricos em ácidos graxos insaturados. Rações com deficiência de nutrientes essenciais, podem acarretar em camarões cultivados em sistemas intensivos um crescimento deficiente, apresentar deformidades ou mesmo serem susceptíveis à doenças (GUILAUME,1997).

Os ingredientes como proteínas, lipídeos, carboidratos, vitaminas, minerais e água nas rações para camarões, são utilizados para a construção, manutenção dos tecidos e o suprimento de energia. As proteínas, os lipídeos, os minerais e a água são usados pelo animal

para a formação dos tecidos. Já os carboidratos, bem como os lipídeos e proteínas, podem ser oxidados para promover energia, enquanto que as vitaminas e os minerais solúveis na água atuam como componentes funcionais de coezimas (CUZON et al., 2004).

Os crustáceos, como todos os outros animais, necessitam de proteínas na forma de aminoácidos essenciais para o crescimento e a reprodução. As proteínas são nutrientes indispensáveis no crescimento e na formação do tecido muscular de todos os organismos, incluindo o camarão (SHIAU, 1998). Os níveis de proteínas exigidos para o desenvolvimento dos camarões variam em cada fase do desenvolvimento. Na fase de pós-larvas, os níveis ficam em torno de 30-35% e para os juvenis está em 30% (CUZON et al., 2004).

A determinação do tipo de aminoácidos necessários na alimentação do camarão é baseada na composição dos encontrados no músculo do camarão, sendo dez aminoácidos considerados essenciais (arginina, leucina, histidina, isoleucina, lisina, metionina, fenilalanina, treonina, triptosina, valina) (AKIYAMA et al. , 1991). A farinha de peixe é a principal fonte protéica dietária que satisfaz as exigências dos aminoácidos essenciais e não essenciais na produção de ração para a aquicultura, sendo o maior constituinte em rações para espécies onívoras/detritívoras de camarões marinhos (TACON, 2006; FAO, 2007). Uma das vantagens do seu uso é o alto teor de lisina e metionina comparados a outras rações. Além disso, outros componentes como as vitaminas do complexo B e os minerais, cálcio e fósforo dos ossos, e ainda iodo, zinco, ferro, selênio e flúor, levam à escolha da farinha de pescado para uso em formulações especiais (GUILLAUME, 1997).

A maioria das farinhas comerciais de peixe é produzida a partir de várias espécies de peixes e pode ser rotulada em função da cor (branca ou marrom), espécie de pescado, procedimento de manufatura ou país de origem. A qualidade destas farinhas depende de vários fatores, tais como, temperatura no momento da captura do pescado, método de captura, temperatura e tempo de estocagem antes do processamento, e composição do pescado capturado (OLIVEIRA, 2002). Apesar de ser um ingrediente de alto valor protéico, a sua grande participação na composição dos custos das rações tem conduzido ao interesse contínuo na identificação e desenvolvimento de novas fontes alternativas de proteínas.

A utilização de fontes protéicas de origem vegetal na formulação de rações para camarões marinhos já vem sendo realizada com sucesso (DAVIS E ARNOLD, 2000; SUDARYONO et al. 1999). Dentre as fontes de proteína de origem vegetal, a soja *Glycine Max* (L) é considerada uma das principais fontes alternativas de proteína que venha substituir a farinha de peixe na formulação das rações comerciais. A proteína da soja apresenta alto teor de proteíco, baixo teores de carboidratos e fibras, alta digestibilidade, e bom padrão de

aminoácidos essenciais quando comparados a outras fontes de proteína vegetal (ALAN et al., 2005).

No entanto, de acordo com Samocha et al. (2004), a soja tem uma utilização comercial limitada devido a problemas potenciais associados com níveis insuficientes de aminoácidos essenciais como lisina e metionina. Além disso, a presença de determinados carboidratos afetam a sua palatabilidade, e fatores antinutricionais comprometem a sua digestibilidade. Porém, durante o processamento da soja muito desses fatores podem ser removidos com a aplicação de solvente (álcool aquoso) ou através de lixiviação isoelétrica, produzindo um produto com até 65% de proteína bruta (STOREBAKKEN et al.,2000). Tais procedimentos tornam seu emprego na carcinicultura promissor, uma vez que se tornam mais assimiláveis para o consumo dos camarões cultivados.

Os lipídeos são componentes orgânicos que incluem ácidos graxos, fosfolipídios, triglicérides e esteróis e funcionam como fonte de energia para o camarão que são adicionados na ração em forma de óleo de peixe e óleo de soja (SHIAU, 1998). No entanto, os camarões não toleram dietas com níveis de lipídeos maiores que 10% (CUZON e GUILAUME, 1997).

Os carboidratos incluem os açúcares, amido e fibras (SHIAU, 1998). Os organismos diferem na habilidade de usar os carboidratos como fonte de energia. Os camarões, com hábito onívoros e herbívoros, utilizam carboidratos eficientemente. Contudo, se o carboidrato presente no alimento estiver em baixa concentração, o camarão tem a capacidade de utilizar as proteínas como fonte de energia (ROSAS et al., 2002).

Vitaminas são componentes orgânicos necessários para o camarão em quantidades mínimas para a manutenção do metabolismo e crescimento (VELASCO, 2000). A deficiência de vitamina C está associada com a síndrome “Black Death”, que é caracterizada pela melanização do tecido subticular (SHIAU, 1998).

Os minerais, componentes inorgânicos, que normalmente estão incluídos na ração são: cálcio, magnésio, fósforo, sódio, potássio e cloro. O cálcio é necessário para a formação do exoesqueleto, músculo e para a osmorregulação. O camarão é capaz de absorver o cálcio diretamente da água e por isso, aqueles que são criados em água salgada, não necessitam de suplementação de cálcio na dieta (DAVIS e LAWRENCE, 1997). Por outro lado, o camarão cultivado em baixa salinidade retira os minerais necessários para a sua osmorregulação da água e alimentos, tendo então a ração um importante papel com fonte de minerais para o animal (VALENÇA e MENDES, 2003).

2.4 Sistemas de cultivo

Segundo Wasielesky et al. (2006a) a carcinicultura esteve fundamentada em três sistemas de cultivo: extensivo, semi-intensivo e intensivo. O sistema de produção extensivo é baseado na utilização de viveiros com grandes áreas (100 ha), baixa densidade de estocagem (2,5-5 camarões/m2), baixa renovação de água (0-5% renovação de água/dia), sem aeração artificial, pouca fertilização e produtividades médias em torno de 50-500 Kg/ha/ano alimentado exclusivamente pela biota natural dos viveiros.

Subsequentemente, a produção de camarões em sistema semi-intensivo incorporou novas estratégias de cultivo e melhores tecnologias, entre elas protocolos de fertilização, uso de bandejas de alimentação, aumento das densidades de estocagem (10-20 camarões/m2), uso parcial de aeração artificial - ao final do cultivo, aumento da taxa de renovação de água (5-20% renovação de água/dia) e produtividade variando de 500-5.000 Kg/ha/ano (CORREIA et al., 2002; NUNES, 2003; TACON et al., 2004; WASIELESKY et al., 2006a).

Com os avanços no manejo de cultivo a carcinicultura adotou densidades de cultivo mais elevadas e alta intensificação dos sistemas como o uso de aeração constantes, rações de alta qualidade proteica e preocupação com a qualidade da água (WURMANN e MADRID, 2006). Segundo Hopkins et al. (1993) estes sistemas utilizam elevadas taxas de troca d'água que variam de 5 a 30% do volume do viveiro por dia e, estima-se que para produzir 1kg de camarão seja necessário de 39 a 199 metros cúbicos de água, dependendo do nível de intensificação do sistema de cultivo. As águas de efluentes do sistema de produção intensiva de camarão são tipicamente caracterizadas por altas cargas de nitrogênio, fósforo, carbono orgânico, partículas de sólidos em suspensão (PÁEZOSUNA, 2001; PIEDRAHITA, 2003; COHEN et al., 2005). Entretanto, sua liberação direta no ambiente, sem tratamento prévio, representa uma perda de nutrientes valiosos, consequentemente, reduzindo a rentabilidade dos cultivos (SMITH et al., 2002). Além das questões econômicas, as frequentes perdas de agua podem gerar grandes impactos ambientais, ocasionando a eutrofização dos corpos de água que recebem estes efluentes.

Desta forma, a carcinicultura vem buscando práticas de produção que visam aumentar a biossegurança do cultivo, optando por sistemas de produção com limitada ou sem troca d'água (BROWDY et al., 2001; SAMOCHA et al., 2004; HANDY et al, 2004; BURFORD et al., 2004). Tais sistemas vem sendo classificados como sistemas superintensivos (SAMOCHA, 2009).

Os sistemas de cultivo superintensivos que adotam a tecnologia de bioflocos apresentam muitos benefícios sobre os sistemas tradicionais, já descritos. As principais

vantagens desses sistemas são a redução do uso da água e de efluentes e, consequentemente, redução de possíveis impactos ambientais. Destaca-se ainda o aumento da conversão alimentar e controle dos níveis dos compostos nitrogenados inorgânicos através da proteína microbiana produzida endogenicamente (BROWDY et al., 2001; WASIELESKY et al., 2006b; AZIM et al., 2008; AVNIMELECH, 2009). Além disso, reduz o risco de introdução e disseminação de enfermidades, promovendo os benefícios a dieta dos animais cultivados através da produtividade natural dos viveiros (McINTOSH, 2000; BURFORD et al., 2003a; WASIELESKY et al., 2006b).

O cultivo em regime de limitada ou zero troca d'água combinam o tratamento de água com a reciclagem de alimento artificial não consumido, a partir de uma biota predominantemente aeróbica e heterotrófica (AVNIMELECH et al., 1994; CHAMBERLAIN e HOPKINS, 1994; BURFORD et al., 2003a; AVNIMELECH, 2006; WASIELESKY et al., 2006b). Esse tipo de cultivo é conhecido como *Biofloc Technology* (BFT), *Activated Suspension Technique* (AST), *Active Suspension Pond* (ASP), *Zero exchange, aerobic, heterotrophic* (ZEAH), entre outros termos (McINTOSH, 1999; McNEIL, 2000; ERLER et al., 2005; WASIELESKY et al., 2006a; AVNIMELECH, 2007; DE SCHRYVER et al., 2008).

O cultivo em sistema heterotrófico é realizado mediante o desenvolvimento e o controle da densidade de flocos microbianos na coluna da água através da adição de fontes de carboidratos (AVNIMELECH, 2007; CRAB et al., 2009) e, tem como característica trabalhar em condições superintensivas. Nestas, os viveiros são revestidos com manta de polietileno de alta densidade, possuem pouca ou nenhuma renovação de água e altas taxas de aeração (> 25 CV/ha). As densidades de estocagens são elevadas muito embora as rações utilizadas com baixo teor protéico (< 30% de proteína bruta), elevadas densidades de estocagem de camarões (300 a 500 camarões/m2) (BOYD e CLAY, 2002; BURFORD et al., 2003a, 2004; SAMOCHA et al., 2007).

A comunidade heterotrófica desenvolve-se após 7 a 8 semanas, pela adição de uma fonte de carbono orgânico, e caracteriza-se por flocos compostos de células bacterianas embutidas em uma matriz de cálcio e silicato (AVNIMELECH, 1999; McINTOSH, 2000). O consumo destes flocos pelos camarões pode contribuir para nutrição e reciclagem dos nutrientes no ambiente de cultivo (McNEIL, 2000). O aporte de carbono nos sistemas heterotróficos pode ocorrer de fontes ricas em C orgânico (açúcares, amido, celulose, glucose, acetato, glicerol, etc) (HARI et al., 2006; DE SCHRYVER et al., 2008; AVNIMELECH, 2009) com destaque para o melaço provido da cana-de-açúcar, empregado como promotor de

crescimento bacteriano em viveiros de cultivo no Brasil e no mundo (WASIELESKY et al., 2006b).

As bactérias heterotróficas possuem a habilidade de sintetizar proteína do carbono orgânico e da amônia. Entretanto, é essencial que a relação C/N seja adequada para utilização das bactérias. Misturas balanceadas de Carbono:Nitrogênio são constantemente estudadas por diversos autores que buscam a proporção ideal para o sistema de cultivo (SCHNEIDER et al.,2006; WASIELESKY et al., 2006b; FONTENOT et al., 2007). Entretanto, a relação mais aceita é a proporção 20:1, na qual o carbono será digerido mais facilmente pelas bactérias (CHAMBERLAIN et al., 2001b).

A correta manutenção da relação Carbono:Nitrogênio no desenvolvimento das bactérias heterotróficas em cultivos intensivos e semi-intensivos, resulta na conversão de compostos nitrogenados inorgânicos em células microbianas ricas em proteína (ASADUZZAMAN et al., 2008). O nitrogênio inorgânico é imobilizado em células bacterianas quando os substratos orgânicos têm uma alta relação C/N (AZIM et al., 2008; CHAMBERLAIN et al., 2001a), e com isso são utilizados para sintetizar proteínas bacterianas em novas células que podem ser utilizadas como fonte de alimento por peixes e camarões (AVNIMELECH, 1999; McGRAW, 2002; HARI et al., 2004; AZIM et al., 2008). Segundo Burford et al. (2003b) os microrganismos melhoram a qualidade da água e, na forma de partículas floculadas ou "flocos", fornecem uma fonte suplementar de alimento para os camarões. Os flocos bacterianos são formados durante o ciclo de produção e são constituídos principalmente de bactérias, microalgas, fezes, exoesqueletos, restos de organismos mortos, cianobactérias, protozoários, pequenos metazoários e formas larvais de invertebrados, entre outros (DECAMP et al., 2002; BURFORD et al., 2003a; WASIELESKY et al., 2006b; RAY et al., 2009).

Normalmente, os flocos são compostos de 45% de proteína, que é quase o dobro do nível de proteína dos alimentos das rações utilizadas em viveiros de camarões (McINTOSH, 2000). Do ponto de vista nutricional, Azim e Little (2008) concluíram que o biofloco contém 38% de proteína, 3% de lipídios, 6% de fibra, 12% de cinzas e 19 kJ/g de energia bruta. A presença do floco microbiano rico em proteína, minerais, lipídios e vitaminas, pode reduzir substancialmente o requerimento de proteína nas rações (CHAMBERLAIN et al., 2001b), podendo ocasionar uma diminuição nos custos com alimentação, o que representa mais de 50% das despesas totais de produção (TAN e DOMINY, 1997; MARTINEZ-CORDOVA et al., 2003; SHIAU e BAI, 2009).

3. OBJETIVOS

3.1 Geral

Avaliar o desempenho zootécnico e a fisiologia digestória do *Litopenaeus vannamei* submetidos a cultivos heterotróficos e autotróficos.

3.2 Específicos

- Analisar o efeito da substituição da farinha de peixe por concentrado protéico de soja (SPC) sobre o desempenho das enzimas digestivas do *L. vannamei*.
- Analisar os efeitos de dietas com diferentes níveis protéicos de proteína de peixes sobre o desempenho zootécnico e das atividades de proteases digestórias do *L. vannamei.*
- Avaliar a influência dos cultivos autotróficos e heterotrófico sobre a as enzimas digestórias e sobre a histologia do hepatopâncreas do *L. vannamei*.

CAPÍTULO 1

Influence of biofloc and autotrophic systems on the digestive protease activity and histomorphology of the hepatopancreas from marine shrimp *Litopenaeus vannamei* (Boone, 1931)

A ser submetido ao periódico Aquaculture

Influence of biofloc and autotrophic systems on the digestive protease activity and histomorphology of the hepatopancreas from marine shrimp *Litopenaeus vannamei* (Boone, 1931)

Janilson Felix da SILVA[1], Gabriele Rodrigues de LARA[2], Danielle da Silva ALANIZ[2], Arthur Tenório Ribeiro CLARK[3], Luis Alberto ROMANO[2], Wilson Francisco Britto WASIELESKY JUNIOR[2], Eduardo Isidoro Carneiro BELTRÃO[3], Karina RIBEIRO[4], Ranilson de Souza BEZERRA[1,3]*

[1]Enzimologia (LABENZ), Departamento de Bioquímica e Laboratório de Imunopatologia Keizo Asami (LIKA), Universidade Federal de Pernambuco, Cidade Universitária, 50670-420, Recife-PE, Brazil.

[2]Instituto de oceanografia, Universidade Federal do Rio Grande, Cassino, 96210-030, Rio Grande-RS, Brazil.

[3]Setor de Patologia do Laboratório de Imunpatologia Keizo Asami (LIKA), Universidade Federal de Pernambuco, Cidade Universitária, 50670-420, Recife-PE, Brazil.

[4]Unidade Acadêmica Especializada em Ciências Agrárias – EAJ/UFRN, Macaíba, RN, Brazil.

Corresponding author:

Ranilson S. Bezerra

Laboratório de Enzimologia (LABENZ), Departamento de Bioquímica e Laboratório de Imunopatologia Keizo Asami (LIKA), Universidade Federal de Pernambuco, Cidade Universitária, 50670-420, Recife-PE, Brazil.

Tel, +55 81 21268540; Fax, +55 81 21268576

email: ransoube@uol.com.br

ABSTRACT

The effects of the autotrophic and bioflocs systems on the activities of digestive proteases and hepatopancreatic tissue of *Litopenaeus vannamei* (adults) were studied after 20 days. Three experimental units (60 individuals/tank) were adopted, one autotrophic system with application feed (T1) and two bioflocos systems, one with added of feed (T2) and the other without feed (T3). The feed consisted of a commercial diet with 35% crude protein (CP). At the end of culture, the final weight of shrimp, hepatosomatic index (HSI) and hepatopacreatic tissue (H&E) were analyzed. The activities of digestive proteases from the hepatopancreas were assessed using Azocasein (protease total), BApNA (trypsin), SApNA (quimitripsina), Leu-p-Nan (leucine aminopeptidase) as substrates. The enzymes were also analyzed by zymogram. The data showed that the animals presented higher final weight T2 followed by T1 and T3, respectively. The SHI obtained from the hepatopancreas collected in animals from T2 presented the highest percentage followed by T1 and T3, respectively. In T1, the hepatopancreatic ducts showed R, F and B cells. In T2 and T3 were found B and F cells. Analyzes for the identification of total proteases activities showed greater activity in T1 compared to T2 and T3. Studies using BApNA substrate showed specific activity with greater intensity in the T2 and T3. The use of SApNA showed higher enzyme activity in T2. However, the T3 presented higher specific activity response to leucine aminopeptidase. Through zymogram three bands with high enzyme activity were observed at T1, while the T2 and T3, six bands were identified with high intensity. These results showed that the bioflocs systems exerted influence on the digestive proteases of *Litopenaeus vannamei*, increasing their activities, when compared with animals cultured in autotrophic system.

Keywords: biofloc, histology, *Litopenaeus vannamei*, protease

1. Introduction

Pacific white shrimp, *Litopenaeus vannamei*, native to the western coast of the Mexico, is most widely cultured penaeid shrimps in many parts of the world. Because of its rusticity in culture, this tropical species had been widely cultured in extensive, semi-intensive e intensive systems. However, in last decade, because of the environmental impact of marine shrimp farms, associated with the incidence of diseases, has led to the development of production systems with little or no water exchange (Baloi et al., 2013).

The application of biofloc technology (BFT), denomination proposed by Avnimelech (2007), in zero-exchange shrimp culture systems has gained popularity because it offers a practical solution to maintain water quality and recycle feed nutrients simultaneously (Xu & Pan, 2013). In such system, dense microbial communities are managed to control ammonia released mainly by the cultivated organisms. Ammonia can be absorbed by microalgae or heterotrophic bacteria, or be transformed by nitrifying bacteria (Ebeling et al., 2006). These organisms usually grow in the form of microbial flocs (bioflocs) that can be used as feed for the cultivated species (Schryver et al., 2008). In addition to algae and bacteria, the bioflocs are composed of aggregates of organic matter, protozoa, rotifers, and nematodes (Avnimelech, 2009). These organisms can be consumed as a supplemental food source by the shrimp, creating a nutrient recycling process within the systems and subsequently increasing feed utilization efficiency (Burford et al., 2003; Schneider et al., 2006; Hargreaves, 2006; Wasielesky et al., 2006).

Over the past decade, the production of *L. vannamei* based in the application of BFT had become popular and achieved sustainable. Several studies noted that the bioflocs could improve growth performance of cultured shrimp (Tacon & Cody, 2002; Wasielesky et al. 2006; Xu & Pan 2012a; Xun & Pan 2012b; Xun & Pan, 2013; Baloi et al., 2013; Luo et al.,

2013; Schveitzer et al., 2013). However, the influence of this system on the physiology of farmed shrimp is still poorly understood, especially the effects on the digestive process.

Thus, this study was conducted to evaluate the effects of the biofloc and autotrophic systems on the digestive protease activity and histomorphology of the hepatopancreas from *L. vannamei*.

2. Materials and Methods

2.1 Experimental culture

The culture were performed from October 28 until November 17, 2011 in the Estação Marinha de Aquacultura (EMA), Instituto de Oceanografia, Universidade Federal do Rio Grande-FURG (Rio Grande, Brazil). Three units were developed in experimental tanks with a capacity of 1000L under controlled parameters (pH around 8.0; salinity 30‰, photoperiod 12L:12D, feeding once a day at a rate of 8% of the biomass calculated at the beginning of culture). Three experimental systems were adopted: one autotrophic system with application of feed (T1) and two bioflocs systems one with (T2) and the other without the addition of feed (T3). The organic fertilization of the last two treatments was based on the methods proposed by Avnimelech (1999) and Ebeling et al. (2006). The group T1 was stocked with salt water and it occurred to exchange 80% of its volume every 48 hours. In all treatments there was a regime of strong aeration. *L. vannamei* specimens with initial weight of 10.34 g (± 1.69) were acclimated and then randomly stocked at a density of 43 shrimp/m2 (60 individuals/tank) in culture for a period of twenty days. The feed consisted of a commercial feed (Guabi ®, Campinas, São Paulo, Brazil) with 35% crude protein (CP). After the cultivation experiment, 10 animals per tank were randomly collected. Then were weighted and dissected to remove the hepatopancreas. Subsequently were placed in cryogenic tubes and frozen at -80 °C for

three days and then were placed in refrigerated cooler and shipped to the Laboratório de Enzimologia, Universidade Federal de Pernambuco (UFPE), for subsequent enzyme assays.

2.2 Histology of the hepatopancreas and hepassomatic index

For the histological description, five hepatopancreas were collected each treatment for 24 hours and fixed in Davidson's solution (ethanol, formaldehyde, acetic acid and water) (Bell and Lightner, 1988). Afterwards, the material passed by dehydrated process in increasing ethanol solution, and finally parafinizado. 4mm sections were stained with hematoxylin-eosin. The tissues were stained with hematoxylin and evaluated in light optical microscope Olympus (Olympus, Tokyo, Japan). The contents hepato (HSI) values were determined by the following equation: HSI = (HW / SW) X 100, where HW and SW refer to the weight and shrimp hepatopancreas respectively.

2.3. Crude extract

Ten hepatopancreas of each tank were homogenized in buffer (0.01 M Tris-HCl, pH 8, 0, with the addition of 0.15 M NaCl) at a concentration of 5 ml for each 1g of viscera (w / v). Then, the homogenate was centrifuged at 10,000 g for 25 min at 4 ° C to remove tissue fragments. The supernatants obtained (crude extracts) were collected and stored at -25 ° C for later analysis. The measurement of total soluble protein in crude extracts was determined as described by Bradford (1976) using bovine serum albumin as the standard protein.

2.4 Enzyme activity

2.4.1. Total proteolytic activity

The total enzymatic activity of proteases present in crude extracts was performed using 1% azocasein as substrate, prepared in 10 mM Tris-HCl, pH 8 0. Aliquots containing 30

μL of the crude extract were incubated with 50 μL of substrate solution for 1 hour at 25 °C. Then it was added 240 μL of 10% trichloroacetic acid to stop the reaction. After 15 minutes the mixture was centrifuged at 8,000 xg for 5 minutes. The supernatant was collected and 70 μL of it was mixed in 130 μL 1M sodium hydroxide solution (revealing solution) in microplates. The absorbance was measured on a microplate reader (Bio-Rad 680) at a wavelength of 450 nm. A negative control (blank) was performed, replacing the enzyme extract by a solution of 10 mM Tris-HCl, pH 8.0 with added 0.15 M NaCl. The activities were carried out in triplicate and one unit (U) of enzyme activity was defined as the amount of enzyme required to hydrolyze azocasein and produce a change of 0.001 units of absorbance per minute.

2.4.2. Specific proteolytic activities

The enzymatic activities of trypsin, chymotrypsin and leucine aminopeptidase, were determined in microplates with the use of Nα-benzoyl-DL-arginine-p-nitroanilide (BApNA), succinyl phenylalanine proline alanine aminotransferase pnitroanilide (SApNA) and pnitroanilide-leucine (Leu-p-Nan) as specific substrates, respectively (Bezerra et al., 2005). These substrates were dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 8 mM. All assays were performed in triplicate. The enzyme extracts (30 μL) were incubated with 140 μL of buffer Tris-HCl 0.1 M, pH 8.0, and 30 μL of the substrate for a period of 15 minutes. Soon after, the absorbance readings were measured and recorded by using a microplate reader (Bio-Rad 680). The wavelength used in the measurements was 405 nm. One unit (U) of activity was defined as the amount of enzyme required to produce one mole of p-nitroaniline per minute. The specific activity was expressed as units per milligram of protein.

2.5 Zymogram

Proteolitic zymogram was initiated by electrophoresis (SDS-PAGE) under immersion in an ice bath. Separation gel was used at 12.5% (w/v) and concentration gel at 4% (w / v). Enzyme preparations of each treatment (30 μg of protein) were applied to the concentration gel. After electrophoresis, the gels were immersed in 100 mL of Triton X-100 2.5%, diluted in Tris-HCl 0.1 M, pH 8.0, for 30min at 4 ° C to remove the SDS. Then Triton X-100 was removed by washing the gel with Tris-HCl 0.1 M, pH 8.0. Afterward, the gel was incubated in 100 mL of casein 3% (w / v) diluted in Tris-HCl 0.1 M, pH 8.0, for 30 minutes at 4° C to determine the proteolytic activity. After that, the gel was kept in the same casein solution at 25° C for 90 minutes to allow the digestion of casein by active fractions. Finally, the gel was stained with a solution containing Coomassie Brilliant Blue 0.01%, 25% methanol, 10% acetic acid and after 24 hours it was bleached in a solution with the same composition but without the presence of the dye.

2.6 Statistical analysis

The data were analyzed using one-way analysis of variance (ANOVA) complemented with Tukey's test. Differences were reported as statistically significant when $P < 0.05$, using the program MicrocalTM OriginTM version 8.0 (Software, Inc, U.S.).

3. Results

During twenty days of culture, there was no mortality. The water quality parameters such as temperature, dissolved oxygen, nitrogen and salinity did not show large variations favoring the cultured environment.

The animals collected show similar size among all treatments, with a mean of 12 ± 1cm. However the data presented in Table 1 showed that animals from the biofloc system with additional feed (T2) presented higher weight (12.29 ± 1.90 g), but were significantly

similar to the group T1, represented by autotrophic system (11.38 g ± 1.38). The shrimps originating from the system biofloc without addition of feed (T3) had lower weight (10.48 ± 1.38) and was significantly different (P <0.05) from the other groups.

The relative weight of the hepatopancreas in the different treatments was calculated as percentages of the total body weight of the animals by determining the hepatosomatic index (HSI). The HSI obtained from the hepatopancreas in animals collected from the T2 had a higher percentage (4.69%) followed by T1 (3.86%) and T3 (2.49%), respectively (Table 1).

Histological analysis in hepatopancreas in shrimp subjected to different treatments showed a gland containing ducts formed by different cell types. In treatment T1, ducts in star shaped lumen can be commonly found. The cells that make up this duct cells are mostly R and F. It was also observed in this organ, the presence of B cells. Its tubules showed a great amount of B-cells and the presence of F cells can be commonly observed. The hepatopancreatic tissue from animals submitted to treatment T3 showed ducts in rounded shape and subtly star shaped. The walls of these ducts are mostly formed by B cells and F cells are also found (Figure 1). Tubules of the hepatopancreas of animals stemmed T2 and T3 showed digestive fluids inside them.

The analysis for the identification of total proteolytic activity showed statistical difference ($P < 0,05$) from T1 compared to T2 and T3 (Figure 1A). The actions of trypsin, chymotrypsin and leucine aminopeptidase were identified in the different treatment in which it was observed that the specific activities varied between them. Thus, the studies with the substrate BApNA showed higher specific activity in the T2 and T3, observing a statistical difference when compared to T1 (Figure 1B). The use of SApNA showed higher enzyme activity (P <0.05) in T2 compared to T1 and T3 (Figure 1C). However, the T3 had higher specific activity for leucine aminopeptidase compared to T1 and T2 (Figure 1D).

Through the analysis of the zymograms was found active forms of enzymes in T1, T2 and T3. Such activities demonstrated the presence of proteolytic bands in different numbers in different treatments. Relative to T1, three bands were observed with high enzymatic activity and the presence of a band of low intensity. In the groups T2 and T3, six bands were observed with high intensity and thus high activity was observed in these treatments.

3. Discussion

Over the years, shrimp farming has been developed with the aim of enhancing the systems and the production techniques. However, this expansion did not minimize the environmental impacts generated by traditional autotrophic systems (Samocha et al., 2007).

Bioflocs systems have been developed and discriminated as a biotechnological alternative for raising the production. These systems have several characteristics such as, for example, little or no play of water (Boyd and Clay 2002; Samocha et al. 2007). Thus, the water quality is one of the limiting factors to the success of the production.

During culture the water quality parameters remained under appropriate conditions in all treatments, indicating favorable conditions for culture of the animals, which strengthen the results of 100% survival. As seen in heterotrophic system, the results obtained from the survival in autotrophic system showed the proper maintenance of nitrogen compounds during the cultivation time. However, the large volume of water used in this system is disadvantageous for the intensive culture system due to the production of effluents with high loads nitrogen, phosphorus and suspended solids, creating serious environmental problems (Cohen et al. 2005) This is fact minimized in the bioflocs systems (Samocha et al., 2007).

The performances of the growth and health of shrimps depend on the quantity and quality of food ingested and assimilated (Becerra-Dorrame et al., 2012). The food used in the cultivation of aquatic organisms is one of the most important factors of culture. Balanced diet

is based on animal protein that represents the most expensive ingredient of the diet, about 60% of the total cost of production (Shiau, 1998; Lemos, 2003). Autotrophic systems conventionally use supplementary diets with higher protein concentrations during cultivation. This fact causes high cost in the production. Bioflocs systems, in turn, may reduce the costs of feed through of the intake the microorganisms present in the flocs by the shrimps. These micoorganismos may contribute to the nutrition of animals, in addition to recycle nutrients from the growing environment (McNeil, 2000).

The results obtained at the end of the experiment showed that the animals had a similar rate of growth were not statistically different (P <0.05) between treatments. However, it is observed that animals from T2 had a higher weight gain followed by T1 and T3. These results show that although the bioflocs systems assists in food supplementation in the culture environment, the use of feed is an important factor in intensive systems, since the animals submitted to T3 showed a weight gain insignificant relative to their initial weight. Authors demonstrated that although organic solids present in bifloc system, be highly efficient for the cultivation of marine shrimp, makes necessary the use of diets with low protein supplementing the diet of shrimp. Thus, the cultivation becomes effective with the zootechnical parameters from high growth rates (Decamp & Tacon, 2002; Moss, 2002; Becerra-Dorame et al., 2012). The hepatopancreas is one belonging to the midgut which has the function of metabolizing energy reserve of product (Garcia et al., 2002). This reserve is used during processes of growth and gonadal maturation (Cavalli et al., 2001; Millamena and Pascual, 1990). Thus, the relative weight of the hepatopancreas represents the assimilation of food and mobilized energy expended probably during somatic growth of the animal (Dahll and Moriaty, 1983; Skinner, 1985; Sage and Ra'anan, 1988). The HSI found among treatments showed that treatment T2 had the highest SHI compared to the other treatments, suggesting that the hepatopancreas is accumulating nutrient reserve mobilization according to

the demand of the animal's metabolism, thus advocated for crustaceans by Dahll and Moriaty (1983). It was thus observed that treatment T2 favored in animal weight gain and maintenance of energy reserves, indicating that this culture system will facilitate nutritionally the quality of life of the animal.

Digestion in penaeid shrimps is facilitated by the hepatopancreas which has a key role in the secretion of digestive enzymes, digestion, nutrient uptake, storage reservation and release of non-assimilable (Al-mohana et al, 1985; Vogt, 1993). This bilobate organ consists of numerous blind-ending tubules (Gibson and Baker, 1979). These tubules are structured by tubular epithelial cell comprised of four different cell types, E cells, F cells, B cells and R cells (Jacob, 1928). However, F cells were found in the hepatopancreas from animals of the three experimental treatments. These cells are involved in the secretion of digestive enzymes e and present vesicles in the apical cytoplasm. Studies with *Penaeus semisulcatus* suggest that such vesicles are zymogen precursor (Al-Mohana et al, 1985). The accumulation of these vesicles promotes a morphological change in the cell F and become B cell (Al-Mohana and Nott, 1989). This cell type was also observed in the hepatopancreas of animals from different treatments and is involved in the absorption of nutrients stemming from hepatopancreatic tubule (Franceschin-Vicentine et al., 2009). They are also responsible for intracellular digestion, concentrating and absorbing materials in a large vacuole that compresses the nucleus to the basal region of the cell (Sousa and Petriella, 2000). Thus, a large number of B cells present in the hepatopancreas tubule, may show the influence of the treatment on the nutrient profile and production of digestive enzymes of animals. Thus, one can justify the greatest evidence of B cells present in the hepatopancreas T2 which in turn has a higher rate of SHI, suggesting that this treatment has a positive answer for nutrition animal. Thus, a large number of B cells present in the hepatopancreas tubule, may show the influence of the treatment on the nutrient profile and production of digestive enzymes of animals. Another cell

type, cell R, was observed in hepatopancreatic tubules animals undergoing treatment T1. R cells are responsible for storing glycogen and lipids. These reserves can be mobilized to produce energy during periods of starvation, moulting and breeding (Sousa and Petriella, 2000; Al-Mohana and Nott, 1989).

During digestion, the fluids secreted by the digestive enzymes remain in the lumen of the tubules (Al-Mohana and Nott, 1989). Thus, shrimp fed presented digestive fluid and fine particles emanating from the gastric mill, a fact, seen most evident in the hepatopancreatic structures of animals of the treatments T2 and T3. However, studies that may elucidate the changes in the size of the tubules of the hepatopancreas in response to food are scarce.

Nutritional conditions of organisms cultivation is not only a consequence of the consumption of diet during the culture, but also of their digestive physiology (Becerra-Dorame, 2012). The digestive response is influenced by many intrinsic and extrinsic factors such as food and metamorphic changes, respectively (Zhou et al, 2009). Several studies have demonstrated the importance of digestive enzymes such as proteases in the digestion process crustaceans (Córdova-Murueta et al., 2003; López-López, 2005; Gaxiola et al., 2005; Buarque et al., 2009, 2010). It was observed that the enzymatic activities for total protease and trypsin were significantly higher for treatments linked to bioflocs systems. Regarding chymotrypsin and leucinaaminopeptidase highest activities occurred for animals belonging to biofloc system with feed and without feed, respectively. Although the activities for chymotrypsin and leucine aminopeptidase has not been differentiated from findings in animals stemmed autotrophic system, the enzymatic activities in general were positively influenced by biflocs systems. Studies suggest that the enzyme activity may be related not only with the increase of protein in the diet of animals cultured, but also due to the presence of heterotrophic bacteria (Ong and Johnston, 2006; Becerra-Dórame et al., 2012). This information is reinforced by the fact that studies describe that the presence of bacteria with their respective exogenous enzymes may

stimulate the production of endogenous enzymes by shrimp (Zhou et al, 2009; Ziaei-Nejade et al., 2006; Kurokawa et al., 1998). Thus, in this study the increase in protease activity and trypsin in T2 and T3 could be related to the presence of heterotrophic bacteria.

The zymogram technique is used as an important tool for the identification of active enzyme forms, thus allowing analysis of digestive enzymes. In the present study, the proteolytic zymograms showed bands caseinolityc in extracts obtained from animals in all treatments. However, the bands are more evident and active in greater number in samples obtained from animals stemmed from bioflocs systems. The data can be linked with the activities of the proteases studied in vitro, which also suffered influences of the systems. This corroborates the information already highlighted and described previously that proteins present in the diet and the presence of heterotrophic bacteria can positively influence the enzymatic activity of proteases.

4. Conclusion

The current study showed that the bioflocs systems exerted influence on the digestive proteases of *Litopenaeus vannamei*, increasing their activities, when compared with animals cultured in autotrophic system. This response may reflect the ability of these animals to adapt to different environments. Furthermore, the results suggest that the bioflocs system supported with external feed is the optimal management to improve the zootechnical parameters of shrimp cultured in super-intensive systems.

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References

Al-Mohanna, S. Y., Nott, J. A., & Lane, D. J. W. (1985). M-'midget'cells in the hepatopancreas of the shrimp, Penaeus semisulcatus De Haan, 1844 (Decapoda, Natantia). *Crustaceana*, 260-268.

Al-Mohanna, S.Y. e Nott, J.D. Function citology of the hepatopancreas of *Penaeus*

Avnimelech, Y., 2007. Feeding with microbial flocs by tilapia in minimal discharge bio-flocs technology ponds. Aquaculture 264 (1–4), 140–147.

AVNIMELECH, Y. 2009. Biofloc Technology - A Practical Guide Book. Baton Rouge, Louisiana, United States. The World Aquaculture Society. 181p.

Baloi, M., Arantes, R., Schveitzer, R., Magnotti, C., & Vinatea, L. 2012. Performance of Pacific white shrimp *Litopenaeus vannamei* raised in biofloc systems with varying levels of light exposure. Aquacult. Eng.

Becerra-Dórame, M. J., Martínez-Porchas, M., Martínez-Córdova, L. R., Rivas-Vega, M. E., Lopez-Elias, J. A., & Porchas-Cornejo, M. A. (2012). Production Response and Digestive Enzymatic Activity of the Pacific White Shrimp *Litopenaeus vannamei* (Boone, 1931) Intensively Pregrown in Microbial Heterotrophic and Autotrophic-Based Systems. *The Scientific World Journal*, *2012*.

Bell, T.A., Lightner, D.V., 1988. A handbook of normal shrimp histology. Special Publication No. 1. World Aquaculture Society, Baton Rouge, LA, 114 pp.

Boyd, C. E., and J. W. Clay. 2002. *Evaluation of Belize aquaculture, Ltd: a superintensive shrimp aquaculture system in Belize.* World BankB/NACA/WWF/FAO Consortium Program on Shrimp Farming and the Environment.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein binding. Anal. Biochem. 72, 248-254.

Buarque, D. S., Castro, P. F., Santos, F. M. S., Amaral, I. P. G., Oliveira, S. M., Alves, K. B., ... & Bezerra, R. S. (2010). Digestive proteinases and peptidases in the hepatopancreas of the southern brown shrimp (*Farfantepenaeus subtilis*) in two sub-adult stages. *Aquaculture Nutrition*, *16*(4), 359-369.

Buarque, D. S., Castro, P. F., Santos, F. M. S., Lemos, D., Júnior, L. B. C., & Bezerra, R. S. (2009). Digestive peptidases and proteinases in the midgut gland of the pink shrimp *Farfantepenaeus paulensis* (Crustacea, Decapoda, Penaeidae). *Aquaculture Research*, *40*(7), 861-870.

Burford, M. A., Thompson, P. J., McIntosh, R. P., Bauman, R. H., & Pearson, D. C. (2003). Nutrient and microbial dynamics in high-intensity, zero-exchange shrimp ponds in Belize. *Aquaculture*, *219*(1), 393-411.

CAVALLI, R. O. ; LAVENS, P. ; SORGELOOS, P. . Reproductive performance of *Macrobrachium rosenbergii* females in captivity. Journal of the World Aquaculture Society[JCR], Baton Rouge, EUA, v. 32, n.1, p. 60-67, 2001.

Cohen, J., Samocha, T.M., Fox, J.M., Lawrence, A.L., 2005. Biosecured production of juvenile Pacific white shrimp in an intensive raceway system with limited water discharge. Aquacult. Eng. 32 (3–4), 425–442.

Córdova-Murueta, J. H., García-Carreño, F. L., & Navarrete-del-Toro, M. D. L. A. (2003). Digestive enzymes present in crustacean feces as a tool for biochemical, physiological, and ecological studies. *Journal of experimental marine biology and ecology*, *297*(1), 43-56.

DAHLL, W. & MORIATY, D.J.W. Functional aspects of nutrition and digestion: The midgut. In: BLISS, D.E.; MANTEL L.H. (eds). The biology of Crustacea, Vol. 5: International anatomy and physiological regulation. Academic Press, p. 215-251. 1983.

Ebeling J.M., Timmons M.B. & Bisogni J.J. (2006) Engineering analysis of the stoichiometry of photoautotrophic, autotrophic, and heterotrophic removal of ammonia-nitrogen in aquaculture systems. Aquaculture 257, 346–358.

Franceschini-Vicentini, I. B., Ribeiro, K., Papa, L. P., Marques Junior, J., Vicentini, C. A., & Valenti, P. M. C. M. (2009). Histoarquitectura del Hepatopáncreas del Camarón de la Amazonia *Macrobrachium amazonicum*. *International Journal of Morphology*, *27*(1), 121-128.

Gaxiola, G., Cuzon, G., García, T., Taboada, G., Brito, R., Chimal, M. E., ... & Van Wormhoudt, A. (2005). Factorial effects of salinity, dietary carbohydrate and moult cycle on digestive carbohydrases and hexokinases in *Litopenaeus vannamei* (Boone, 1931). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *140*(1), 29-39.

Hargreaves, J.A., 2006. Photosynthetic suspended-growth systems in aquaculture.Aquacultural Engineering 34, 344–363.

Jacobs, W. (1928) Untersuchungen über die Cytologie der Seretbildung in der mitteldarmdrüse von *Astacus leptodactylus*. In: Icely, J.D. e Nott, J.A. Digestion and Absorption: digestive system and associated organs. *Microscopic Anatomy of Invertebrates Volume 10: Decapod Crustacea*, p. 147-201, 1992.

Lemos, D., Rodríguez, A., 1998. Nutritional effects on body composition, energy content and trypsin activity of *Penaeus japonicus* during early postlarval development. Aquaculture 160, 103 - 116.

Lopez-Lopez, S., Nolasco, H., Villarreal-Colmenares, H., & Civera-Cerecedo, R. (2005). Digestive enzyme response to supplemental ingredients in practical diets for juvenile freshwater crayfish *Cherax quadricarinatus*. *Aquaculture nutrition*, *11*(2), 79-85.

Luo, G. Z., Avnimelech, Y., Pan, Y. F., & Tan, H. X. (2012). Inorganic nitrogen dynamics in sequencing batch reactors using biofloc technology to treat aquaculture sludge. *Aquacultural Engineering*.

McNeil, R., 2000. Zero exchange, aerobic, heterotrophic systems: key considerations.

Millamena, O. M., & Pascual, F. P. (1990). Tissue lipid content and fatty acid composition of Penaeus monodon Fabricius broodstock from the wild. *Journal of the world Aquaculture society*, *21*(2), 116-121.

Moss, S.M., 2002. Dietary importance of microbes and detritus in Penaeid shrimpaquaculture. In: Lee, C.S., O'Bryen, P. (Eds.), Microbial Approaches to Aquatic Nutrition Within Environmentally Sound Aquaculture Production Systems.World Aquaculture Society, Baton Rouge, USA, pp. 1–18.

Sagi, A., Ra'anan, Z. Morphotypic differentiation of males of the freshwater prawn *Macrobrachium rosenbergii*: changes in the midgut glands and the reproduction system. Journal of Crustacean Biology. v.8, n.1, p.43-47, 1988.

Samocha, T. M., Patnaik, S., Speed, M., Ali, A. M., Burger, J. M., Almeida, R. V., ... & Brock, D. L. (2007). Use of molasses as carbon source in limited discharge nursery and grow-out systems for *Litopenaeus vannamei*. *Aquacultural Engineering*, *36*(2), 184-191.

Schneider, O., Sereti, V., Machiels, M.A.M., Eding, E.H., Verreth, J.A.J., 2006. Thepotential of producing heterotrophic bacteria biomass on aquaculture waste.Water Research 40 (14), 2684–2694.

Schryver, P.D., Crab, R., Defoirdt, T., Boon, N., Verstraete, W., 2008. The basics of bio-flocs technology: the added value for aquaculture. Microbial Ecology 277,125–137.

Schveitzer, R., et al., Effect of different biofloc levels on microbial activity, water quality and performance of *Litopenaeus vannamei* in a tank system operated with no water exchange. Aquacult. Eng. (2013), http://dx.doi.org/10.1016/j.aquaeng.2013.04.006

Shiau, S.Y. Nutrient requirements of penaeid shrimps. *Aquaculture*, v. 168, p. 77-93, 1998.

SKINNER, D.M. Molting and regeneration. In: BLISS, D.E.; MANTEL L.H. (eds). The biology of Crustacea, Vol. 5: International anatomy and physiological regulation. Academic Press, p. 44-128, 1985.

Souza, L.G. e Petriella, A.M. Histology of the hepatopancreas of the freshwater prawn *Palaemonetes argentinus* (crustacean, caridea). *Biocell*, 24(3):189-195. 2000.

Wasielesky Jr., W., Atwood, H., Stokes, A., Browdy, C.L., 2006. Effect of naturalproduction in a zero exchange suspended microbial floc based super-intensiveculture system for white shrimp *Litopenaeus vannamei*. Aquaculture 258,396–403.

Xu W.J., Pan L.Q., Sun X.H. & Huang J. (2012a) Effects of bioflocs on water quality, and survival, growth and digestive enzyme activities of *Litopenaeus vannamei* (Boone) in zero-water exchange culture tanks. Aquaculture Research. doi:10.1111/j.1365-2109.2012.03115.x.

Xu W.J., Pan L.Q., Zhao D.H. & Huang J. (2012b) Preliminary investigation into the contribution of bioflocs on protein nutrition of *Litopenaeus vannamei* fed with different dietary protein levels in zero-water exchange culture tanks. Aquaculture. doi:doi:10.1016/j.aquaculture.2012.04.003.

Xu, W. J., & Pan, L. Q. (2013). Dietary protein level and C/N ratio manipulation in zero-exchange culture of *Litopenaeus vannamei*: Evaluation of inorganic nitrogen control, biofloc composition and shrimp performance. Aquac. Res.

Zhou, X. X., Wang, Y. B. and Li, W. F. 2009. "Effect of probiotic on larvae shrimp (*Penaeus vannamei*) based on water quality, survival rate and digestive enzyme activities," *Aquaculture*, vol. 287, no. 3-4, pp. 349–353.

Ziaei-Nejad, S., Rezaei, M. H., Takami, G. A., Lovett, D. L., Mirvaghefi, A. R., & Shakouri, M. (2006). The effect of *Bacillus* spp. bacteria used as probiotics on digestive enzyme

activity, survival and growth in the Indian white shrimp *Fenneropenaeus indicus*. *Aquaculture*, *252*(2), 516-524.

Figure caption

Figure 1. Transversal sections hepatopancreatic tissues (H&E) of *Litopenaeus vannamei* adult submitted to autotrophic and bioflocs systems. A (100x) e B (400x) represent the autotrophic system with application of feed (T1); C (100x) e D (400x), bioflocs systems with additional feed (T2); E (100x) e F (400x), bioflocs systems without the addition of feed (T3).

Figure 2. Enzymatic activity of the hepatopancreas of *Litopenaeus vannamei* cultured in autotrophic and bioflocs systems. T1, T2 and T3 represent the autotrophic system, bioflocs system with additional feed and bioflocs system without additional feed, respectively. A - Total proteolytic activity using azocasein 1%, B - Activity trypsin using 8 mM BApNA; C - chymotrypsin activity using 8 mM SApNA, D - leucine aminopeptidase activity using 8 mM Leu-p-Nan. The means ± standard deviations were obtained from in vitro assays in triplicate. Different superscript letters indicate statistical differences in the activity of proteases between treatments (Tukey, $P < 0.05$).

Figure 3. Proteolytic zymogram of crude extracts of the hepatopancreas of L. vannamei cultured in cultured in autotrophic and bioflocs systems. T1, T2 and T3 represent the autotrophic system, bioflocs system with additional feed and bioflocs system without additional feed, respectively.

Table 1. Initial weight, final weight, survival, length parameters and hepatossomatic index (HIS) of *Litopenaeus vannamei* submitted to autotrophic system with application of feed (T1), bioflocs systems with additional feed (T2) and bioflocs systems without the addition of feed (T3).

Groups	Initial weight (g)	Final weight (g)	Survival (%)	Length (cm)	HSI (%)
T1	10.34±1.69[a]	11.38±1.38[a]	100	12.0±1.0	3.86[a]
T2	10.30±1.63[a]	12.29±1.90[a]	100	12.0±1.0	4.69[a]
T3	10.31±1.67[b]	10.14±1.48[b]	100	12.0±1.0	2.49[b]

Notes: Values are mean ± S.E. (N = 40). Means with different superscripts represent significantly different (P <0.05).

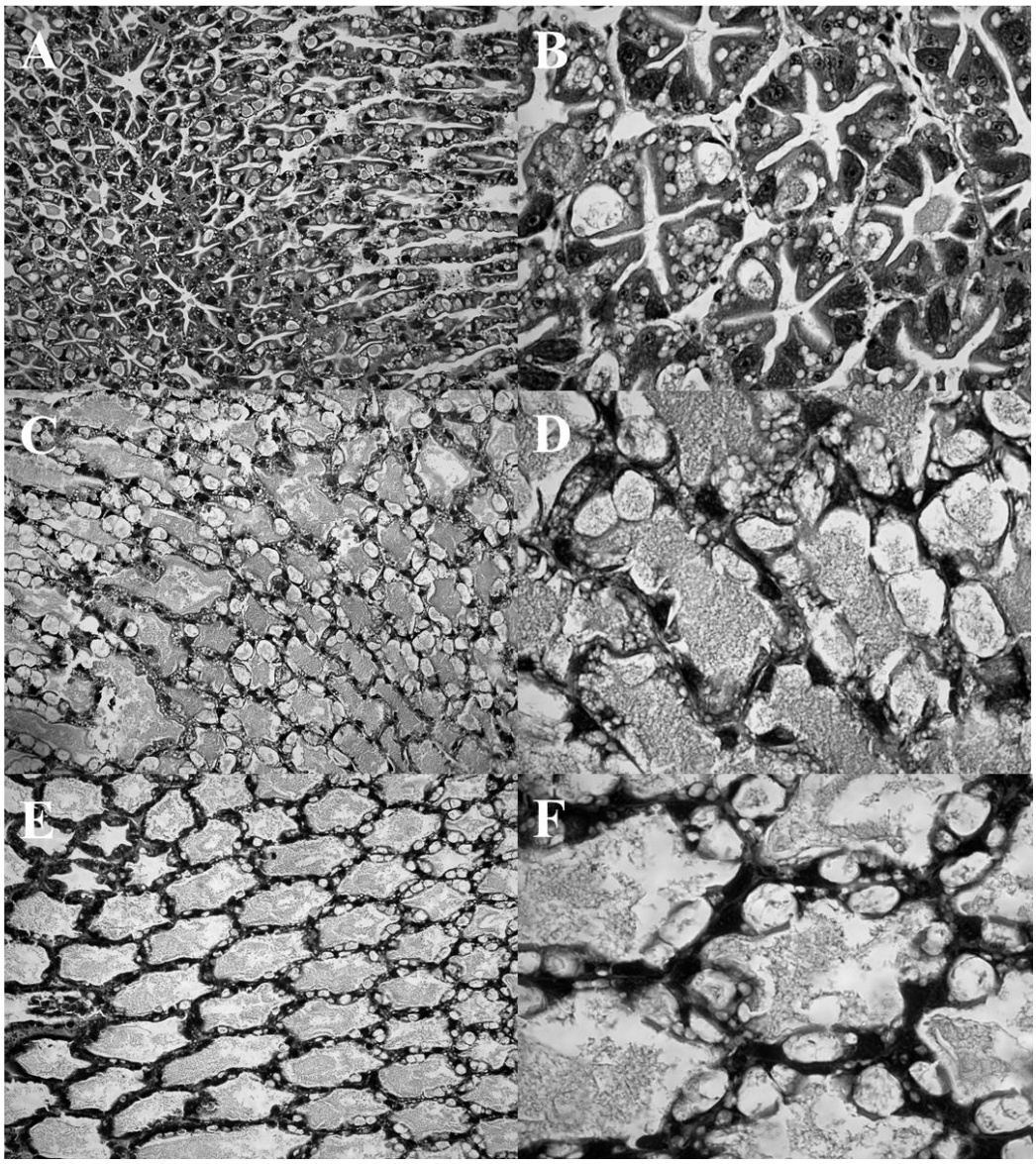

Figure 1

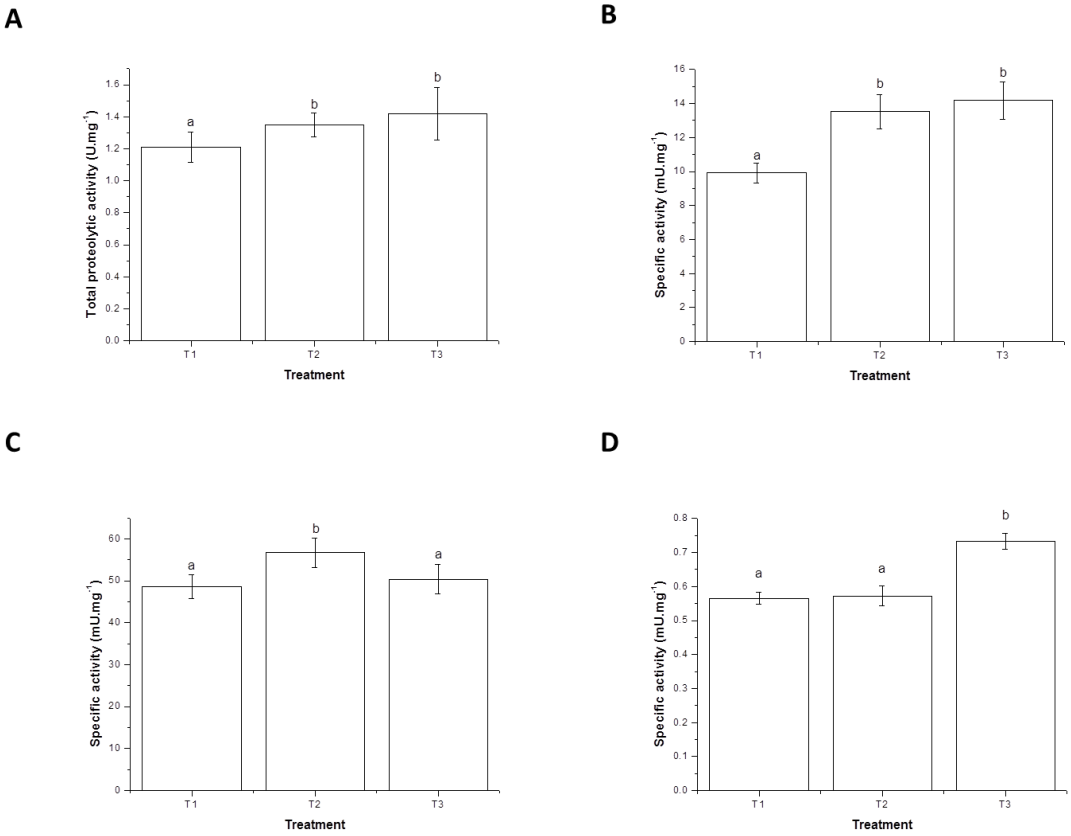
A
Total proteolytic activity (U.mg-1)
a
b
b
T1
T2
T3
Treatment
B
Specific activity (mU.mg-1)
a
b
b
T1
T2
T3
Treatment
C
Specific activity (mU.mg-1)
a
b
a
T1
T2
T3
Treatment
D
Specific activity (mU.mg-1)
a
a
b
T1
T2
T3
Treatment

Figure 2

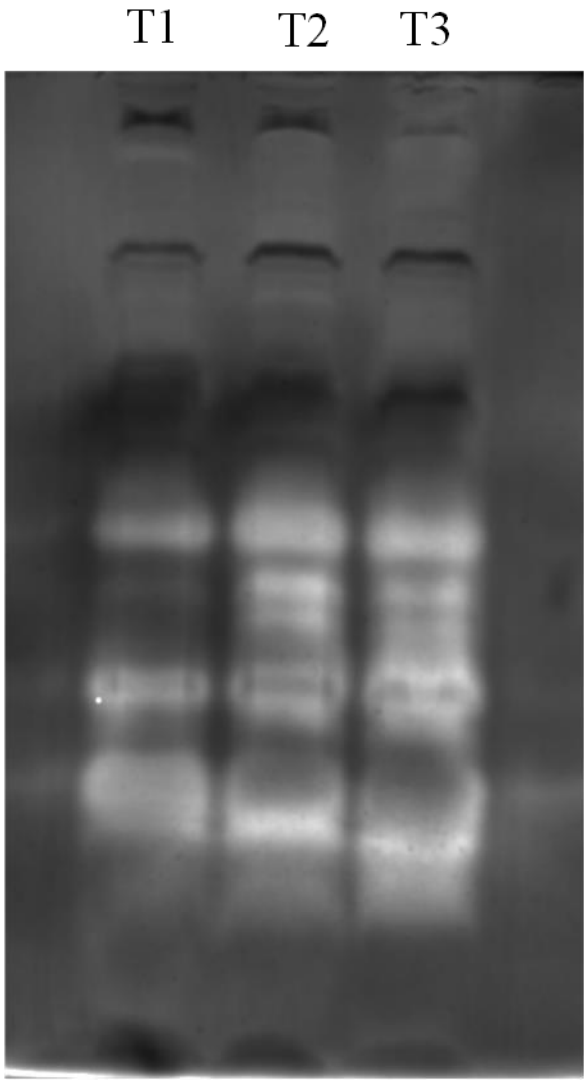

Figure 3

CAPÍTULO 2

Effect of diets with different protein levels on zootechnical performance and digestive protease activity of marine shrimp *Litopenaeus vannamei* **in biofloc system**

A ser submetido ao periódico Aquaculture

Effect of diets with different protein levels on zootechnical performance and digestive protease activity of marine shrimp *Litopenaeus vannamei* **in biofloc system**

Janilson Felix da SILVA[1], Charles Rosemberg do NASCIMENTO JUNIOR[1], Fabiana Penalva de MELO[2], Maria Gabriela Padilha FERREIRA[2], João Paulo Viana de LIMA[2, 3], Karina RIBEIRO[4], Eudes de Souza CORREIA[2], Ranilson de Souza BEZERRA[1]*

[1]Laboratório de Enzimologia (LABENZ), Departamento de Bioquímica e Laboratório de Imunopatologia Keizo Asami (LIKA), Universidade Federal de Pernambuco, Cidade Universitária, 50670-420, Recife-PE, Brazil.

[2]Universidade Federal Rural de Pernambuco, Departamento de Pesca e Aquicultura, Laboratório de Sistemas de Produção Aquícola (LAPAq), 52171-900, Recife, PE, *Brazil.*

[3]Instituto Agronômico de Pernambuco (IPA), C.P. 1022, 50761-000, Recife, PE, Brazil.

[4]Unidade Acadêmica Especializada em Ciências Agrárias – EAJ/UFRN, Macaíba, RN

Author to whom the correspondence should be addressed:

Ranilson S. Bezerra

Laboratório de Enzimologia (LABENZ), Departamento de Bioquímica e Laboratório de Imunopatologia Keizo Asami (LIKA), Universidade Federal de Pernambuco, Cidade Universitária, 50670-420, Recife-PE, Brasil.

Phone: +55 81 21268540; Fax, +55 81 21268576

e-mail: ransoube@uol.com.br

ABSTRACT

It was evaluated the effects of different protein levels in diets on the zootechnical performance and digestive protease activity of *L. vannamei* cultured in biofloc system. Juveniles (1.55±0.1 g) were culture for 50 days at Estação de Aquicultura Continental Professor Johei Koike (UFRPE). It was used a completely randomized design with four treatments in triplicate involving different levels of protein in commercial pelleted ration (20, 25, 30 and 35%) offered four times per day. At the end of experiments it was evaluated the final weight gain, biomass gain and survival. Water temperature, dissolved oxygen, pH and salinity were measured daily, while NH_3-N, NO_2-N, NO_3-N, PO_4-P and alkalinity, weekly. For enzymatic assays, the hepatopancreas of ten shrimp of each conditioned tank were collected, homogenized and centrifuged to obtain crude extracts. Proteolytic activity was determined using azocasein (total protease), BApNA (trypsin), SApNA (chymotrypsin) and Leu-p-Nan (leucine-amino-peptidase). SDS-PAGE and protease zymogram were carried out with 13.5% separating gels. There was no significant difference in water quality parameters except for nitrite concentration, influenced by protein levels (P <0.05). In the same way, no statistical difference was found in zootechnical parameters. Higher total protease (1.54±0.11 $U.mg^{-1}$) and trypsin (15.0±0.75 $mU.mg^{-1}$) activities were observed for 20% treatment. For chymotrypsin, 35% treatment activity (0.16±0.03 mU.mg-1) was significantly higher than other groups. Leucineaminopeptidase activity was higher in 30% group (0.26±0.03 $mU.mg^{-1}$). Seventeen bands were present in SDS-PAGE for 35, 30 and 20%, while 25% presented sixteen; zymogram showed twelve bands with protease activity for 35 and 30% and nine for 25 and 20%. Physiological adaptations possible resulted in similar performance for all treatments. This data suggests that the use of biofloc system on peneids farming with no loss of production is possible, even with crude protein levels diet lower than the usual 35%.

Keywords: Digestive proteases, biofloc system, *Litopenaeus vannamei*.

1.Introduction

The challenge faced by aquacultural nutritionists is mainly to decrease the feed costs, increase the food efficiency and minimize environmental impacts caused by nutrient rich effluents, produced by provided formulated feeds (Chamberlain et al., 2001a; Funge-Smith e Briggs, 1998).

The use of high water exchange rates in intensive systems is a general procedure to maintain the water quality (Hopkins et al., 1993). However, in aquaculture systems, the water income is the main way of pathogen introduction in culture environment (Lotz and Lightner, 1999).

In order to minimize the amount of nutrients released in coastal ecosystems and production loss due to diseases, shrimp culture with limited or zero water exchange has taken a important step in this direction. (Samocha et al., 2007). This system combines water treatment and recycle of not consumed food by aerobic and heterotrophic microbiotics degradation (Avnimelech et al., 1994; Burford et al., 2003a; Wasielesky et al., 2006a).

Heterotrophic bacterial growth is stimulated by addition of an organic carbon source (sugarcane molasses) (Avnimelech 1999; Burford et al., 2003b). These microorganisms improve water quality and act as a food source, as flocculated shaped particles which are consumed by shrimps (Burford et al., 2004). However, the use of microbial protein depends on the animal skill in capture and metabolize such protein (Avnimelech, 1999).

In culture systems based in biofloc technology (BFT), these particles are formed in the production cycle and are composed by a large variety of microorganisms, feed remains, faeces, debris, exoskeletons, other dead animals, etc. (Decamp et al., 2002; Burford et al., 2003a; Wasielesky et al., 2006b; Ray et al., 2009). According to McIntosh (2000), bacterial flocs are composed by aproximately 45% protein, which corresponds to almost two times the amount of protein in feeds designed for aquaculture animals.

The introduction of protein and aminoacids rich microbial flocs can greatly reduce the protein requirement in rations (Chamberlain et al., 2001b), and thereafter decrease the costs with feed formulation, which represents more than 50% of operational costs in aquaculture systems (Tan e Dominy, 1997; Martinez-Cordova et al., 2003; Shiau e Bai, 2009).

Many works report *L. vannamei* farming using low levels of crude protein in feed and water with high primary and secondary food production, reducing the amount ofwater needed for exchange, as the natural available food complements a low protein diet. (McIntosh, 2000; Burford et al., 2004; Hari et al., 2004; Wasielesky et al., 2006a; Ballester et al., 2010). On the other hand, there is still a huge gap regarding nutritional aspects, relative to microorganisms and farmed animals, which need more investigation. The aim of this work was evaluate the effects of diets with different proteic levels on the zootechnical performance and digestive protease activity of marine shrimp *Litopenaeus vannamei* in biofloc system.

2. Materials and methods

2.1 Experimental sites and conditions

Experimental work was carried out at Estação de Aquicultura da Universidade Federal Rural de Pernambuco (UFRPE), Recife-PE, Brazil, and was performed using twelve circular fiber glass tanks, located in open area, provided with 800 L of salt water (30 g.L-1) aerated individually with four porous air stone (Ø 1"), maintained with a radial compressor 2 CV (CR 5, Ibram Ltda, SP, Brazil).

2.2 Water supply and tank managements

Water used in the experiments was previously filtered and provided by Laboratório de Maricultura Netuno. At aquaculture station, water was chlorinated at 10 ppm chlorine with sodium hypochlorite and dechlorinated by aeration. Tanks were supplied with a mixture of

300 L of clean salt water (30g.L-1) and 500 L of salt water from other culture (30 g.L-1). After storage, sugarcane molasses was added daily to the culture water, as a substrate for bacteria development and control the ammonia levels. The amount of molasses added was calculated by the C:N 6:1 ratio, where 6 g of organic carbon immobilizes 1 g of ammoniac nitrogen found in culture water, as recommended by Avnimelech (1999) and Samocha et al. (2007). During the whole culture there was no water renewal, only weekly water replacement, with chlorinated freshwater in order to offset the evaporation loss.

2.3 Experimental design and shrimp culture

A completely randomized design with four treatments comprised with different protein level in pelleted commercial ration (20, 25, 30 and 35%). *L. vannamei* post-lavae in PL12 stage (0.002 g) were obtained from a provider in Rio Grande do Norte and transfered to Estação de Aquicultura (DEPAq/UFRPE), where nursery culture was carried out for 48 days. At this stage, post-larvae were stocked at 1500 PL m^{-3}, feed four times a day with commercial ration 450 g kg^{-1} PB (INVE, EPAC®) and *Artemia sp.* nauplius newly hatched (40 nauplius PL dia^{-1}), for one week. At the end of this nursery phase, juveniles of *Litopenaeus vannamei* were produced with average weight of 1.55±0.1 g, which were stocked randomly in culture tanks, density of 300 animals m^{-3} (240 per tank), for a period of 50 days.

Diets used in this study were manufactured by IRCA Produção Animal (IRCA, PE, Brazil) containing crude protein at 200, 250, 300 and 350 g kg^{-1} ration. Once a week, a random 24 shrimps samples of each experimental unit was collected and weighted, using digital balance 0.01 g (Shimadzu®, BL 3200 H, SP, Brazil). After weighing, animals were replaced to their respective culture tanks.

Feed was offered four times a day (8:00, 11:00, 14:00 e 17:00 h) in feeding trays, which allowed a preview of feed debris and daily adjustment of provided ration. Feed amount

was calculated based on data obtained in biometrics, seeing weekly growth of 1 g, feed convention ratio 1.5 and estimated mortality of 0.5% per week, according to Samocha et al. (2007).

2.4 Water quality analysis

During experimental period, water temperature, dissolved oxygen, pH and salinity were recorded in each tank, twice a day (8:00 and 16:00 h), with the aid of multiparameter probe YSI 556 MPS (YSI Incorporation, Ohio, USA). Weekly, water samples of each tank was collected in order to measure levels of total ammonia nitrogen (TAN), nitrite-N (NO_2-N), nitrate-N (NO_3-N), orthophosphate (PO_4) and total alkalinity. Prior to analysis, samples were filtered using analytical filter 0.45 µm. Nitrogenous compounds were measured methods TNT 830 (salicylate method), 8507 (diazotization method) and 8539 (cadmium reduction) for NH_3-N, NO_2-N and NO_3-N, respectively. Orthophosphate concentration was measured using PhosVer®3 8048 method (ascorbic acid). Samples were read in digital spectrophotometer Hach DR 2800 (Hach Company, Colorado, USA). Total alkalinity was determined by volumetric titration (APHA, 1995). Sodium bicarbonate was added when alkalinity levels kept below 100 mg L^{-1}, in order to alkalinity maintain above 150 mg $CaCO_3$ L^{-1}.

2.5 Obtention of *L. vannamei* hepatopancreas enzymatic extract

Ten animals were sacrificed in ice bath and desiccation of cephalothorax was carried out to remove hepatopancreas, which was homogenized in Tris-HCl 0.01 M pH 8.0 buffer containing 150 mM NaCl, with 5 mL of solution for each gram of viscera, using a tissue homogenator at 500 rpm for 1 min in ice. Afterwards, the extracts were centrifuged at 10,000 g(force) for 25 min at 4°C. Following this procedure, supernatant (crude extract) was

collected and stored at -20°C for later use. Protein determination was performed by the method of Bradford (1976).

2.6 Determination of proteolytic activity using unspecific substrate

Proteolytic unspecific activity of crude extract was measured using azocasein as substrate. Aliquots in triplicate of 30 µL were incubated in micro tubes with 50µL of azocasein 1% (m/v), pH 8,0, for 1 h, at room temperature (25 °C). Blank was carried out in same conditions but replacing extract for buffer (Tris-HCl 0.01 M + 0.9% NaCl). After that, 240 µL of 10 % (m/v) trichloroacetic acid was added and the mixture was allowed to rest for 15 min, then centrifuged at 8.000xg for 5 min. Aliquots were taken from supernatant (70 µL) and placed in 96-wells plate containing 130 µL of 1 M sodium hydroxide and then immediately read using a microplate spectrophotometer at 450 nm (Alencar et al., 2003).

2.7 Protease activity measured using specific substrates

For specific protease assays we used BApNA (Benzoil-D,L-Arginine-p-Nitroanilide), Leu-p-Nan (leucine-p-nitroanilide) and SApNA **(**succinil- alanine-phenilalanine-p-nitroaniline), with final concentration of 0.2 mM. The assay was caried out in 96-well plates adding 140 µL of Tris-HCl 0.1 M pH 8.0, 30 µL of crude extract and 30 µL of substrate. Blank was performed with 30 µL substrate and 170 µL buffer. After 15 min incubation time, absorbance was taken in a microplate spectrophotometer at 405 nm (Alencar et al., 2003).

2.8 Electrophoresis and zymogram

Sodium dodecil-sulfate polyacrylamide gel electrophoresis was carried out according to the methods of Laemmli (1970) with 13,5% resolving gels. Samples and a molecular weight standard protein pool were submitted to electrophoresis and after migration gel was stained with a solution of Comassie Brilliant Blue in order to revel protein bands and

destained with the same solution without Comassie. The methods for zymogram are described by García Carreño et al. (1993): briefly, a 4% stacking, and 13,5% resolving was used. After electrophoretic migration, gels were immersed in a solution containing 2,5% Triton X-100 and 0.1M Tris-HCl pH 8.0 for 30 min at 4°C in order to remove remaining SDS. After that, a solution of 3% casein was added to the recipient containing the gel for 30 min at 4°C and then incubated for 90 min to develop caseinolitic bands. Gels were stained with Comassie Brilliant Blue R250 solution and after 24 h destained with the same solution without Comassie to reveal clear proteolytic bands.

2.9 Statistical analysis

Shrimp growth variables (final weight, final biomass, survival, specific growth rate and feed conversion ratio), water quality parameters and enzymatic activity data were analyzed using ANOVA in order to evaluate effects of protein levels (35, 30, 25 and 20%) with 5% significance. When Tukey's post test was applied when difference was detected to compare groups using the software Microcal^{TM} Origin^{TM} 8.0 (Software, Inc, USA).

3. Results and discussion

Water temperature, dissolved oxygen, pH, salinity, ammonia nitrogen, nitrate, orthophosphate and total alkalinity were not affected by different protein levels ($P \geq 0,05$) (Table 1). According to Davis et al. (2004) *L. vannamei* is a species found in waters with large range of salinity, from 1 to 40 g L^{-1}, altough develops better in environments between 15 and 25 g L^{-1}, parameters maintained in this work. Li et al. (2007) reported that *L. vannamei* adapts to a wide range of salinity, but are more susceptible to ammonia toxicity and spend more energy to offset the cost spent with osmoregulation in low salinity environments.

Dissolved oxygen presented an average value of 5,89 mg L^{-1}, higher than ideal levels considered and reported by BOYD e CLAY (2002), where super intensive culture systems must have dissolved oxygen levels above 4 mg L^{-1}. Sometimes, pH levels were recorded over 7.0, ranging between 7.69 to 7.75. According to WASIELESKY et al. (2006b), due to intense animal breathing, there is a natural tendency to decrease of pH below 7.0 in culture environment. However, in culture systems with bioflocs pH levels below 7.0 affect negatively the growth of *L. vannamei* (WASIELESKY et al., 2006a).

During experimental period, pH variation can also be attributed to total alkalinity levels (> 100 mg $CaCO_3$ L^{-1}), constantly hulled by addition of sodium bicarbonate in order to increase the buffering capacity of the water.

Levels of dissolved inorganic nitrogen and orthophosphate (TAN, NO_2-N, NO_3-N e PO_4) during experimental period are presented in Table 1. The protein levels did not influenced the ammoniacal nitrogen, or nitrate neither orthophosphate ($P \geq 0,05$). Concentrations of NH_3-N were found below 50 mg L^{-1}, for the whole period in all treatments ($P \geq 0,05$) (Table 1). BURFORD et al. (2003a) showed that shrimp farm tanks coated with canvas and operated with high density had levels of ammoniacal nitrogen up to 0,56 mg L^{-1} which did not interfere in production.

Nitrite was strongly influenced by protein levels in feed ($P < 0,05$). CORREIA et al. (2010) evaluated the effects in diets with high (400 g kg^{-1}) and low (300 g kg^{-1}) levels of crude protein in *L. vannamei* nursery and reported that shrimps could endure sever days or more exposed to high concentrations of nitrite, between 17.7 and 34 mg L^{-1}, with no undesirable effects on survival. Systems with no water exchange the decrease of decomposition rate by heterotrophic bacteria results in accumulation of inorganic nitrogen (ammonia, nitrite, nitrate) (McINTOSH, 2000).

Levels of orthophosphate (PO_4) exhibited a tendency of increase with culture time, with levels above 15 mg L^{-1}. McIntosh (2001) reported that culture systems without water exchange levels of total phosphorous increased with time and reach values above 25 mg L^{-1}. Hopkins et al. (1993) working in farms with limited water swap recorded levels of orthophosphate significantly higher than tanks with no water exchange. Burford et al. (2003a) reported that systems with limited water swap higher accumulation of orthophosphate occur in tanks coated with canvas (no sediment) when compared to ground tanks, for phosphorous bind to the sediment. According to Hopkins et al. (1993), water swap is more effective in avoid accumulation of this nutrient in culture environment.

Protein levels in diets also did not significantly influenced (*P≥0,05)* growth (Table 2). At the end of 50 days of culture shrimps presented final weight of 7.17±0.4 g, which correspond to an average weight gain of 5.62 g, and the lowest values found to treatment 20% (6.61 and 5.06 g, respectively). Samocha et al. (2004) evaluated the performance of *L. vannamei* fed with diets containing different levels of protein (210 and 310 g kg^{-1}) with bioflocs and observed that shrimps feeded with 310 g.kg-1 had increased final weight but performance was not influenced by addition of bacterial supplement.

The average survival was 80.2±6.9%. Fróes et al. (2007), studied the influence of different crude protein levels in ration on survival of shrimp *Farfantepenaeus paulensis,* and recorded a variation of 81,62 to 93,80%, values similar to that found in this work. Gomez-Jimenez et al. (2005) did not find difference on survival, final weight and weight gain in *L. vannamei* submitted to intensive system and commercial feeds with different levels of crude protein (250, 300, 350 or 400 g kg^{-1}) with no water exchange. Samocha et al. (2007) work with shrimp growth and limited water exchange obtained higher survival using diets with 30% level.

Shrimps presented continuous growth along 50 culture days, with similar development varying between 0.71 to 0.83 g $week^{-1}$ with different treatments. The variation was also included in the specific growth rate, but with no statistical difference among treatments

(*p≥0,05*). Huai et al. (2010) reported that the growth of *L. vannamei* feeded with a diet of low level protein diet and lower fish powder supplemented with synthetic amino acids can achieve a growth performance similar to that feed with high protein levels and high concentration of fishmeal. Ballester et al. (2010) cultured *Farfantepenaeus paulensis* in intensive system with microbial flocs and no water swap observed that a diet containing 35% of crude protein allowed increased final weight, weight gain and specific growth rate.

Many authors reported a reduction of protein levels in diets with no negative effects on shrimp growth, when there is presence of natural food as microbial flocs, acting as an important source of available protein for shrimps (McIntosh, 2000; Burford et al., 2003a; Wasielesky et al., 2006b).

Intensive systems with limited water exchange allow a better control of the flocculated material in water column (Samocha et al., 2007). According to Burford et al. (2003a) these flocs provide a supplemental source of food for shrimps. Despite the high stocking density (300 shrimps m^{-3}) in this work, the feed conversion ratio (FCA) varies from 1.79 to 2.71 (*P≥0.05*). Higher FCA reported in 20% treatment causes the low survival, biomass gain and low final biomass in this treatment. Samocha et al. (2007) using different concentrations of molasses in intensive culture of *L. vannamei* reported a FCR from 1.59 to 1.95, lower than the present work. Ballester et al. (2010) observed significantly differences after 45 cultured days in FCR, weight gain and final weight with *Farfantepenaeus paulensis* feeded with diets containing 25% and 30% of crude protein.

Digestion of proteins in peneids occurs mainly in hepatopancreas, where enzymes such as trypsin, chymotrypsin and aminopeptidases are found (Garcia-Carreño et al., 1993; Cuzon et al., 2004). All together, these enzymes break peptide bonds releasing amino acids which are aborted in the digestive tract. In vitro quantification of total protease activity of viscera enzymatic extract is commonly achieved using the synthetic substrate azocasein

(Garcia-Carreño et al., 1993). In this work, this unspecific substrate (1% azocasein in Tris-HCl buffer) assay showed the higher activity value in 25% crude protein in ration (1.63 ± 0.20 $U \cdot mg^{-1}$), followed by 20% (1.54 ± 0.11$U \cdot mg^{-1}$), 30% (1.40 ± 0.07 $U \cdot mg^{-1}$) and 35% (1.33 ± 0.02 $U \cdot mg^{-1}$). However, no statistical difference (P<0,05) was found between groups (Figure 1A).

Specific protease activities were carried out using the synthetic substrates BApNA, SApNA and Leu-p-Nan to determinate the activity of trypsin-like, chymotrypsin-like and leucineamino peptidase, respectively. Tryptic activity was higher in treatments 20% (15.00 ± 0.75 $mU \cdot mg^{-1}$) and 25% (14.02 ± 0.74 $mU \cdot mg^{-1}$). However, despite show higher enzymatic performance, they were not statistical different when compared to treatments ($p < 0.05$) 35% (12.60 ± 1.24 $mU \cdot mg^{-1}$) and 30% (12.06 ± 1.50 $mU \cdot mg^{-1}$) (Figure 1). Trypsin is usually the most abundant protease in digestive tract of crustaceans (Fernández Gimenez et al., 2002). Some authors had emphasized the importance of this enzyme in peneids and there is a estimative that its contribution to protein digestion be about 60% (Sánchez-Paz et al., 2003). Moreover, it is important in others biological processes such as zymogen activation and mediation between food intake and absorption of nutrients (Sainz et al., 2004).

Regarding the proteolytic activity, the second most abundant protease in shrimp hepatopancreas is most likely chymotrypsin (Garcia-Carreño et al., 1994). The analysis of this serino protease activity using 8 mM SApNA showed that activities in treatment 35% (0.16 ± 0.03 $mU \cdot mg^{-1}$) was significantly higher than other treatments like 20% (0.03±0.01 $mU \cdot mg^{-1}$), 25% (0.02 ± 0.00 $mU \cdot mg^{-1}$) and 30% (0.02 ± 0.00 $mU \cdot mg^{-1}$). So, group 35% was statistically different ($p < 0,05$) from others which did not present differences in the activities.

Still regarding specific substrates, the assays carried out with 8 mM Leu-p-Nan showed higher leucineaminopeptidase activity in group 30% (0.26 ± 0.03 $mU \cdot mg^{-1}$).

Nevertheless, the activity of this group was not different ($p < 0.05$) when compared to groups 20% (0.22 ± 0.03 mU·mg^{1}), 25% (0.25 ± 0.06 mU·mg^{-1}) and 35% (0.21 ± 0.03 mU·mg^{-1}) .

L. vannamei is one versatile animal with great capacity of adaptation to many conditions in which one is submitted. Variations shown in proteolytic activities are most likely suggested to be adjustments in its physiology in response to the provided diets. Furthermore, heterotrophic systems provides microorganisms in bioflocs like bacteria, particularly of *Bacillus* genus, that secrets a large array of enzymes that possible have a positive influence in shrimp digestive capacity (Wang, 2007).

The crude extract profile of the four treatments was analyzed by SDS-PAGE (Figure 2A). Treatments 35%, 30% and 20% showed 17 proteic bands while 25% presented 16 bands, all of them between molecular weight from 6.9 to 198.8 kDa. According to the work of Lemos et al., (2000), enzymes like trypsin and chymotrypsin can be involved in such profiles, since trypsin-like enzymes present molecular weight between 14.6 to 21.7 kDa and chymotrypsin 28.9 to 37.7 kDa.

Through zymogram it is possible to verify the number of active forms of a enzyme class in the studied species. The proteolytic activities of *L. vannamei* enzymes are shown in Figure 2B. Treatments 25% and 20% presented 9 bands with protease activity while 35% and 30% presented 12. It was possible to observe in all treatments bands with intensity below 29 kDa, which suggests that there is most trypsin activity in the extracts.

The different enzymatic activities showed in this study reflect the capacity of physiological adaptation of *L. vannamei* in order to utilize the energetic source available without development losses. This characteristic of this species make it one of the main shrimp farmed worldwide.

Other studies carried out in intensive culture shows that the heterotrophic biota contributes not only for the maintenance of water quality variables (Thompson et al. 2002;

Samocha et al. 2007), but also represents a important source of nutrients for development of aquatic animals. (Burford et al., 2004; Wasielesky et al., 2006b).

4. Conclusion

Intensive culture system for shrimp *Litopenaeus vannamei*, with addition of bioflocs as an alternative food source, is likely to reduce the protein levels in feed from 35 to 20%, without affect growth, feed conversion ratio and survival. Microorganisms present in bioflocs perform an important role regarding water quality maintenance and as essential nutrients suppliers for the animals. This information shows that heterotrophic system can likely provide lesser protein amounts in feed and yet obtain zootechnical results similar to common more-protein rations.

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References

APHA (American Public Health Association), 1995. Standard Methods for the Examination of Water and Wastewater. 19th ed. APHA, Washington, DC, USA. 1082 pp.

AVNIMELECH, Y.; KOCHVA, M., DIAB, S. Development of controlled intensive aquaculture systems with a limited water exchange and adjusted carbon to nitrogen ratio. *Aquaculture Bamidgeh*, v.46, n.3, p.119-131, 1994.

AVNIMELECH, Y. Carbon/nitrogen ratio as a control element in aquaculture systems. *Aquaculture*, v. 176, p. 227-235, 1999.

BOYD, C.E., 2001. *Manejo da qualidade de água na aqüicultura e no cultivo do camarão marinho*. Tradução Josemar Rodrigues. Associação Brasileira de Criadores de Camarão (ABCC), Recife, PE, 157 pp.

BOYD, C.E.; CLAY, J.W. Evaluation of Belize Aquaculture, Ltd: A Superintensive Shrimp Aquaculture System. Report prepared under the World Bank, NACA, WWF and FAO Consortium Program on Shrimp Farming and the Environment. Work in Progress for Public Discussion. Published by the Consortium. 17 pp. 453, 2002.

BALLESTER, E.L.C., ABREU, P.C., CAVALLI, R.O., EMERENCIANO, M., DE ABREU, L. & WASIELESKY, W. Effect of practical diets with different protein levels on the performance of *Farfantepenaeus paulensis* juveniles nursed in a zero exchange suspended microbial flocs intensive system. *Aquaculture Nutrition*, v. 16, p. 163–172, 2010.

BURFORD, M. A.; THOMPSON, P. J.; McINTOSH, R. P.; BAUMAN, R. H.; PEARSON, D. C. Nutrient and microbial dynamics in high-intensity, zero-exchange shrimp ponds in Belize. *Aquaculture*, v. 219, p. 393-411, 2003a.

BURFORD, M. A.; THOMPSON, P. J.; BAUMAN, R. H.; PEARSON, D. C. Microbial communities affect water quality, shrimp performance at Belize Aquaculture. *The global Aquaculture Advocate*, p.63-65, 2003b.

BURFORD, M.A., SMITH D.M., TARBRETT S.J., COMAN F.E., THOMPSON P.J., BARLLAY M.C. & TOSCAS P.J. The effect of dietary protein on the growth and survival of the shrimp *Penaeus monodon* in outdoor tanks. *Aquaculture Nutrition*, v. 10, p. 15-23, 2004.

BROWDY, C.L., BRATVOLD, D., STOKES, A.D. & MCINTOSH, R.P. 2001. Perspectives on the application of closed shrimp culture systems. In: JORY, E.D. e BROWDY, C.L. (Eds.) *The new Wave, Proceedings of the Special Session on Sustainable Shrimp Culture*. World Aquaculture Society, p. 20–34.

CHABERLAIN, G.; AVNIMELECH, Y.; McINTOSH, R. P.; VELASCO, M. Advantages of aerated microbial reuse systems with balanced C:N - I: Nutrient transformation and water quality benefits. *The Global Aquaculture Advocate*, p. 53-56, 2001a.

CHAMBERLAIN, G.; AVNIMELECH, Y.; McINTOSH, R.; VELASCO, M. Advantages of aerated microbial reuse systems with balanced C:N. III: Practical Applications. *The global Aquaculture Advocate*, v.4, n.5, p.50-54, 2001b.

CORREIA, E.S.; SAMOCHA T.M.; WILKENFELD, J.S.; MORRIS, T.C; WEI, L. 2010 Intensive nursery of the pacific white shrimp *Litopenaeus vannamei* in greenhouse-enclosed raceways using low and high protein diets under no water exchange. In: AQUACULTURE 2010. San Diego, 1-5/mar./2010. *Anais*... p225.

DAVIS, D.A., SAMOCHA, T.M., BULLIS, R.A., PATNAIK, S., BROWDY, C., STOKES, A., ATWOOD, H. 2004 Practical diets for *Litopenaeus vannamei*, (Boone. 1931): working towards organic and/or all plant production diets. In: AVANCES EN NUTRICION ACUICOLA VII. MEMORIAS DEL VII SIMPOSIUM INTERNACIONAL DE NUTRICION ACUICOLA. *Anais*... Hermosillo, 16-19/nov./2004. *Anais*… p.202–214.

DECAMP, O.; CONQUEST, L.; FORSTER, I.; TACON, A.G.J. 2002. The nutrition and feeding of marine shrimp within zero-water exchange aquaculture production systems: Role of eukaryotic microorganisms. In: LEE, C.S. e O`BRYEN, P. (Eds.). *Microbial approaches to aquatic nutrition within environmentally sound aquaculture production systems*, World Aquaculture Society 2002, Baton Rouge, Louisiana USA, 2002, p.79-86.

FUNGE-SMITH, S.J.; BRIGGS, M.R.P. Nutrient budgets in intensive shrimp ponds: implications for sustainability. *Aquaculture*, v.164, p.117-133, 1998.

FRÓES, C.N., CAVALLI, R.O., ABREU, M.P., WASIELESKY, W., PRENTICE-HERNÁNDEZ, C. Efeitos de dietas práticas com diferentes níveis de proteína bruta na sobrevivência e crescimento do camarão-rosa *Farfantepenaeus paulensis* (Pérez-Farfante, 1967). *Atlântica*, v.29, n.1, p.25-34, 2007.

GOMEZ-JIMENEZ S., GONZALEZ-FELIX M.L., PEREZ-VELAZQUEZ M., TRUJILO-VILLALBA D.A., ESQUERRA-BRAUER I.R., BARRAZA-GUARDADO R. Effect of dietary protein level on growth, survival and ammonia efflux rate of *Litopenaeus vannamei* (Boone) raised in a zero water exchange culture system. *Aquaculture Research*, v. 36, p. 834–840, 2005.

HARI, B.; MADHUSOODANA KURUP, B.; VARGHESE, J.T.; SCHRAMA, J.W.; VERDEGEM, M.C.J. Improved sustainability in extensive shrimp culture systems: control of carbon nitrogen ratio through addition of carbohydrate to the pond. *Aquaculture*, v.241, p.179-194, 2004.

HOPKINS, J.S.; HAMILTON, R.D.; SANDIFER,P.A,; BROWDY, C.L.; STOKES, A.D. Effect of water exchange rate on production, water quality, effluent characteristics and nitrogen budgets of intensive shrimp ponds. *Journal of the World Aquaculture Society*, v.24, n.3, 1993.

HUAI M.Y., LIU Y.J., TIAN L.X., DENG S.X., XU A.L., GAO W., YANG H.J. Effect of dietary protein reduction with synthetic amino acids supplementation on growth performance, digestibility, and body composition of juvenile Pacific white shrimp, *Litopenaeus vannamei*. *Aquaculture*, v. 18, p. 255–269, 2010.

INMET, Instituto Nacional de Meteorologia. Disponível em: <http://www.inmet.gov.br/>. Acesso em: 11 de março de 2011.

LI, E.; CHEN, L. ZENG, C.; CHEN, X.; YU, N.; LAI, Q.; QIN, J.G. Growth, body composition, respiration and ambient ammonia nitrogen tolerance of the juvenile white shrimp, *Litopenaeus vannamei*, at different salinities. *Aquaculture*, v.265, 385-390, 2007.

LOTZ, J.M.; LIGHTNER, D.V. 1999. Shrimp biosecurity: pathogens and pathogen exclusion. pp. 67-74 in: R.A. Bullis and G.D. Pruder (Eds.) *Controlled and Biosecure Production Systems*. Evolution and Integration of Shrimp and Chicken Models. Proceedings of a Special Session, World Aquaculture Society. Sydney, Australia, April 27-30, 1999.

MARTINEZ-CORDOVA, L.R.; CAMPAÑA-TORRES, A.; e PROCHAS-CORNEJO, M.A. Dietary protein level and natural food management in the culture of blue (*Litopenaeus stylirostris*) and white shrimp (*Litopenaeus vannamei*) in microcosms. *Aquaculture Nutrition*, v. 9, p. 155–160, 2003.

MCINTOSH, R.P. Changing paradigms in shrimp farming: V. Establishment of heterotrophic bacterial communities. *The Global Aquaculture Advocate*, v. 3, p. 52–54, 2000.

MCINTOSH, R.P. Changing paradigms in shrimp farming: V. Establishment of heterotrophic bacterial communities. *The Global Aquaculture Advocate*. v.4, p.53-58, 2001.

RAY, A.J.; SHULER, A.J.; LEFFLER, J.W.; BROWDY, C.L. 2009. Microbial ecology and management of biofloc systems. In: BROWDY, C.L. e JORY, D.E. (Eds.). *The rising tide, Proceedings of session on sustainable shrimp Farming*, World Aquaculture Society 2009, Baton Rouge, Louisiana USA, 2009, p.255-266.

SAMOCHA, T., DAVIS, D.A., SAOUD, I.P., DEBAULT, K. Substitution of fish meal by co-extruded soybean poultry by-product meal in practical diets for the Pacific white shrimp, *Litopenaeus vannamei*. *Aquaculture*, v. 231, p. 197-203. 2004.

SAMOCHA, T.M., PATNAIK, S., SPEED, M., ALI, A.-M., BURGER, J.M., ALMEIDA, R.V., AYUB, Z., HARISANTO, M., HOROWITZ, A. & BROCK, D.L. Use of molasses as

carbon source in limited discharge nursery and grow-out systems for *Litopenaeus vannamei*. *Aquacultural Engineering*, v. 36, p. 184–191, 2007.

SHIAU, S.Y. Nutrient requirements of penaeid shrimps. *Aquaculture*, v. 168, p. 77-93, 1998.

SHIAU, S.Y e BAI, S. Micronutrientes in shrimp diets. In: BROWDY, C.L. e JORY, D.E. (Eds.). **The rising tide, Proceedings of session on sustainable shrimp Farming**, World Aquaculture Society 2009, Baton Rouge, Louisiana USA, 2009, p.126-132.

TAN, R.K.H.; DOMINY, W.G. Commercial pelleting of crustacean feeds. In: D'ABRAMO, LR.; CONKLIN, D.E.; e AKIYAMA, M.D. (Ed.). *Crustacean Nutrition*. World Aquaculture Society, Baton Rouge, 1997, p. 520-549.

THOMPSON, F.L., ABREU, P.C. & WASIELESKY, W. Importance of biofilm for water quality and nourishment in intensive shrimp culture. *Aquaculture*, v.203, p.263–278. 2002

WASIELESKY, W. JR, ATWOOD, H., STOKES, A.; BROWDY, C.L. Effect of natural production in a zero exchange suspended microbial floc based super-intensive culture system for white shrimp Litopenaeus vannamei. *Aquaculture*, v. 258, p. 396–403, 2006a.

WASIELESKY, W.; EMERENCIANO, M.; BALLESTER, E.; SOARES, R.; CAVALLI, R.; ABREU, P.C. Cultivos em meios com flocos microbianos: um novo caminho a ser percorrido. *Panorama da Aquicultura*, v.16, n.96, p.14-23, 2006b.

ZAR, J.H., 1996. *Biostatistical analysis*. Prentice Hall, New Jersey. 622 pp.

Figure Chapter

Figure 1. Enzymatic activity in *Litopenaeus vannamei* hepatopancreas of animals cultures in heterotrophic system. A – Total proteolytic activity using 1% azocasein; B – Trypsin-like activity using 8 mM BApNA; C – Chymotrypsin-like activity using 8 mM SapNA; D – Leucineamino peptidase-like activity using 8 mM Leu-p-Nan. Treatments 35%, 30%, 25% and 20% correspond to the crude protein levels in commercial feed offered to animals. The values of average± standard deviation were obtained on *in vitro* assays in triplicate. Different letters superscript represent statistical differences in protease activities between treatments (Tukey; $p < 0.05$).

Figure 2. SDS-PAGE (A) and zymogram (B) from hepatopancreas crude extract of *L. vannamei* cultured in heterotrophic system. Treatments 35%, 30%, 25% and 20% correspond to crude protein in ration provided to the animals. P – molecular weight standard.

Table 1. Effect of different levels of protein on water quality parameters through experimental period (means±S.E*)

Variables	Protein level (% Crude protein)			
	20	25	30	35
Temperature (°C)	29,09±0,15	29,23±0,15	29,09±0,14	29,02±0,14
Dissolved Oxygen (mg L^{-1})	6,01±0,06	5,93±0,06	5,77±0,07	5,86±0,07
Ph	7,74±0,02	7,79±0,01	7,70±0,02	7,76±0,02
Salinity (g L^{-1})	22,46±0,15	22,15±0,17	22,09±0,18	22,63±0,16
NH_3-N (mg L^{-1})	0,21±0,04	0,22±0,04	0,23±0,04	0,24±0,03
NO_2-N (mg L^{-1})	5,51±2,48	4,42±0,97	4,10±1,40	2,48±0,95
NO_3-N (mg L^{-1})	24,28±3,62	20,85±3,48	25,96±4,45	26,18±3,07
PO_4-P (mg L^{-1})	25,12±1,90	28,68±2,12	27,72±2,07	30,98±2,38
Total alkalinity (mg $CaCO_3$ L^{-1})	158,9±10,02	159,1±9,92	142,4±10,36	151,0±12,31

*Different letters in the same line indicates statistical difference ($P<0,05$) between treatments (Tukey).

Table 2. Effect of different protein levels on growth of *Litopenaeus vannamei* cultured for 50 days with no water exchange. (means±S.E).

Variables	Protein levels			
	35	30	25	20
Final weight (g)		7,34 ± 0,64	7,24 ± 0,68	6,61 ± 0,56
Weight gain (g)		5,79 ± 0,65	5,69 ± 0,68	5,06 ± 0,56
Biomass gain (g)		1072,6 ± 253,4	1078,93 ± 148,2	833,75 ± 358,61
Final biomass ($g.m^{-2}$)		1445,0 ± 252,9	1451,6 ± 149,3	1205,8 ± 358,4
Survival (%)		81,67 ± 10,57	83,54 ± 4,65	75,55 ± 20,52
Specific growth rate (% day^{-1})		3,10 ± 0,18	3,07 ± 0,19	2,89 ± 0,17
Food conversion factor		1,94 ± 0,51	1,79 ± 0,36	2,71 ± 1,85

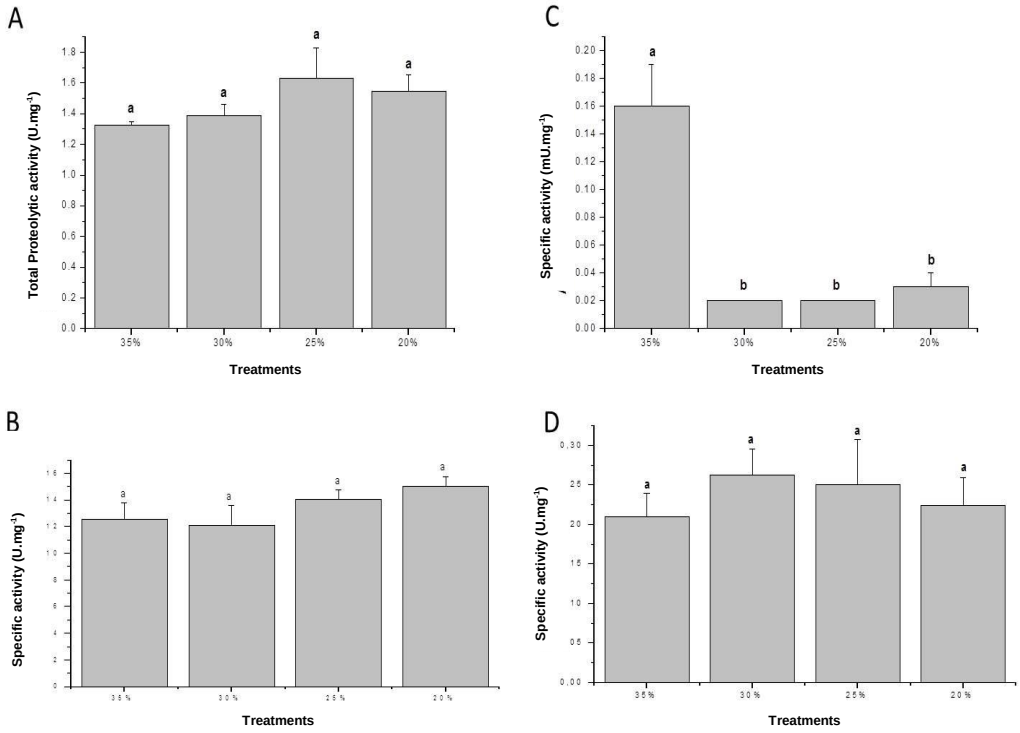
A
Total Proteolytic activity (U.mg-1)
a
a
a
a
35%
30%
25%
20%
Treatments
C
Specific activity (mU.mg-1)
a
b
b
b
35%
30%
25%
20%
Treatments
B
Specific activity (U.mg-1)
Treatments
D
Specific activity (U.mg-1)
a
a
a
a
35%
30%
25%
20%
Treatments

Figure 1.

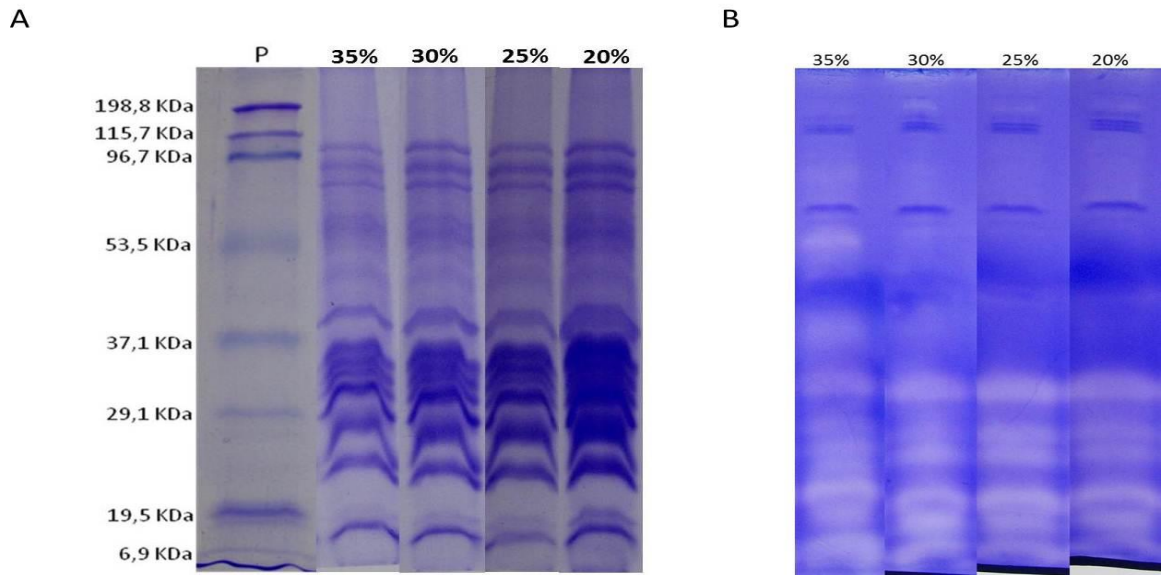
A
P
35%
30%
25%
20%
198,8 KDa
115,7 KDa
96,7 KDa
53,5 KDa
37,1 KDa
29,1 KDa
19,5 KDa
6,9 KDa
B
35%
30%
25%
20%

Figure 2.

CAPÍTULO 3

Digestive enzymes of the white shrimp *Litopenaeus vannamei* fed under diets based on soy protein concentrate in replacement of fishmeal

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Digestive enzymes of the white shrimp *Litopenaeus vannamei* fed under diets based on soy protein concentrate in replacement of fishmeal

Douglas H. H. Andrade[a], Janilson F. Silva[a], Augusto C. V. F. Junior[a], Alberto J. P. Nunes[c], Patrícia F. Castro[b], Ranilson S. Bezerra[a]

[a]Laboratório de Enzimologia (LABENZ), Departamento de Bioquímica e Laboratório de Imunopatologia Keizo Asami (LIKA), Universidade Federal de Pernambuco, Cidade Universitária, 50670-420, Recife-PE, Brazil

[b]Embrapa Meio-Norte, Caixa Postal 341, 64200-970, Parnaíba - PI, Brazil

[c]Instituto de Ciências do Mar (LABOMAR), Universidade Federal do Ceará, 60165-081, Fortaleza, - CE, Brazil

Corresponding author:

Ranilson S. Bezerra

Laboratório de Enzimologia (LABENZ), Departamento de Bioquímica e Laboratório de Imunopatologia Keizo Asami (LIKA), Universidade Federal de Pernambuco, Cidade Universitária, 50670-420, Recife-PE, Brazil.

Tel, +55 81 21268540; Fax, +55 81 21268576

email: ransoube@uol.com.br

ABSTRACT

This work aimed to evaluate the effect of replacing fishmeal by soybean protein concentrate (SPC) at levels of 0% (C), 30% (S_{30}), 60% (S_{60}) and 100% (S_{100}) on the performance of digestive enzymes from *Litopenaeus vannamei*. Juvenile specimens (2.02 ± 0.51 g) were subjected to experimental diets during ten weeks. Then midgut glands from shrimps of each treatment were collected and enzyme activities were analyzed by *in vitro* assays, using long-chain substrates (1% azocasein and 2% starch), p-nitroanilide (BApNA, SApNA and Leu-p-Nan) and β-naphthylamide (alanine, arginine, leucine, tyrosine, serine, glycine, isoleucine, and histidine). Moreover, there were performed SDS-PAGE and proteolytic and amylolytic zymograms. The S_{100} group showed higher proteolytic (1.18 ± 0.01 $U.mg^{-1}$) and amylolitic activity (5.04 ± 0.33 $U.mg^{-1}$). Major activities of chymotrypsin (13.78 ± 1.61 $U.mg^{-1}$) and leucine aminopeptidase enzymes (0.45 ± 0.03 $U.mg^{-1}$) were observed in the control group, while the highest trypsin activity (13.13 ± 0.53 $U.mg^{-1}$) was observed for the S_{30} treatment. There were higher levels of aminopeptidasic activity for arginine and alanine was seen in all treatments, mainly in the S_{30} that also showed increased activity in the presence of glycine (1.05 ± 0.08 $U.mg^{-1}$). It was noted that for serine, the aminopeptidasic activity was reduced gradually as the level of SPC in the shrimp diets increased. The S_{60} treatment showed higher aminopeptidasic activity for isoleucine (0.69 ± 0.02 $U.mg^{-1}$) and histidine (0.85 ± 0.04 $U.mg^{-1}$). In relation to leucine and tyrosine, the aminopeptidasic activity was statistically equal for all dietary variations. SDS-PAGE revealed 26 protein bands between 6.9 and 198.8 kDa for all treatments. The protease zymogram exhibited two similar profiles, one with eighteen (C and S_{30}) and another with twelve proteolytic bands (S_{60} and S_{100}). While the amylolytic zymogram revealed five bands for all treatments. The average body weight gain of shrimps showed the highest value using the S_{30} diet (8.48±1.03 g), however did not evidenced significant differences ($p<0.05$) among treatments. The results indicate that the substitution of

fishmeal by SPC at 30, 60 e 100% in the diets of farmed shrimps provided a positive effect on animals performance. These results provide important information about the potential use of lower levels of protein from animal sources while formulating feeds for white shrimp.

Keywords: *Litopeneaus vannamei*, ration diet, soybean protein, proteases, amylase

1. Introduction

The production of aquatic organisms has increased substantially in recent decades due to the growing demand for new food sources. Shrimps farming features among the best developed aquaculture activities, being associate to high commercial market value (FAO, 2003). In Latin America, about 90% of the penaeid cultivated corresponds to the white shrimp *Litopenaeus vannamei* (Boone, 1931), a shrimp native to the Pacific Ocean (Wurmann et al., 2004). The quest for increased productivity has stimulated numerous studies aimed at determining various ideal zootechnical parameters for optimal performance in captivity of this shrimp (Nunes et al, 2006; Araneda et al., 2008, Esparza-Leal et al., 2010, Neal et al., 2010).

Nowadays feed remains the main obstacle for producers, since it represents about 60% of the total cost of shrimp production (Roy et al., 2009), being protein the most expensive component of the animals' diet (Lemos et al., 2003). The main ingredient in shrimp diet is the fishmeal, which is rich in quality protein and has a balanced amino acids and fatty acids composition that is suitable for the rapid growth of marine organisms (Cruz-Suárez et al. 2000). However, the use of fishmeal is affected by economic, ecological and market factors, raising its cost and restricting its use (Amaya et al., 2007). Thus, the substitution of fishmeal by alternative protein sources such as: meals from fisheries by-products, livestock, and plant ingredients have been increasingly include Keywords: bioflocs, digestive proteases, soy protein, *Litopenaeus vannamei* in commercial diets formulations (Samocha et al., 2004; EAPA, 2006; Amaya et al., 2007; Swick, 2007; Roy et al., 2009).

Nevertheless, it is important to note that the presence of anti-nutritional factors or deficiency of some essential amino acids may lead to reduced growth, since certain dietary components are not properly absorbed by the animal (Davis et al., 2004). According to Fernández et al., (2001), biochemical information about the enzymatic arsenal of an organism can be useful in selecting ingredients for use in animal feed, since their enzymatic profile is

closely related to feeding habits and the diets that are submitted. Furthermore, the specific activity of enzymes in the digestive tract can be used to illustrate the ability of crustaceans to explore various diets in order to supplement their nutritional requirements (Johnston and Freeman, 2005).

In this sense, the present study aimed to evaluate the effect of replacing fishmeal by soybean protein concentrate (SPC) on the performance of the digestive enzymes of *L. vannamei*.

2. Material and Methods

2.1. Reagents

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

2.2. Experimental culture

Specimens of *L. vannamei*, weighing 2.2 ± 0.51 g, were farmed in 50 circular tanks with total capacity of 500 L each, under continuous water recirculation and density of 70 animals / m² (40 shrimp / tank). The cultivation was conducted at the Institute of Marine Sciences at the Federal University of Ceará, Brazil (LABOMAR - UFC) for a period of 10 weeks. For the feeding of shrimps four isonitrogenous diets (38% crude protein) and isoenergetic (15.9 MJ / kg, dry matter basis) (Tables 1 and 2) were produced in the laboratory. For the group of four diets with the same level of inclusion of fish oil, the fishmeal was gradually replaced by soy protein concentrate (SPC) in 0% (control), 30%, 60% and 100%. The treatments were performed in triplicate. As inclusion of SPC increased, the level of dietary soybean oil (SBO) was also increased in order to balance the lipids and energy content

of the diets, and they were also supplemented with synthetic sources of methionine and lysine. The diets were offered twice a day according to the appetite of the animals. At the end of cultivation, biometry was performed using 15 shrimp/tank for each treatment. The length measurement was limited to distance from the eyeball to the end of telson. To body weight of the shrimp was measured by the model: Average weight gain (WG) in grams obtained by the difference between the final average weight (AW_f) and the initial weight (W_I): $WG = AW_f - W_i$.

2.3. Preparation of crude extract and determination of total soluble protein

Fifteen shrimps per treatment were collected for the removal of the midgut glands. The midgut glands were packed in dry ice and transported to the Laboratory of Enzymology at the Federal University of Pernambuco, Brazil (Labenz-UFPE), where they were thawed and homogenized at 5 mg / mL concentration (w / v) of tissue in a solution of 0.01 M Tris-HCl, pH 8 0, plus 0.15 M NaCl. Then the homogenate was centrifuged at 10,000 g for 25 min at 4 °C to remove tissue debris. The supernatants obtained (crude extracts) were collected and stored at -25 °C for further analysis. The dosage of total soluble protein in crude extracts was determined as described by Bradford (1976), using bovine serum albumin as standard protein.

2.4. Enzymatic assays

2.4.1. Total proteolytic activity

The total enzymatic activity of proteases present in crude extracts was performed using 1% azocasein as substrate, prepared in 10 mM Tris-HCl, pH 8 0. Aliquots containing 30 μL of the crude extract were incubated with 50 μL of substrate solution for 1 hour at 25 °C. Then it was added 240 μL of 10% trichloroacetic acid to stop the reaction. After 15 minutes the mixture was centrifuged at 8,000 xg for 5 minutes. The supernatant was collected and 70

μL of it was mixed in 130 μL 1M sodium hydroxide solution (revealing solution) in microplates. The absorbance was measured on a microplate reader (Bio Rad xMark™) at a wavelength of 450 nm. A negative control (blank) was performed, replacing the enzyme extract by a solution of 10 mM Tris-HCl, pH 8.0 with added 0.15 M NaCl. The activities were carried out in triplicate and one unit (U) of enzyme activity was defined as the amount of enzyme required to hydrolyze azocasein and produce a change of 0.001 units of absorbance per minute.

2.4.2. Specific proteolytic activities

The enzymatic activities of trypsin, chymotrypsin and leucine aminopeptidase, were determined in microplates with the use of Nα-benzoyl-DL-arginine-p-nitroanilide (BApNA), succinyl phenylalanine proline alanine aminotransferase pnitroanilide (SApNA) and pnitroanilide-leucine (Leu-p-Nan) as specific substrates, respectively (Bezerra et al., 2005). These substrates were dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 8 mM. All assays were performed in triplicate. The enzyme extracts (30 μL) were incubated with 140 μL of buffer Tris-HCl 0.1 M, pH 8.0, and 30 μL of the substrate for a period of 15 minutes. The absorbance was measured at 405 nm with a microplate reader (Bio Rad xMark™). One unit (U) of activity was defined as the amount of enzyme required to produce one mole of p-nitroaniline per minute. The specific activity was expressed as units per milligram of protein.

For the determination of aminopeptidasic activities 8 amino acids were used as specific substrates (Alanine, Arginine, Glycine, Histidine, Isoleucine, Leucine, Serine, Tyrosine). The assay was performed in microcentrifuge tubes at 37 °C. Each substrate (40 μL) was incubated with 40 μL of distilled water, 40 μL of enzyme extract and 480 μL of sodium phosphate buffer 0.05 M, pH 7.0. After incubation, the reaction was stopped by adding 200

μL of Garnet reagent prepared in sodium acetate buffer 0.2 M, pH 4.2, containing 10% Tween 20 (v / v). Posteriorly 200 μL of the mixture was transferred to a microplate. The absorbance was measured at 525 nm with a microplate reader (Bio Rad xMark™).

2.4.3. Amylolytic activity

The total amylase activity was based on the method of Bernfeld (1955), using 2% starch solution (w / v) as substrate. The reaction consisted in the incubation of 20 μL of the crude extract with 125 μL of buffer 0.1 M Tris-HCl, pH 8.0 and 125 μL of the substrate at 37 °C for 10 minutes. Then 30 μL of incubated solution was added to 300 μL of 3,5-dinitrosalicylic acid (DNSA) at 100 °C for 10 minutes to stop the reaction. Soon after its cooling, 200 μL of the solution were transferred to microplate and the absorbance was measured at 570 nm using a microplate reader (Bio Rad xMark™). One unit of enzyme activity was expressed as mg released maltose at 37 °C per minute per milligram of protein. To determine the concentration of released maltose, a calibration curve was prepared using comercial maltose.

2.5. SDS-PAGE

The polyacrylamide gel electrophoresis containing sodium dodecyl sulfate (SDS-PAGE) was performed according to the methods of Laemmli (1970), using 12.5% (w / v) separation gel and 4% (w / v) stacking gel. Samples containing 100 μg of protein were applied into the gel, along with a standard solution of defined molecular mass containing the following proteins: Myosin (198.8 kDa), β-galactosidase (115.7 kDa), Bovine serum albumin (96.7 KDa), ovalbumin (53.5 kDa), Carbonic anhydrase (37.1 kDa), Soybean trypsin inhibitor (29.1 kDa), Lysozime (19.5 kDa), Aprotinin (6.9 kDa). The gel was stained with a solution

composed of Coomassie Brilliant Blue 0.01% (w / v), methanol 25% (v / v) and acetic acid 10% (v / v) and after 24 hours was destained in solution with the same composition but devoid of the dye for visualization of bands.

2.6. Zymograms

Zymograms were performed to determine the proteolytic activity (Garcia-Carreño et al., 1993) and amylolytic activity (Fernández et al., 2001). Both zymograms were initiated by electrophoresis (SDS-PAGE) under immersion in an ice bath. The separation staking gels used were of 12.5% and 4% (w / v) respectively, being applied 30 μg of protein into the gel.

2.7. Statistical analysis

Data of enzyme activity were analyzed using one-way analysis of variance (ANOVA) complemented with Tukey's test. Differences were reported as statistically significant when $P<0.05$, using the program MicrocalTM OriginTM version 8.0 (Software, Inc, U.S.).

3. Results

In vitro assays were performed with the use of long-chain substrates to determine the action of enzymes present in extracts of the midgut glands of *L. vannamei* cultured with different diets. The results related to these activities are shown in Figure 1. The three dietary treatments, that consisted on the replacements of 30% (S_{30}), 60% (S_{60}) and 100% (S_{100}) of fishmeal by soybean protein concentrate (SPC), did not show any significant differences ($p <0.05$) in the total proteolytic activity between them, using 1% azocasein as substrate. However, it was observed that the experimental diets differed significantly ($p <0.05$) of the control group (0.90 ± 0.03 U.mg^{-1}) (Figure 1A). Regarding the performance of amylase, the

treatment S_{100} (5.04 ± 0.33 $U.mg^{-1}$) was more efficient in the hydrolysis 2% starch solution, differing significantly ($p < 0.05$) of the control (4.01 ± 0.32 $U.mg^{-1}$) (Figure 1B).

Analyzing the specific activities of these proteases in the presence of p-nitroanilide substrates (Figure 2) it was revealed that the S_{30} (13.13 ± 0.53 $mU.mg^{1}$) and S_{60} (11.82 ± 0.21 $mU.mg^{-1}$) treatments had the highest trypsin activity. These groups did not show significant differences. Moreover, these treatments were statistically different of the control (9.23 ± 0.52 $mU.mg^{-1}$), and S_{100} diet (9.09 ± 0.40 $mU.mg^{-1}$) (Figure 2A). With the SApNA substrate was assessed the activity of enzymes chymotrypsin and showed that animals submitted to diet composed only with fish protein (C) showed higher activity for chymotrypsin (13.78 ± 1.61 $mU.mg^{-1}$) and was significantly different ($p < 0.05$) compared to S_{60} and S_{100} treatments. The lowest chymotrypsin activity was found in the S_{60} treatment (4.28 ± 0.64 $mU.mg^{-1}$), which exhibited no statistical differences in relation to diet with 100% SPC. The S_{30} treatment (10.77 ± 1.26 $mU.mg^{-1}$) showed no statistical difference to both the control group and the S_{100} treatment (Figure 2B). The activity of leucine aminopeptidase using Leu-p-Nan substrate was the highest in C treatment (0.45 ± 0.03 $mU.mg^{-1}$). This control group was significantly different ($p < 0.05$), when compared to experimental diets, which proved to be similar among them. Thus, it was observed that the catalytic action of this enzyme decreased with the increase of the soy protein concentration in the diets (Figure 2C).

Variations in nutrients of animal and vegetable origin in the diets of shrimp also affected their aminopeptidase activity. The assays were performed in the presence of β-naphthylamide substrates, showing activity for all amino acids used (Figure 3). The total replacement of fish protein for soy in the diet of penaeid induced a decrease in aminopeptidasic activity when using the nonpolar amino acid (Ala-) as substrate (Figure 3A). Using the basic substrate (Arg-), the highest aminopeptidasic activity was found for S_{30} diet, but it did not statistically differed ($p < 0.05$) from the control group. The increase in the level

of substitution of animal protein by vegetable (S_{60} and S_{100}) also resulted in decreased aminopeptidasic activity (Figure 3B). For nonpolar (Leu-) and neutral polar (Tyr-) substrates, the action of aminopeptidases was unmoved statistical variations diets (Figure 3C and 3D). It was noted that for neutral polar (Ser-) substrate the activity of the aminopeptidase of shrimps subjected to experimental diets gradually decreased (Figure 3E). The aminopeptidasic activity of cultured animals, when the neutral polar amino acid (Gly-) were used as substrate, reached the highest value in the S_{30} treatment (1.05 ± 0.08 $U.mg^{-1}$), revealing significant differences ($p < 0.05$) when compared to the control group (0.80 ± 0.02 $U.mg^{-1}$) (Figure 3F). With the use of nonpolar amino acid (Ile-), the aminopeptidasic activity was higher in S_{60} (0.69 ± 0.02 $U.mg^{-1}$) followed by the control group (0.68 ± 0.01 $U.mg^{-1}$). Both were not statistically different between them, but showed significant differences with the other treatments (Figure 3G). For the basic amino acid (His-), the S_{60} diet showed the highest value of aminopeptidasic activity (0.85 ± 0.04 $U.mg^{-1}$). The group C showed statistical differences when compared to the experimental diets (Figure 3H).

Proteins from the midgut glands of cultured *L. vannamei* were analyzed by SDS-PAGE (Fig. 4 A). A common pattern was observed in the number of bands in each treatment. There were detected twenty-six bands ranging from 6.9 kDa to 198.8 kDa. The proteolityc zymogram revealed differences in the number and intensity of bands. Eighteen bands (C and S_{30}) were seen, these with greater intensity, and twelve for both S_{60} and S_{100}. The zymogram of amylase revealed five bands with amylase activity for all treatments (Figure 4).

The analysis of average body weight gain of shrimps showed the highest value when used the S_{30} diet (8.48±1.03 g), however did not evidenced significant differences ($p<0.05$) between treatments (Figure 5).

4. Discussion

Since one of the premises of sustainable aquaculture is to minimize the use of resources of limited availability, several studies evaluating the replacement of the fishmeal by alternative protein sources in the production of feeds for aquatic organisms has been reported (Tidwell et al., 1993; Webster and Lim, 2002). The effect of alternative protein sources on digestive enzymes of penaeid has also been reported (Gimenez et al., 2009).

In this study, assays employing of long-chain substrates (azocasein and starch) showed increased enzymatic activity as the fishmeal was replaced by SPC in the diets. Although fishmeal contain a supply of high quality protein and a balance of fatty acids and amino acids suitable for the rapid growth of marine organisms (Cruz-Suarez et al., 2000; Hertrampf; Piedad-Pascual, 2000), the inclusion of SPC in diets for *L. vannamei* showed a positive effect on digestion of both proteins and carbohydrates. As is well known, the presence of a high content of endo and exoproteases renders protein digestion more efficient. A digestive adaptation to new food preferences may be occurring in this period.

The analysis of specific proteolytic activities in the presence of p-nitroanilide substrates, revealed high values for both trypsin and chymotrypsin, compared to the activity using the substrate Leu-p-Nan. These results are consistent with the literature, because generally, the crustacean digestive system presents a high concentration of serine proteases, mainly trypsin and chymotrypsin (Fernández et al., 1997). Trypsin also plays an important role in digestion through the activation of zymogens of both itself and other endopeptidases (Natalia et al., 2004).

Despite the intense trypsin activity observed in the midgut glands of cultured animals, occurred a variation of these activities due to a change in diet composition. The replacement of fishmeal by soy protein concentrate at 30 and 60% provided an increase of trypsin activity compared to other treatments (C and S_{100}). As the literature reports, the trypsin activity in *L.*

vannamei can be strongly modulated by the quality and quantity of dietary protein (Lee et al., 1984). The increase of trypsin activity can be suggested as a consequence of an adjustment mechanism to low protein content of the diet or low availability of dietary protein because of relatively poor digestibility. (Le Vay et al., 1993; Rodríguez et al., 1994; Kumlu and Jones, 1995; Lemos and Rodríguez, 1998).

The chymotrypsin and leucine aminopeptidase activities from midgut glands of cultured *L. vannamei* decreased as fishmeal was replaced by soybean protein concentrate in diets. These results indicate the adaptation in *L. vannamei* of theses digestive enzymes to the quality of dietary protein. However, possible factors limiting enzymatic hydrolysis may be suggested, as the presence of inhibitors or deficiency of certain nutrients in the diet. The effect of alternative sources of protein on the activity of chymotrypsin in penaeid was also reported by Gimenez et al., (2009), highlighting the achievements in researchs, involving the replacement of fishmeal by soy protein in diets for shrimp.

Several authors have reported the study of aminopeptides in fish (Sabapathy and Teo, 1993; Tengjaroenkul et al., 2000; 2002; Natalia et al., 2004; Refstie et al., 2006). This demonstrates the importance of understanding the role of these enzymes in the digestion of aquatic organisms. However, there is little information available on aminopeptidases in shrimp.

In this study also were analyzed the aminopeptidasic activities of midgut glands of the farmed shrimp, through β-naphthylamide substrates. Elevated levels of aminopeptidasic activity were observed in presence of arginine and alanine. This may be related to the efficient digestion and incorporation of these essential nutrients (Lemos and Nunes, in press). Moreover the results corroborate the requirements described in the literature for arginine, since this essential amino acid is described as one of the most limiting in commercial shrimp

diets (Fox et al., 1995). Heu et al. (2003) also found high activities of aminopeptidases to arginine in the residues of processing in *Pandalus borealis* and *Trachypena curvirostris*.

Although the enzymatic activity for substrates (Ala- and Arg-) to be considered high, its values decreased as the fishmeal was gradually replaced by levels of SPC. A similar result was observed with the use of serine as substrate. Studies Ezquerra et al. (1999) demonstrated the influence of diet composition on aminopeptidasic activity in *L. vannamei.* In their experiments, the activity of aminopeptidase also decreased when the shrimps were subjected to the diet with soy protein.

As is known, the nutritional value of protein ingredients, usually defined by protein and amino acids in the composition may influence the enzymatic hydrolysis of aminopeptidases. However, other nutritional parameters such as availability of minerals, carbohydrates, lipids and presence of antinutritional factors could also affect the digestive system of shrimp.

The analysis of the extracts of midgut glands of cultured *L. vannamei* showed no differences by SDS-PAGE in the number of proteolytic bands between treatments. However, the zymogram of proteases showed a decrease in the intensity of proteolytic bands as fishmeal was gradually replaced by soy protein in diets. Although the amylase activity to have revealed significant differences between treatments, the zymogram of amylase was not able to highlight those differences.

The levels of substitution of fishmeal by SPC at 30, 60 and 100% in diets for *L. vannamei* provided a positive effect on animals performance mainly relationship to body weight gain. Similar result was found by Samocha et al. (2004), where *L. vannamei* were fed practical diets containing 32% CP (crude protein) and 100% of the fishmeal was replaced by co-extruded soybean poultry by-product meal. Commercial shrimp feeds are commonly reported to include fishmeal at levels between 25% and 50% of the total diet (Dersjant-Li,

2002; Tacon and Barg, 1998). However, recent studies have shown that commercial shrimp feeds containing 30 - 35% crude protein can include levels as low as 7.5 - 12.5% fishmeal without compromising shrimp performance (Fox et al., 2004). The successful replacement of animal protein sources with plant proteins in shrimp feeds also has been achieved by Davis et al. (2004).

5. Conclusion

It was possible to determine the influence of diet on the *L. vannamei* digestive enzymes. The differences in enzyme activities of midgut glands of the farmed shrimp provided important information about the potential of white shrimp (*L. vannamei*) to use alternative food formulations with lower levels of animal protein sources. Given the results above, it was concluded that the substitution of fishmeal by SPC at levels of 30, 60 e 100% in diets for *L. vannamei* offered a positive effect on shrimps performance. This fact corroborates with the information that *L. vannamei* can be fed with vegetable protein sources to replace fishmeal without affecting the development of the animal. It is expected, with determining the feasibility of partial or total substitution of animal protein for vegetable protein, contribute to reducing the cost of feed, without reducing the productivity of production systems. Also, are expected ecological benefits, such as preservation of species of marine fish and recovery of the balance of the marine environment.

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References

Amaya, E., Davis, D.A., Rouse, D.B., 2007. Alternative diets for the Pacific white shrimp *Litopenaeus vannamei*. Aquaculture 262, 419-425.

Araneda, M., Pérez E.P., Gasca-Leyva, E., 2008. White shrimp *Penaeus vannamei* culture in freshwater at three densities: Condition state based on length and weight. Aquaculture 283, 13-18.

Bernfeld, P., 1955. Amylases, α and ß. IN Colowick, S.P. & Kaplan, N.O. (Eds.) Methods in Enzymology. New York, Academic Press.

Bezerra, R.S., Lins, E.J.F., Alencar, R.B., Paiva, P.M.G., Chaves, M.E.C., Coelho, L.C.B.B., Carvalho Júnior, L.B., 2005. Alkaline proteases from intestine of Nile tilapia (*Oreochromis niloticus*). Process Biochem. 40, 1829-1834.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein binding. Anal. Biochem. 72, 248-254.

Cruz-Suárez, L.E., Ricque-Marie, M.; Nieto-López, M.; Tapía-Salazar, M., 2000. Revisión sobre calidad de harinas y aceites de pescado para la nutrición del camarón. *In*: Civera-Cerecedo, R.; Pérez-Estrada, C.J.; Ricque-Marie, D.; Cruz-Suárez, L.E. (Eds.) Avances em Nutrición Acuícola IV. Memorias del IV Simposium Internacional de Nutrición Acuícola 1998. México.

Davis, D.A., Samocha, T.M., Bullis, R.A., Patnaik, S., Browdy, C., Stoke, A., Atwood, H., 2004. Practical diets for *Litopenaeus vannamei*, (Boone, 1931): Working towards organic and/or all plant production diets. Avances en Nutricion Acuicola. 16-19. Hemosillo, Sonora, México.

Dersjant-Li, Y., 2002. The use of soy protein in aquafeeds. Avances em Nutricion Acuicola VI. Memorias del VI Simposium Internacional de Nutricion Acuicola. 3-6 de Septiembre del 2002. Cancun, Quintana Roo, Mexico.

EAPA (European Animal Protein Association), 2006. Sustainable resources secure the future of aquaculture: The use of natural animal proteins in fish feed to develop a more environmentally responsible and ecologically sustainable aquaculture. Int. Aquafeed vol. 9, n. 4, p. 20-23.

Esparza-Leal, H.M., Ponces-Palafox, J.T., Aragón-Noriega, E.A., Arredondo-Figueroa, J.L., Gómez, M.G.U., Valenzuela-Quiñonez, W., 2010. Growth and performance of the whiteleg shrimp *Penaeus vannamei* (Boone) cultured in low-salinity water with different stocking densities and aacclimation times. Aquac. Res. 41, 878-883.

Ezquerra, J.M., Garcia-Carreño, F.L., Arteaga, G., Haard, N.F., 1999. Effect of feed diet on aminopeptidase activities from the hepatopancreas of white shrimp (*Penaeus vannamei*). J. Food Biochem. Volume: 23 Issue: 1 Pages: 59-74.

FAO (Food and Agriculture Organization of the United Nation). 2003, http: //www.fao.org/fi/statist/fisoft/FISHPLUS.aps.

Fernández, I., Moyano, F.J., Díaz, M., Martínez, T., 2001. Characterization of [alpha]-amylase activity in five species of Mediterranean sparid fishes (*Sparidae, Teleostei*). J. Exp. Mar. Biol. Ecol [S.I.], v. 262, n. 1, p. 1-12.

Fernández, I., Oliva, M., Carrillo, O., van Wormhoudt, A., 1997. Digestive enzymes of *Penaeus notialis* during reproduction and moulting cycle. Comp. Biochem. Physiol. 118A, 1267-1271.

Fox, J.M., Lawrence, A.L., Li-Chan, E., 1995. Dietary requirement for lysine by juvenile *Penaeus vannamei* using intact and free amino acid sources. Aquaculture 131, 279–290.

Fox, J.M., Lawrence, A.L., Smith, F., 2004. Development of a low-fish meal feed formulation for commercial production of *Litopenaeus vannamei*. Avances en Nutricion Acuicola VII. Memorias del VII Simposium Internacional de Nutricion Acuicola. 16–19 Noviembre, 2004. Hermosillo, Sonora, Mexico.

Garcia-Carreño, F.L., Dimes, L.E., Haard, N.F., 1993 Substrate-gel electrophoresis for composition and molecular weight of proteinases or proteinaceous proteinase inhibitors. Anal. Biochem. 214, 65-69.

Gimenez, A.V.F., Diaz, A.C., Velurtas, S.M., Fenucci, J.L., 2009. Partial Substitution of Fishmeal by Meat and Bone Meal, Soybean Meal, and Squid Concentrate in Feeds for the Prawn, *Artemesia longinaris*: Effect on Digestive Proteinases. Isr. J. Aquac. - Bamidgeh, Vol: 61 Issue: 1, 48-56.

Hertrampf, J.W.; Piedad-Pascual, F., 2000. Handbook on Ingredients for Aquaculture Feeds. Holanda: Kluwer.

Heu, M.S., Kim, J.S., Shahidi, F., Jeong, Y., Jeon, Y.J., 2003. Extraction, fractionation and activity characteristics of proteases from shrimp processing discards. J. Food Biochem. 27, 221 - 236.

Johnston, D.; Freeman, J., 2005. Dietary preference and digestive enzyme activities as indicators of trophic resource utilization by six species of crabs. Biol. Bull. [S.I.], v. 208, p. 36-46.

Kumlu, M., Jones, D.A., 1995. The effect of live and artificial diets on growth, survival, and trypsin activity in larvae of *Penaeus indicus*. J. World Aquac. Soc. 26, 406 - 415.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Le Vay, L., Rodríguez, A., Kamarudin, M.S., Jones, D.A., 1993. Influence of live and artificial diets on tissue composition and trypsin activity in *Penaeus japonicus* larvae. Aquaculture 118, 287 - 297.

Lee, P.G., Smith, L.L., Lawrence, A.L., 1984. Digestive proteases of *Penaeus vannamei* Boone: relationship between enzyme activity, size and diet. Aquaculture 42, 225–239.

Lemos, D, Nunes, A.J.P., in press. Prediction of culture performance of juvenile *Litopenaeus vannamei* by *in vitro* (pH-stat) degree of feed protein hydrolysis with species-specific enzymes.

Lemos, D., 2003. Testing quality of feeds and feed ingredients: in vitro determination of protein digestibility with enzymes from the target species. Int. Aquafeed.

Lemos, D., Rodríguez, A., 1998. Nutritional effects on body composition, energy content and trypsin activity of *Penaeus japonicus* during early postlarval development. Aquaculture 160, 103 - 116.

Natalia, Y., Hashim, R., Ali, A., Chong, A., 2004. Characterization of digestive enzymes in a carnivorous ornamental fish, the Asian bony tongue *Scleropages formosus* (*Osteoglossidae*). Aquaculture 233, 305 - 320.

Neal, R.S., Coyle, S.D., Tidwell, J.H., Boudreau, B.M., 2010. Evaluation of Stocking Density and Light Level on the Growth and Survival of the Pacific White Shrimp, *Litopenaeus vannamei*, Reared in Zero-Exchange Systems. J. World Aquac. Soc. 41. Issue: 4, 533-544.

Nunes, A.J.P., Sá, M.V.C., Carvalho, E.A., Neto, H.S., 2006. Growth performance of the white shrimp *Litopenaeus vannamei* reared under time- and rate-restriction feeding regimes in a controlled culture system. Aquaculture 253, 646-652.

Refstie, S., Glencross, B., Thor, L., Sorensen, M., Lileeng, E., Hawkins, W., Krogdahl, A., 2006. Digestive fuction and intestinal integrity in Atlantic salmon (*Salmo salar*) fed kernel

meals and protein concentrates made from yellow or narrow-leafed lupins. Aquaculture 261, 1382 - 1395.

Rodríguez, A., Le Vay, L., Mourente, G., Jones, D.A., 1994. Biochemical composition and digestive enzyme activity in larvae and postlarvae of *Penaeus japonicus* during herbivorous and carnivorous feeding. Mar. Biol. 118, 45 - 51.

Roy, L.A., Bordinhon, A., Sookying, D., Davis, D.A., Brown, T.W., Whitis, G.N., 2009. Demonstration of alternative feeds for the Pacific white shrimp, *Litopenaeus vannamei*, reared in low salinity waters of west Alabama. Aquac. Res. 40, 496-503.

Sabapathy, U., Teo, L.H., 1993. A quantitative study of some digestive enzymes in the rabbitfish, *Siganus canaliculatus* and the sea bass (*Lates calcarifer*). J. Fish Biol. 42, 595 - 602.

Samocha, T., Davis, D.A., Saoud, I.P., De Bault, K., 2004. Substitution of fish meal by co-extruded soybean poultry by-product meal in practical diets for the Pacific white shrimp, *Litopenaeus vannamei*. Aquaculture 231, 197–203.

Swick, R.A., 2007. Soybean meal and soy protein concentrate for shrimp production. Int. Aquafeed, Mar-Apr.

Tacon, A.G.J., Barg, U.C., 1998. Major challenges to feed development for marine and diadromous finfish and crustacean species. In: De Silva, S.S. (Ed.), Tropical Mariculture. Academic Press, San Diego, CA, USA, pp. 171–208.

Tengjaroenkul, B., Smith, B.J., Caceci, T., Smith, S.A., 2000. Distribution of intestinal enzyme activities along the intestinal tract of cultured Nile tilapia, *Oreochromis niloticus* L. Aquaculture 182, 317 - 327.

Tengjaroenkul, B., Smith, B.J., Smith, S.A., Chatreewongsin, U., 2002. Ontogenic development of intestinal enzymes of cultured Nile tilapia, *Oreochromis niloticus* L. Aquaculture 211, 241 - 251.

Tidwell, J.H., Webster, C.D., Yancey, D.H., D'Abramo, L.R., 1993. Partial and total replacement of fish meal with soybean meal and distillers' by-products in diets for pond culture of the freshwater prawn (*Macrobrachium rosenbergii*). Aquaculture 118, 119 - 130.

Webster, C.D., Lim, C.E., 2002. Nutrient Requirements and Feeding of Finfish for Aquaculture. CAB International, New York, NY.

Wurmann, C., Madrid, R.M., Brugger, A.M., 2004. Shrimp farming in Latin America: currents status, opportunities, challenges and strategies for sustainable development. Aquac. Econ. Manag. 8, 117–141.

Figure captions

Figure 1. Proteolytic (A) and amylase activity (B) in the midgut glands of the *Litopenaeus vannamei* using long-chain substrates, 1% azocasein and 2% starch, respectively. The shrimps were fed diets with gradual replacement of fishmeal by soybean protein concentrate in 0% (C), 30% (S_{30}), 60% (S_{60}) and 100% (S_{100}). Different letters show statistical differences ($p < 0.05$).

Figure 2. Specific proteolytic activities in the midgut glands of the *L. vannamei* in the presence of p-nitroanilide substrates. The enzymatic activities of trypsin (A), chymotrypsin (B) and leucine-aminopeptidase (C) were determined with the use of Nα-benzoyl-DL-arginine-p-nitroanilide (BApNA), succinyl phenylalanine proline alanine aminotransferase p-nitroanilide (SApNA) and p-nitroanilide-leucine (Leu-p-Nan) as substrates, respectively. The specimens cultured had changes in their diets where fishmeal was gradually replaced by soy protein at concentrations of 0% (C), 30% (S_{30}), 60% (S_{60}) and 100% (S_{100}). Different letters show statistical differences ($p < 0.05$).

Figure 3. Aminopeptidase activities in the midgut glands of the *L. vannamei*, using β-naphthylamide substrates. Eigth amino acids were employed as specific substrates: Ala (A), Arg (B), Leu (C), Tyr (D), Ser (E), Gly (F), Ile (G), Hist (H). The diet established for cultured penaeid was based on the gradual replacement of fishmeal by soybean protein concentrate in 0% (C), 30% (S_{30}), 60% (S_{60}) and 100% (S_{100}). Different letters show statistical differences ($p < 0.05$).

Figure 4. Polyacrylamide gel electrophoresis - SDS-PAGE of crude extracts in the midgut glands of cultured *L. vannamei* (A). The diet established for cultured penaeid was based on the gradual replacement of fishmeal by soybean protein concentrate in 0% (C), 30% (S_{30}), 60% (S_{60}) and 100%(S_{100}). A standard molecular weight (P) was applied to gel. In (B) zymogram of protease activity and (C) amylase zymogram in the midgut glands of the cultured *L. vannamei*. Both electrophoresis and zymograms was used in an electric current of 11mA.

Figure 5. Average body weight gain of the reared *L. vannamei* for ten weeks in an experimental clearwater system. The shrimps were fed diets with progressive replacement of anchovy fishmeal by soy protein concentrate at fish oil inclusion level of 2%. The shrimps showed initial weight 2.02±0.51g.

Table **1**. Ingredient composition of practical diets for *L. vannamei* used to evaluate the replacement of fishmeal by soy protein concentrate.

Ingredients	Experimental groups			
	C	S_{30}	S60	S100
Soybean meal, 46% CP (Bunge)	33.00	33.00	33.00	33.00
Wheat flour	25.00	25.00	25.00	25.00
Poultry meal, 61% (Nordal)	15.00	15.00	15.00	15.00
Fishmeal, Anchoveta 67% (Copeinca)	12.00	8.50	5.00	0.00
Soy protein concentrate, 62% (Selecta)	0.00	3.84	7.75	13.32
Soybean oil	2.04	2.30	2.79	3.45
Fish oil	1.00	1.00	1.00	1.00
Broken rice	4.15	3.54	2.59	1.27
Vitamin mineral premix, Shrimp SI (DSM)	2.00	2.00	2.00	2.00
Soy lecithin	1.50	1.50	1.50	1.50
Monodicálcico phosphate, 20% (Serrana)	1.30	1.30	1.30	1.30
Salt	1.00	1.00	1.00	1.00
Potassium chloride	1.00	1.00	1.00	1.00
Synthetic binder, Pegabind (Bentoli)	0.70	0.70	0.70	0.70
L-Lysine (Degussa)	0.12	0.13	0.15	0.17
DL-Methionine 99% (Degussa)	0.00	0.04	0.08	0.14
Magnesium sulfate	0.12	0.07	0.07	0.08
Rovimix Stay-C 35% (DSM)	0.07	0.07	0.07	0.07

Table 2. Nutritional composition of experimental diets offered to the shrimp *L.vannamei.*

Ingredients	Experimental groups			
	C	S_{30}	S_{60}	S_{100}
Basic Nutrients				
Ash	5.87	5.52	5.16	4.65
Crude Fat	8.00	8.02	8.25	8.55
Crude Protein	36.00	36.00	36.00	36.00
Crude Fiber	1.61	1.77	1.93	2.16
Moisture	8.70	8.65	8.59	8.50
Aminoacids (%)				
Met + Cys	1.1497	1.1541	1.1589	1.1658
Methionine	0.6706	0.6700	0.6700	0.6700
Lysine	2.2508	2.2508	2.2508	2.2508
Phe + Tyr	2.7711	2.8137	2.8567	2.9180
Alanine	0.0000	0.1044	0.2107	0.3623
Arginine	2.4284	2.4728	2.5177	2.5818
Histidine	0.8392	0.8445	0.8500	0.8578
Phenylalanine	1.6152	1.655	1.6953	1.7528
Isoleucine	1.5658	1.5846	1.6040	1.6316
Leucine	2.6806	2.6998	2.7193	2.7470
Cystine	0.4791	0.4841	0.4889	0.4958
Threonine	1.3246	1.3247	1.3251	1.3257
Tryptophan	0.4215	0.4260	0.4305	0.4370
Tyrosine	1.1541	1.1569	1.1596	1.1634
Valine	1.7354	1.7443	1.7534	1.7663
TSSA	1.1497	1.1541	1.1589	1.1658
Lipids (%)				
Arachidonic (C20:4n6)	0.0177	0.0128	0.0080	0.0010
Docosahexaenoic (C22:6n3)	1.5509	1.1242	0.6975	0.0880
Eicosapentaenoic (C20:5n3)	0.4360	0.3584	0.2808	0.1700
Linoleic (C18:2n6)	1.7172	1.8402	2.0795	2.4056
Linolenic (C18:3n3)	0.2589	0.2542	0.2638	0.2756
Sum n3 EFA	5.6373	4.1427	2,6627	0.5464
Sum n6 EFA	1.2683	1.3526	1.5470	1.8098
Cholesterol	0.2513	0.2513	0.2513	0.2513
Phospholipid	1.4250	1.4250	1.4250	1.4250
Minerals (%)				
Calcium	1.9939	1.9124	1.8308	1.7142
Magnesium	0.1373	0.0800	0.0800	0.0800
Manganese	0.0005	0.0004	0.0002	0.0000
Potassium	1.3301	1.3842	1.4384	1.5157
Sodium	0.5401	0.5243	0.5085	0.4858
Total Phos.	1.1643	1.1378	1.1108	1.0722
Avail. Phos.	1.0185	0.9755	0.9322	0.8705
Chlorine	1.2672	1.2485	1.2296	1.2025
Energy (KJ/kg)				
Gross Energy (Kcal/kg)	4.282	4.266	4.261	4.251
Metabolizable Carbohydrate	5.964	5.925	5.845	5.735
Metabolizable Fat	3.024	3.032	3.119	3.232
Metabolizable Protein	6.048	6.044	6.040	6.034
Metabolizable, Energy	15.036	15.001	15.003	15.000
Other (%)				
Vitamin C (Ascorbic Acid)	0.025	0.025	0.025	0.025

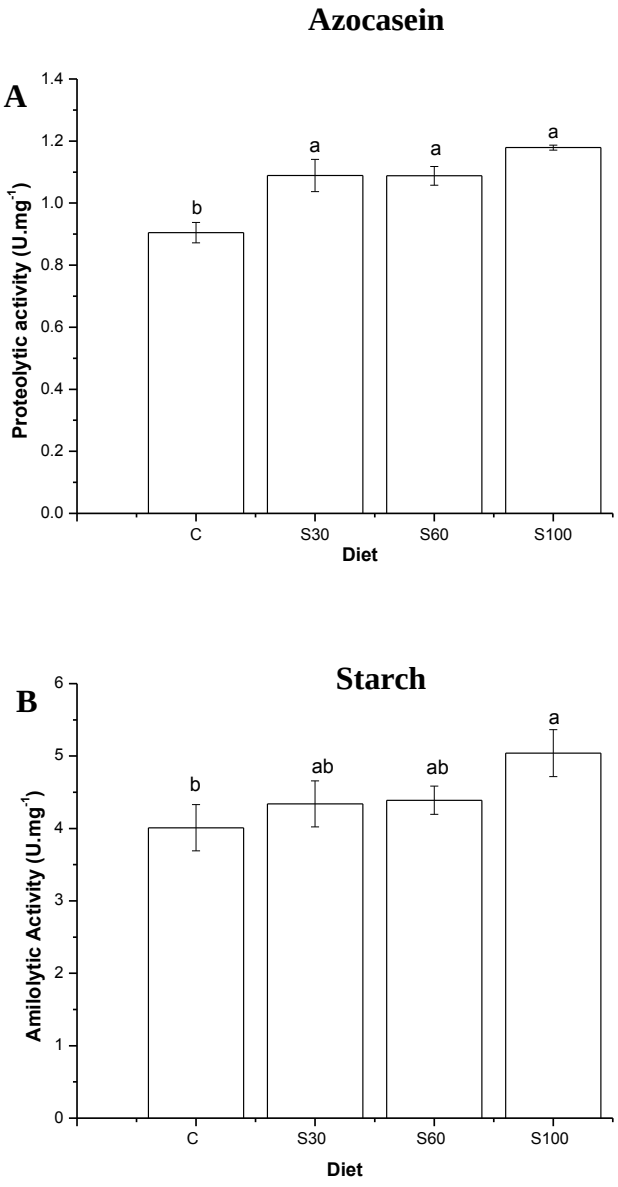
Azocasein
A
Proteolytic activity (U.mg-1)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
b
a
a
a
C
S30
S60
S100
Diet
Starch
B
Amilolytic Activity (U.mg-1)
6
5
4
3
2
1
0
b
ab
ab
a
C
S30
S60
S100
Diet

Figure 1

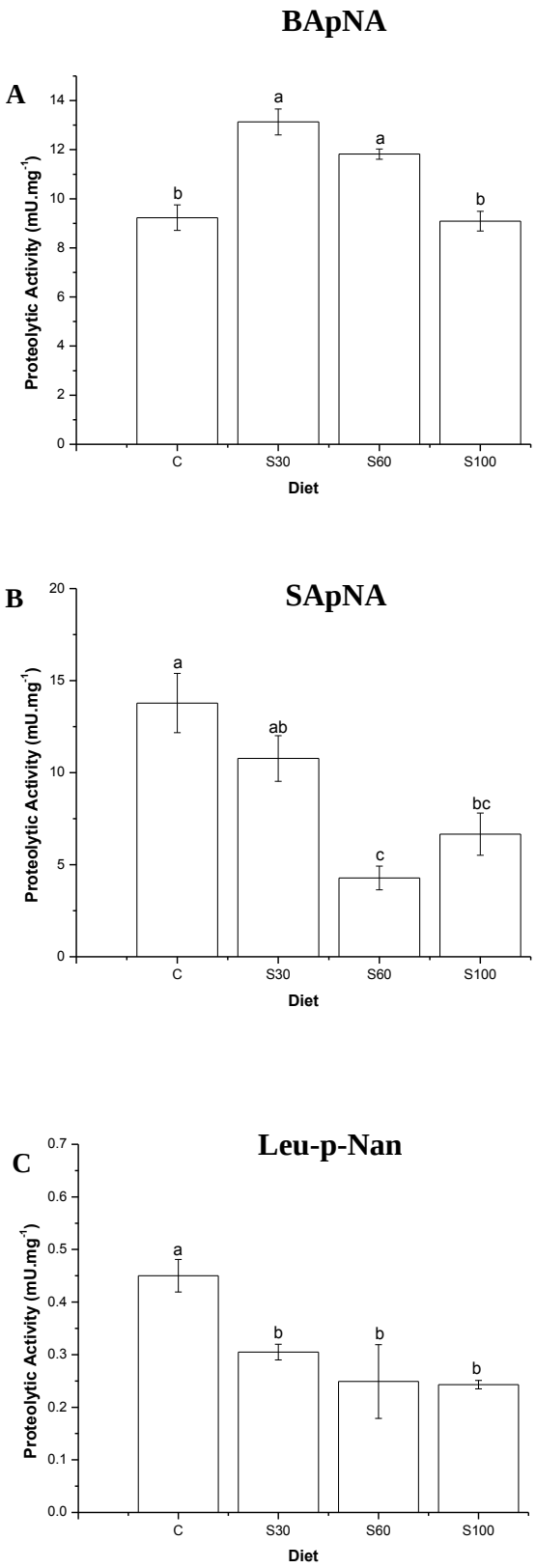
BApNA
A
Proteolytic Activity (mU.mg-1)
0
2
4
6
8
10
12
14
a
a
b
b
C
S30
S60
S100
Diet
B
SApNA
Proteolytic Activity (mU.mg-1)
0
5
10
15
20
a
ab
c
bc
C
S30
S60
S100
Diet
C
Leu-p-Nan
Proteolytic Activity (mU.mg-1)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
a
b
b
b
C
S30
S60
S100
Diet

Figure 2

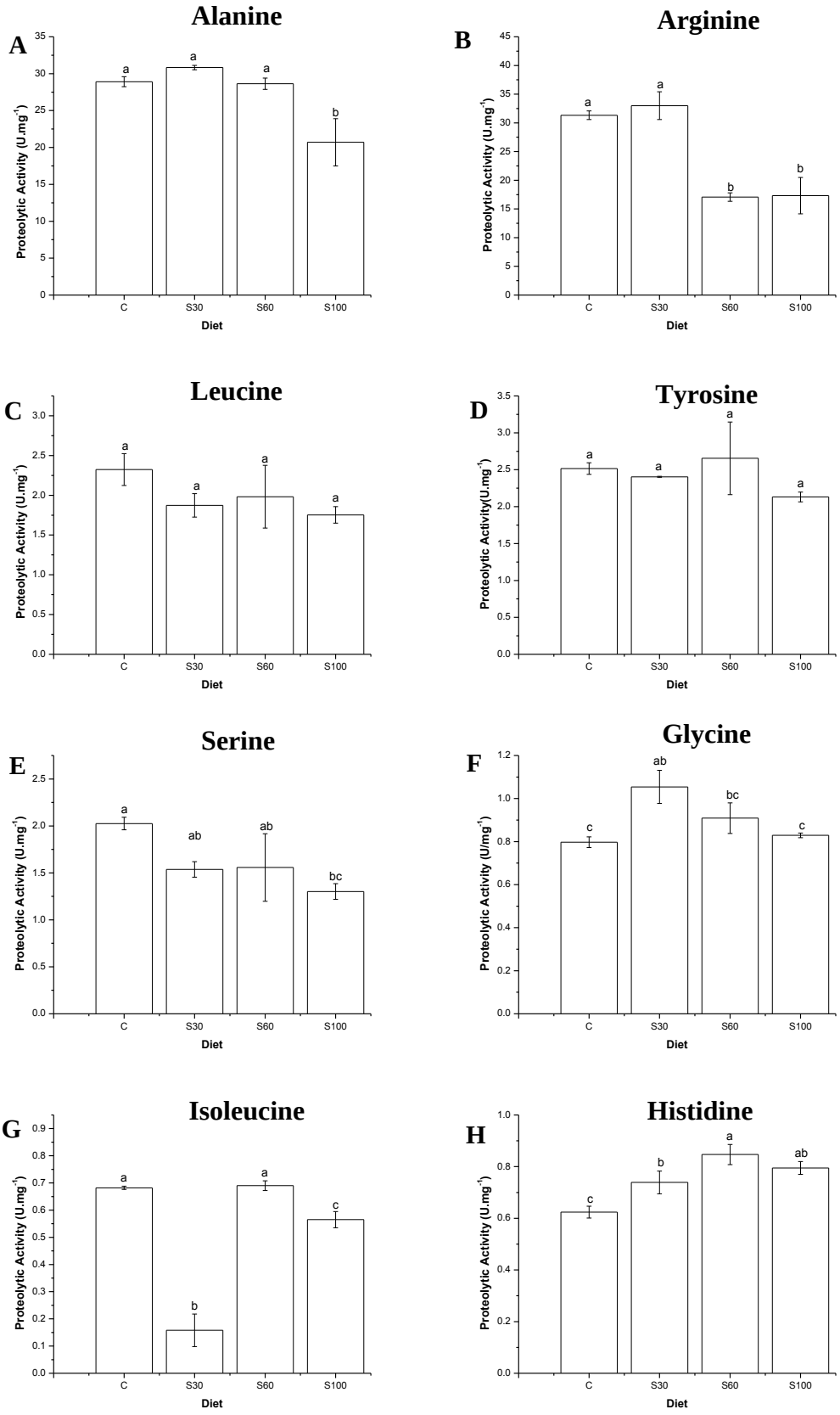
A
Alanine
Proteolytic Activity (U.mg-1)
a
a
a
b
C
S30
S60
S100
Diet
B
Arginine
Proteolytic Activity (U.mg-1)
a
a
b
b
C
S30
S60
S100
Diet
C
Leucine
Proteolytic Activity (U.mg-1)
a
a
a
a
C
S30
S60
S100
Diet
D
Tyrosine
Proteolytic Activity(U.mg-1)
a
a
a
a
C
S30
S60
S100
Diet
E
Serine
Proteolytic Activity (U.mg-1)
a
ab
ab
bc
C
S30
S60
S100
Diet
F
Glycine
Proteolytic Activity (U/mg-1)
c
ab
bc
c
C
S30
S60
S100
Diet
G
Isoleucine
Proteolytic Activity (U.mg-1)
a
b
a
c
C
S30
S60
S100
Diet
H
Histidine
Proteolytic Activity (U.mg-1)
c
b
a
ab
C
S30
S60
S100
Diet

Figure 3

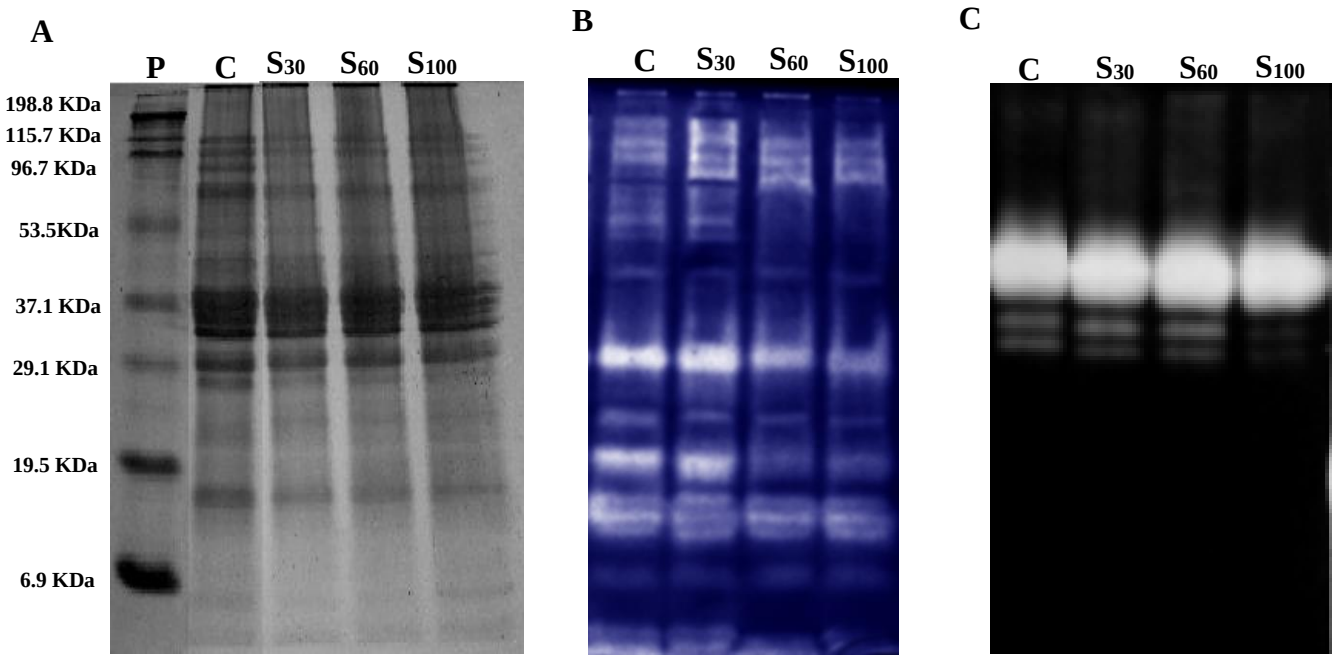
A
P
C
S30
S60
S100
198.8 KDa
115.7 KDa
96.7 KDa
53.5KDa
37.1 KDa
29.1 KDa
19.5 KDa
6.9 KDa
B
C
S30
S60
S100
C
C
S30
S60
S100

Figure 4

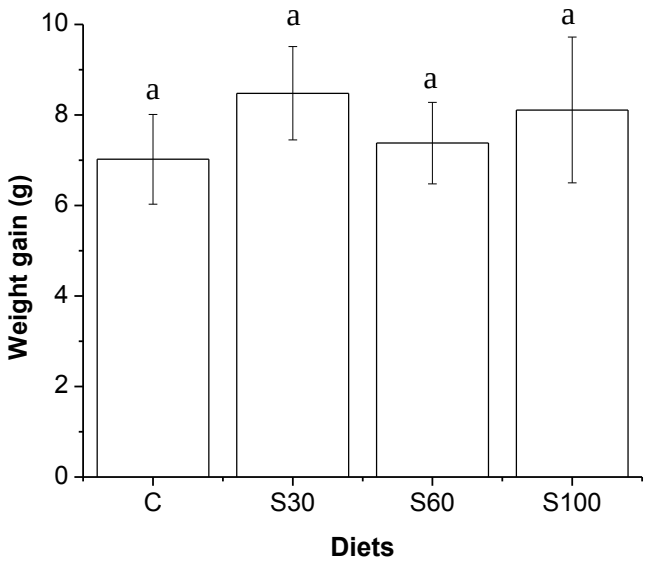

Figure 5

4. CONSIDERAÇÕES FINAIS

Neste estudo, observou-se que os sistemas de bioflocos bem como a substituição da farinha de peixe com SPC nas dietas para *Litopenaeus vannamei* evidenciaram efeito positivo no desempenho deste camarão. As diferenças nas atividades enzimáticas dos hepatopancreas dos camarões cultivados forneceram informações importantes quanto ao potencial desta espécie de utilizar formulações de alimentos com baixos níveis de proteínas e também de fonte proteica alternativa. Espera-se que com a determinação da redução do teor proteico e da substituição parcial ou total da proteína animal por proteína vegetal, contribuir para a diminuição dos custos da ração sem diminuir a produtividade dos sistemas de produção.

REFERÊNCIAS

ABUIN, E., LISSI, E., DUARTE, R. (2004). Distinct effect of a cationic surfactant on transient and steady state phases of 2-naphtyl acetate hydrolysis catalyzed by α-chymotrypsin. **Journal of Molecular Catalysis B: Enzymatic** [S.I.], v. 31, p. 83-85.

AKIYAMA, D. M.; DOMINY, W. G.; LAWRENCE, A. L. Peneid shrimp nutrition for commercial feed industry. **Proceedings of aquaculture Feed Processing and Nutrition Workshop.** American Soybean Association, Singapore, September 19-25,1991,Thailand and Indonesian, p 80-98. 1991.

AKIYAMA, D. M.; WARREN, G. D.; LAWRENCE, A. L. (1992). *Penaeid* Shrimp Nutrition. *In*: FAST, A.; LESTER, L. J. (Eds.) Marine Shrimp Culture: Principles and Practices. **Developments in aquiculture and Fisheries Science**, 23. Holanda: Elsevier.

ALFONSO, E.; COELHO, M. A. Manejo de larvicultura. *In*: CURSO INTERNACIONAL SOBRE PRODUÇÃO DE PÓS-LARVAS DE CAMARÃO MARINHO. Programa Iberoamericano de Ciência y Tecnologia para El Desarrollo. Florianópolis: Cyted, 1997. p. 132.

AL-MOHANNA, S.Y.; NOTT, J.A.; LANE, D.J.W. M-"midget" cells in the hepatopancreas of the shrimp *Penaeus semisulcatus* de Haan 1844 (Crustacea:Natantia). **Crustaceana,** v. 48, p. 260-268, 1985b.

AL-MOHANNA, S.Y.; NOTT, J.A.; LANE, D.J.W. Mitotic E- and secretory F-cells in the hepatopancreas of the shrimp *Penaeus semisulcatus* (Crustacea:Decapoda). **Journal of Marine Biological Association of the United Kingdon**, v. 65, p. 901-910, 1985a.

AL-MOHANNA, S.Y.; NOTT, J.D. B-cells and ingestion in the hepatopancreas of *Penaeus semisulcatus* (Crustacea:Decapoda). **Journal of Marine Biological Association of the United Kingdon**, v. 66, p. 403-414, 1986.

AL-MOHANNA, S.Y.; NOTT, J.D. Function citology of the hepatopancreas of *Penaeus semisulcatus* (Crustacea: Decapoda) during the moult cycle. **Marine Biological,** v. 101, p. 535-544, 1989.

AL-MOHANNA, S.Y.; NOTT, J.D. R-cells and the digestion cycle in *Penaeus semisulcatus* (Crustacea: Decapoda). **Marine Biological**, v. 95, p. 129-137, 1987.

ANDREATTA, E. R.; BELTRAME, E. Cultivo de camarões marinhos. In: POLI, C. R., et al. **Aquicultura: experiências brasileiras.** Florianópolis: Multitarefa, 2004. P. 199-220.

ARANA, L. V. (1999). Aquicultura e o Desenvolvimento Sustentável: Subsídios para a Formulação de Políticas de Desenvolvimento da Aquicultura Brasileira. Florianópolis. Editora da Universidade Federal de Santa Catarina, p. 310.

ASADUZZAMAN, M.; WAHAB, M.A.; VERDEGEM, M.C.J.; HUQUE, S.; SALAM, M.A.; AZIM, M.E. C/N ratio control and substrate addition for periphyton development jointly enhance freshwater prawn *Macrobrachium rosenbergii* production in ponds. **Aquaculture**, v.280, p.117–123, 2008.

AVNIMELECH, Y. Bio-filters: The need for an new comprehensive approach. **Aquacultural Engineering**, v.34, p.172-178, 2006.

AVNIMELECH, Y. Biofloc technology – A Pratical Guide Book. The World Aquaculture Society, Baton Rouge, Louisiana, LA, United States, 182 p., 2009.

AVNIMELECH, Y. Carbon/nitrogen ratio as a control element in aquaculture systems. **Aquaculture**, v. 176, p. 227-235, 1999.

AVNIMELECH, Y. Feeding with microbial flocs by tilapia in minimal discharge bio-flocs technology ponds. **Aquaculture**, v.264, p.140–147, 2007.

AVNIMELECH, Y.; KOCHVA, M., DIAB, S. Development of controlled intensive aquaculture systems with a limited water exchange and adjusted carbon to nitrogen ratio. **Aquaculture Bamidgeh**, v.46, n.3, p.119-131, 1994.

AZIM, M.E.; LITTLE, D.C.; BRON, J.E. Microbial protein production in activated suspension tanks manipulating C:N ratio in feed and the implications for fish culture. **Bioresource Technology**, v.99, p.3590–3599, 2008.

BARBIERE JÚNIOR, R.C. & OSTRENSKY NETO, A. Camarões Marinhos – Engorda, v. II, Aprenda Fácil, Viçosa, 2002.

BARBIERI JÚNIOR, R. C.; OSTRENSKY NETO, A. *Camarões Marinhos (Reprodução, Maturação e Larvicultura)*. Viçosa: Aprenda Fácil Editora, 2001.

BECKER, G. L.; CHEN, C. H.; GREENAWALT, J. W.; LEHNINGER, A. L. Calcium phosphate granulus in the hepatopâncreas of the blue crab *Callinectes sapidus*. J Cell Biol, v. 61, n. 2, p316-326. 1974.

BERG, J. M; STRYER, L.; TYMOCZKO, J. L. (2004). **Bioquímica**. 5 ed. Rio de Janeiro: Guanabara Koogan,. 1104 p.

BOYD, C.E., CLAY, J. Evaluation of Belize Aquaculture Ltd.: Superintensive shrimp aquaculture system. Shrimp Farming and the Environment. Report prepared under the World Bank, NACA, WWF

BROWDY, C.L.; BRATVOLD, D.; STOKES, A.D.; McINTOSH, R.P. Perspectives on the application of closed shrimp culture systems. In: Jory ED, Browdy CL (eds) Proceedings of the special session on sustainable shrimp culture, aquaculture 2001. The World Aquaculture Society, Baton Rouge, LA, p. 20–34, 2001.

BURFORD, M. A.; THOMPSON, P. J.; BAUMAN, R. H.; PEARSON, D. C. Microbial communities affect water quality, shrimp performance at Belize Aquaculture. **The global Aquaculture Advocate**, p.63-65, 2003b.

BURFORD, M. A.; THOMPSON, P. J.; MCINTOSH, R. P.; BAUMAN, R. H.; PEARSON, D. C. Nutrient and microbial dynamics in high-intensity, zero-exchange shrimp ponds in Belize. **Aquaculture**, v.219, p.393-411, 2003a.

BURFORD, M.A.; THOMPSON, P.J.; MCINTOSH, R.P.; BAUMAN, R.H.; PEARSON, D.C. The contribution of flocculated material to shrimp (*Litopenaeus vannamei*) nutrition in a high-intensity, zero-exchange system. **Aquaculture**, v.232, p.525–537, 2004.

CASTILLO-YAÑEZ, F. J., PACHECO-AGUILAR, R., GARCÍA-CARREÑO, F. L., CECCALDI, H. J. Anatomy and physiology of the digestive system. In: D'ABRAMO, L. R. *et al* (Ed.). *Crustacean Nutrition*. Baton Rouge: World Aquaculture Society, 1997. p. 261-291. (Advances in World Aquaculture).

CHABERLAIN, G.; AVNIMELECH, Y.; McINTOSH, R. P.; VELASCO, M. Advantages of aerated microbial reuse systems with balanced C:N - I: Nutrient transformation and water quality benefits. **The Global Aquaculture Advocate**, p. 53-56, 2001a.

CHAMBERLAIN, G. W.; HOPKINS, J. S. Reducing water use and feed cost in intensive ponds. **World Aquaculture,** v. 25, p. 29-32, 1994.

CHAMBERLAIN, G.; AVNIMELECH, Y.; McINTOSH, R.; VELASCO, M. Advantages of aerated microbial reuse systems with balanced C:N. III: Practical Applications. **The global Aquaculture Advocate**, v.4, n.5, p.50-54, 2001b.

CHEN, C. H.; GREENAWALT, J. W.; LEHNINGER, A. L. Biochemical and ultrastructural aspect transport by mitochondria of the hepatopâncreas of the blue crab *Callinectes sapidus*. J Cell Biol, v. 61, n. 2, p301-315. 1974.

COHEN, J.M., SAMOCHA, T.M., FOX, J.M., GANDY, R.L., LAWRENCE, A.L. Characterization of water quality factors during intensive raceway production of juvenile *Litopenaeus vannamei* using limited discharge and biosecure management tools. **Aquaculture**, v.32, p. 425-442, 2005.

CORREA JR., J.D.; FARINA, M.; ALLO I, S. Cytoarchitectural features of *Ucides cordatus* (Crustacea Decapoda) hepatopancreas: structure and elemental composition of electron-dense granules. **Tissue & Cell,** v. 34, n. 5, p. 315-325, 2002.

CORREIA, E.S.; PEREIRA, J.A.; APOLINÁRIO, M.O.; HOROWITZ, A.; HOROWITZ, S. Effect of pond aging on natural food availability and growth of the freshwater prawn *Macrobrachium rosenbergii.* **Aquacultural Engineering**, v.26, p.61–69, 2002.

CRAB, R.; KOCHVA, M.; VERSTRATE, W.; AVNIMELECH, Y. Bio-flocs technology application in over-wintering of tilapia. **Aquacultural Enginnering**, v.40, p.105-112, 2009.

CRUZ-SUÁREZ, L. E., RICQUE-MARIE, D., PINAL-MANSILLA, J. D., WESCHE-EBELLING, P. (1994). Effect of different carbohydrate sources on the growth of *Penaeus vannamei*: economical impact. **Aquaculture** [S.I.], v. 123, n. 3-4, p. 349-360.

CRUZ-SUÁREZ, L.E., RICQUE-MARIE, M.; Nieto-López, M.; Tapía-Salazar, M., 2000. Revisión sobre calidad de harinas y aceites de pescado para la nutrición del camarón. *In*: Civera-Cerecedo, R.; Pérez-Estrada, C.J.; Ricque-Marie, D.; Cruz-Suárez, L.E. (Eds.) Avances em Nutrición Acuícola IV. Memorias del IV Simposium Internacional de Nutrición Acuícola 1998. México.

CUZON, G.; GUILAUME, J. Protein and amino acids. Crustacean Nutrition**.** In: D'ABRAMO, L., CONKLIN, D., AKIYAMA, D.(eds.). **Advances in Word Aquaculture**, vol.6, p. 51-70, 1997.

CUZON,G., LAWRENCE, A., GAXIOLA,G., ROSAS,C., AND GUILLAUME, J. Nutrition of *Litopenaeus vannamei* rared in tanks or in ponds. **Aquaculture**, v. 235 , p 513-551, 2004.

DALL, W.; HILL, B.J.; ROTHLISBERG, P.C. & STAPLES, D.J. Advances in Marine Biology, v.17. Academic Press, 1999.

DAVIS, D.A; LAWRENCE, A.L. Minerals. Crustecean Nutrition. In: D'ABRAMO, L., CONKLIN, D., AKIYAMA, D.(eds.). **Advances in World Aquaculture**, v.6, p.150-15, 1997.

DE SCHRYVER, P.D.; CARB, R.; DEIFOIRDT, T.; BOON, N.; VERSTRAETE, W. The basics of bio-flocs technology: The added value for aquaculture. **Aquaculture**, v.277, p.125-137, 2008.

DE VECCHI, S.; COPPES, Z. (1996). Marine fish digestive proteases - relevance to food industry and the southwest Atlantic region - a review. **Journal of Food Biochemistry** [S.I.], v. 20, p. 193-214.

DECAMP, O.; CONQUEST, L.; FORSTER, I.; TACON, A.G.J. The nutrition and feeding of marine shrimp within zero-water exchange aquaculture production systems: Role of eukaryotic microorganisms. In: LEE, C.S. e O`BRYEN, P. (Eds.). **Microbial approaches to aquatic nutrition within environmentally sound aquaculture production systems**, World Aquaculture Society 2002, Baton Rouge, Louisiana USA, 2002, p.79-86.

DOUGLAS, S. E., MANDLA, S., GALLANT, J. W. (2000). Molecular analysis of the amylase gene and its expression during development in the winter flounder, *Pleuronectes americanus*. **Aquaculture** [S.I.], v. 190, n. 3-4, p. 247-260.

ERLER, D.; SONGSANGJINDA, P.; KEAWTAWEE, T.; CHAIYAKUM, K. Preliminary investigation into the effect of carbon addition on growth, water quality and nutrient dynamics in zero-exchange shrimp (*Penaeus monodom*) culture systems. **Asian Fisheries Science**, v. 18, p. 195-204, 2005.

FACTOR J. R. (1995) The digestive system. In: Factor JR (ed) Biology of the Lobster Homarus americanus. Academic Press, San Diego, pp 395–440.

FACTOR, J. R.; NAAR, M. The digestive system of the lobsters, *Homarus americanus*: 1. Connective tissue of the digestive gland. **Journal of Morphology**, v. 184, p. 311- 321, 1985.

FACTOR, J. R.; NAAR, M. The digestive system of the lobsters, *Homarus americanus*: 2. Terminal arterioles of the digestive gland. **Journal of Morphology**, v. 206, p. 283- 291, 1990.

FERNÁNDEZ GIMENEZ, A. V., GARCÍA-CARREÑO, F. L., NAVARRETE DEL TORO, M. A., FENUCCI, J. L. (2002) Digestive proteinases of *Artemesia longinaris* (Decapoda, *Penaeidae*) and relationship with molting. **Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology** [S.I.], v. 132, n. 3, p. 593-598.

FRANCESCHINI-VICENTINI, I.B.; RIBEIRO, K.; PAPA, L. P., MARQUES JUNIOR, J.; VICENTINI, C.A. Histoarchitectural features of the hepatopancreas of the Amazon river prawn *Macrobrachium amazonicum*. **Anatomia, Histologia, Embryologia,** SUBMITTED, 2007.

FREITAS, P. D. **Estudos de diversidade genética em estoques de reprodutores de camarões Litopenaeus vannamei no Brasil.** 2003. 120 f. Tese (Doutorado em Genética e Evolução) – Centro de Ciências Biológicas e da Saúde, Universidade Federal de São Carlos, São Carlos, 2003.

GARCÍA-CARREÑO, F. L., HERNÁNDEZ-CORTÉZ, M. P., HAARD, N. F. (1994). Enzymes with peptidase and proteinase activity from the digestive systems of a freshwater and a marine decapods. **Journal of Agricultural and Food Chemistry**, 42. 1456-1461.

GIBSON, O. and BARKER, P.L. (1979), The decapod hepatopancreas. *Oceanog. Mar. Biol. Ann. Rev.*, **17**, 285-346.

GONZALEZ, T. & ROBERT-BADOUY, J. (1996). Bacterial aminopeptidases: Properties and functions. **Microbiology Reviews**, 18, p. 319-344.

GUILAUME, J. Protein and amino acids. Crustacean Nutrition. In: D'ABRAMO, L., CONKLIN, D., AKIYAMA, D.(eds.). **Advances in Word Aquaculture**, vol.6, p. 26-50, 1997.

GUILLAUME, J.; CECCALDI, H. J. Physiologie digestive des crevettes. In: GUILLAUME, J. *et al* (Ed.). *Nutrition et alimentation des poissons et crustacés*. Paris: INRA, 1999. Cap.15. p. 297-312.

GUPTA, R.; BEG, Q.K.; LARENZ, P. (2002). Bacterial alkaline proteases: molecular approaches and industrial applications. **Applied Microbiology and Biotechnology**, v. 59, p. 15 32.

GUZMÁN, F. D. (1996). Principales ingredientes a utilizar en dietas para acuacultura. *In*: JARAMILLO, M. P. S.; GÓMEZ, H. R.; CAZA, P. V. (Eds.) Fundamentos de nutrición y

alimentación em acuicultura. Serie Fundamentos Nº 3. Colômbia: Instituto Nacional de Pesca Y Acuicultura.

HANDY, M., SAMOCHA, T.M., PATNAIK, S., McKEE, D.A. Nursery trial compares filtration system performance in intensive raceways. **The Global Aquaculture Advocate**, v.8, p. 77-79, 2004.

HARI, B.; MADHUSOODANA KURUP, B.; VARGHESE, J.T.; SCHRAMA, J.W.; VERDEGEM, M.C.J. Improved sustainability in extensive shrimp culture systems: control of carbon nitrogen ratio through addition of carbohydrate to the pond. **Aquaculture**, v.241, p.179-194, 2004.

HARVEY, R. A., CHAMPE, P. C., FERRIER, D. R. (2009). **Bioquímica Ilustrada**. 4a. ed.: Artmed.

HERTRAMPF, J. W.; PIEDAD-PASCUAL, F. (2000). Handbook on Ingredients for Aquaculture Feeds. Holanda: Kluwer.

HIRSCH, G.C. AND JACOBS, W. Der Arbeitsrhythmus der Mitteldarmdrüse von *Astacus leptodactylus*. I. Teil: Methodik und Technik. 1928. In: ICELY, J.D. AND NOTT, J.A. Digestion and Absorption: digestive system and associated organs. **Microscopic Anatomy of Invertebrates Volume 10: Decapod Crustacea**, 1992. P. 147-201.

HOPKINS, J.S.; SANDIFER, P.A.; BROWDY, C.L. A. Effect of two feed protein levels and feed rate combinations on water quality and production of intensive shrimp ponds operated without water exchange**. Journal of the World Aquaculture Society**, v.26, n.1, 1995.

ICELY, J.D.; NOTT, J.A. (1992), Digestion and absorption: digestive system and associated organs. In-*Microscopic Anatomy of Invertebrates: Decapod, Crustacea,* Vol. 10, eds. F.W. Harrison and A.G. Humes. Wily-Liss Inc., New York, pp 147-201.

JACOBS, W. Untersuchungen über die Cytologie der Seretbildung in der mitteldarmdrüse von *Astacus leptodactylus*. 1928 In: ICELY, J.D.; NOTT, J.A. Digestion and Absorption:

digestive system and associated organs. **Microscopic Anatomy of Invertebrates Volume 10: Decapod Crustacea,** 1992. P. 147-201.

JOHNSTON, D.; FREEMAN, J. (2005). Dietary preference and digestive enzyme activities as indicators of trophic resource utilization by six species of crabs. **Biological Bulletin** [S.I.], v. 208, p. 36-46.

JOHNSTON, D.J., ALEXANDER, C.G. and YELLOWLEES, D. (1998), Epithelial cytology and function in the digestive gland of *Thenus orientalis* (Decapoda, Scyllaridae). *J. Crust. Biol.*, **18** (12), 271-278.

KITANI, H. Larval development of the white shrimp *L. vannamei* (BOONE) reared in the laboratory and the statistical observation of its naupliar stages. **Bulletin of the Japanese Society of Scientific Fisheries**. v. 52 n.7. p. 1131 a 1139, 1986.

KLEIN, B., LE MOULLAC, G., SELLOS, D., VAN WORMHOUDT, A. (1996). Molecular cloning and sequencing of trypsin cDNAs from *Penaeus vanammei* (*Crustacea*, *Decapoda*): Use in assessing gene expression during the moult cicle. **Journal Biochemistry Cell Biology**, 28, p. 551-563.

KOMKLAO, S., BENJAKUL, S., VISESSANGUAN, W., KISHIMURA, H., SIMPSON, B. K. (2007). Purification and characterization of trypsins from the spleen of skipjack tuna (*Katsuwonus pelanis*). **Food Chemistry**, 100, p. 1580-1589.

LE CHEVALIER, P.; VAN WORMHOUDT, A. (1998). Alpha gucosidase from the hepatopâncreas of the shrimp, *Penaeus vannamei* (Crustacea - Decapoda). **Journal of Experimental Zoology** [S.I.], v. 280, p. 384-394.

LEMOS, D. (2003). Testing quality of feeds and feed ingredients: in vitro determination of protein digestibility with enzymes from the target species. **International Aquafeed**, nov. - dez.

LIN, H. Z., GUO, Z., YANG, Y., ZHENG, W., LI, Z. J., 2004. Effect of dietary probiotics on apparent digestibility coefficients of nutrients of white shrimp Litopenaeus vannamei Boone. Aquaculture Research 35, 1441-1447.

LONGAS, M. P. D. (1996). Formulación de dietas. *In*: JARAMILLO, M. P. S.; GÓMEZ, H. R.; CAZA, P. V. (Eds.). Fundamentos de nutrición y alimentación em acuicultura. Serie Fundamentos Nº 3. Colômbia: Instituto Nacional de Pesca Y Acuicultura.

LORENT, S. M.; DEVOS, P. E. Structure and possible functions of the calcospherite-rich cells (R* cells) in the digestive gland of the shore crab Carcinus maenas. Cell Tissue Res, v. 267, n. 1, p. 105-111. 1992.

MARTINEZ-CORDOVA, L.R.; CAMPAÑA, A.T.; PORCHAS-CORNEJO, M.A. Dietary protein level and natural food management in the culture of blue (*Litopenaeus stylirostris*) and white (*Litopenaeus vannamei*) in microcosms. **Aquaculture Nutrition**, v.9, p.155-160, 2003.

McGAW, I. J.; CURTIS, D. L. A review of gastric processing in decapod crustaceans. **J Comp Physiol B**. 2013. v. 183: 443-465.

McGRAW, W. J. Utilization of heterotrophic and autotrophic bacteria in aquaculture. **The Global Aquaculture Advocate**, p. 82-83, 2002.

McINTOSH, R. Changing paradigms in shrimp farming: IV. Low protein feeds and feeding strategies. **The Global Aquaculture Advocate**, v.3, p.45-50, 2000.
McINTOSH, R. P. Changing paradigms in shrimp farming – I: general description. **The Global Aquaculture Advocate**, August/October, p.40-47, 1999.

McNEIL, R. Zero exchange, aerobic, heterotrophic systems: key considerations. **The Global Aquaculture Advocate**, v.3, 72–76, 2000.

NAVARRETE-DEL TORO, M. A., LÓPEZ, M. A. (2006). Purification and biochemical characterization of chymotrypsin from the viscera of Monterey sardine (*Sardinops sagax caeruleus*). **Food Chemistry** [S.I.], v. 99, p. 252-259.
NELSON, D. L.; COX, M. M. (2004). **Lenhinger: Princípios de Bioquímica**. 4 ed. São Paulo: Sarvier, 1119p.

NUNES, A.J.P. Bandejas de alimentação na engorda de camarão marinho. **Panorama da Aquicultura**, v.13, n.80, p.39-47, 2003.

NUNES, A.J.P. O cultivo do camarão *Litopenaeus vannamei* em águas oligohalinas. **Panorama da Aquicultura**, p.17-23. jul/ago. 2001.

OLIVEIRA, S. M., FREITAS JR. J. O., ALVES, K. B., (1999). Rabit kidney aminopeptidases: purification and some properties. **Immunopharmacology**, 45, 215-221.

PÁEZ-OSUNA, F. The environmental impact of shrimp aquaculture: causes, effects, and mitigating alternatives. **Environmental Management**. v.28, n. 1, p. 131-140, 2001.
Penaeus vannamei Boone, 1931, Bull.Am.Mus.Nat.Hist., 63:173.

PIEDRAHITA, R.H. Reducing the potential environmental impact of tank aquaculture effluents through intensification and recirculation. **Aquaculture,** v.226, p. 35–44, 2003.

PRIMAVERA, J. H. A (1998). Sustentabilidade do cultivo do camarão marinho em áreas tropicais. De Silva, S. (Ed.), Tropical mariculture, London: Academic Press, p. 257-289.

PRIMAVERA, J. H. A review of maturation and reproduction in closed *thelycum penarids*. *In*: FIRST INTERNATIONAL CONFERENCE ON THE CULTURE OF PENAEID PRAWNS/SHRIMP. Philippines: lloilo City, 1984.

RAY, A.J.; SHULER, A.J.; LEFFLER, J.W.; BROWDY, C.L. Microbial ecology and management of biofloc systems. In: BROWDY, C.L. e JORY, D.E. (Eds.). **The rising tide, Proceedings of session on sustainable shrimp Farming**, World Aquaculture Society 2009, Baton Rouge, Louisiana USA, 2009, p.255-266.

ROCHA, I. P.; MAIA, E. P. (1998). Desenvolvimento tecnológico e perspectivas de crescimento da carcinicultura marinha brasileira. In: I Congresso Sul-Americano de Aquicultura, 1998. Recife, Anais. Recife: **Associação Brasileira de Aquicultura**, v. 1, p. 213-235.

ROSAS, C., CUZON, G., GAXIOLA, G., ARENA, L., LEMAIRE, P., SOYEZ, C., VAN WORMHOUDT, A. (2000). Influence of dietary carbohydrate on the metabolism of juvenile *Litopenaeus stylirostris*. **Journal of Experimental Marine Biology and Ecology** [S.I.], v. 249, n.2, p. 181-198.

ROSAS, C.; CUZON, G.; GAXIOLA, G.; PASCUAL, C.; TABOADA, G.; ARENA, L.; WORMHOUDT, A.V. An energetic and conceptual model of tha physiological role of dietary carbohydrates and salinity onf *Litopenaeus vannamei* juveniles. **Journal of Experimental Marine Biology and Ecology**. V. 268(1), p 147-67. 2002.

RUPPERT, E. E.; BARNES, R. D. Zoologia dos invertebrados. 4a Ed. Roca. p.1179.

SAINZ, J. C., GARCÍA-CARREÑO, F. L., HERNÁDEZ-CORTÉS, P. (2004). *Penaeus vannamei* isotrypsins: purification and characterization. **Comparative Biochemistry and Physiology Part (B)**, 138, p. 155-162.

SALEH, A. M., AFAF, S. F., TAREK, M. M. (2005). Carbohydrases in camel (*Camelus dromedarius*) pancreas. Purification and characterization of glucoamylase. **Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology** [S.I.], v. 140, n. 1, p. 73-80.

SAMOCHA, T. M.; PATNAIK, S.; SPEED, M.; ALI, A. M.;BURGER, J. M.; ALMEIDA, R. V.; AYUB, Z.; HARISANTO, M.; HOROWITZ, A.; BROCK, D. L., Use of molasses as carbon source in limited discharge nursery and grow-out systems for *Litopenaeus vannamei*. **Aquacultural Engineering**, v.36, p.184-191, 2007.

SAMOCHA, T.M. Advances in shrimp nursery technologies. In: BROWDY, C.L. e JORY, D.E. (Eds.). **The rising tide, Proceedings of session on sustainable shrimp Farming**, World Aquaculture Society 2009, Baton Rouge, Louisiana USA, 2009, p.195-208.

SAMOCHA, T.M.; PATNAIK, S.; GANDY, R.L. Heterotrophic intensification of pond shrimp production. In: The fifth international conference of recirculating aquaculture, 2004. Virginia. **Anais**. 22–25 July 2004, Roanoke, Virginia, USA.

SÁNCHEZ-PAZ, A., GARCÍA-CARREÑO, F., MUHLIA-ALMAZÁN, A., HERNÁNDEZSAAVEDRA, N.Y., YEPIZ-PLASCENCIA, G. (2003). Differential

expression of trypsin mRNA in the white shrimp (*Penaeus vannamei*) midgut gland under starvation condition. **Journal of Experimental Marine Biology and Ecology,** 292, 1–17.

SHIAU, S. – Y. Nutrient requerimets of penaeid shrimps. **Aquaculture**, v. 164, p. 77-93. 1998.

SHIAU, S.Y e BAI, S. Micronutrientes in shrimp diets. In: BROWDY, C.L. e JORY, D.E. (Eds.). **The rising tide, Proceedings of session on sustainable shrimp Farming**, World Aquaculture Society 2009, Baton Rouge, Louisiana USA, 2009, p.126-132.

SIMPSON, B. K. (2000). Digestive Proteases from Marine Animals. In: HAARD, N. F.; SIMPSON, B. K. (eds.), Seafood Enzymes. Marcel Dekker, New York, NY, p. 191– 213.

SMITH, D.M.; BURFOD, M.A.; TABRETT, S.J.; IRVIN, S.J.; WARD, L. The effect of feeding frequency on water quality and growth of the black tiger shrimp (Penaeus monodon). **Aquaculture**, v.207, p.125–136, 2002.

SWICK, R. A. (2007). Soybean meal and soy protein concentrate for shrimp production. **International Aquafeed**, Mar-Apr.

TACON, A.G.J., NATES, S.F.; McNEIL, R.J. 2004. Dietary feeding strategies for marine shrimp: a review. In: Cruz Suárez, L.E., Ricque Marie, D., Nieto López, M.G., Villarreal, D., Scholz, U., González, M. 2004. Avances en nutrición Acuícola VII. **Memorias del VII Simposium Internacional de Nutrición Acuícola**. 16-19 Noviembro, 2004. Homosillo, Sonora, México.

TAN, R.K.H.; DOMINY, W.G. Commercial pelleting of crustacean feeds. In: D'ABRAMO, LR, DE CONKLIN, & MD AKIYAMA (Ed.). **Crustacean Nutrition**. World Aquaculture Society, Baton Rouge, 1997, p. 520-549.

VALENÇA, A.R.; MENDES, G.N. Cultivo de *Litopenaeus vannamei*: Água doce ou oligohalina?.**Panorama da Aqüicultura**, julho agosto, 2003, pág 35-41.

VALLES-JIMENEZ, R.; CRUZ, P.; PEREZ-ENRIQUEZ; R. Population Genetic Structure of Pacific White Shrimp (Litopenaeus vannamei) from Mexico to Panama: Microsatellite DNA Variation Marine Biotechnology, 2005. V 6: 475–484.

VAN WORMHOUDT, A.; FAVREL, P. (1988). Electrophoretic characterization of *Palaemon elegans* (*Crustacea*: *Decapoda*) amylase system: study of amylase polymorphism during the intermolt cycle. **Comparative Biochemistry and Physioliology Part B: Biochemistry and Molecular Biology** [S.I.], v. 89, p. 201-207.

VELASCO, M., LAWRENCE, A.L. and OBALDO, L.G. Determining optimal dietary protein level for *Litopenaeus vannamei* postlarvae. **The Global Advocate**. v.3 Issue 6. December 2000.

VIPARELLI, P., ALFANI, F., CANTARELLA, M. (2001). Experimental validation of a model for α-chymotrypsin activity in aqueous solutions of surfactants aggregate. **Journal of Molecular Catalysis B: Enzymatic** [S.I.], v. 15, p. 1-8.

VOGT, G. (1994), Life-cycle and functional cytology of the hepatopancreas cells of *Astacus astacus* (Crustacea, Decapoda). *Zoomorphology*, **114**, 83-101.

VOGT, G. Transformation of the anterior midgut and hepatopancreas cells by Monodon baculovirus (MBV) in *Penaeus monodon* postlarvae. **Aquaculture**, v. 107, p.239-248, 1992.

VOGT, G.;STÖCKER, W.; STORCH, V.; ZWILLING, R. (1989). Biosynthesis os Astacus protease, a digestive enzyme from crayfish. Histochemistry, 91, 373-381.

WANG, Yan-Bo. Effect of probiotics on growth performance and digestive enzyme activity of the shrimp< i> Penaeus vannamei</i>. **Aquaculture**, v. 269, n. 1, p. 259-264, 2007.

WASIELESKY, W. JR, ATWOOD, H., STOKES, A.; BROWDY, C.L. Effect of natural production in a zero exchange suspended microbial floc based super-intensive culture system for white shrimp Litopenaeus vannamei. *Aquaculture*, v. 258, p. 396–403, 2006a.

WASIELESKY, W.; EMERENCIANO, M.; BALLESTER, E.; SOARES, R.; CAVALLI, R.; ABREU, P. C., Cultivos em meios com flocos microbianos: um novo caminho a ser percorrido. **Panorama da Aquicultura**, v.16, n.96, p.14-23, 2006b.

WHITAKER, J. R. (1994). **Principles of Enzymology for the Food Sciences**, 2nd ed. Marcel Dekker, New York, NY, p. 63–115.

WURMANN, G.; MADRID, R.M. O desenvolvimento da salmonicultura no Chile: lições de um modelo vigoroso e sua possível aplicação na indústria do cultivo do camarão no Brasil. **Panorama da Aquicultura**. v.16, n.93, p.14-23, 2006.

ANEXOS

AQUACULTURE
An International Journal

AUTHOR INFORMATION PACK

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DESCRIPTION

Aquaculture is an international journal for **freshwater** and **marine** researchers interested in the exploration, improvement and management of all **aquatic food resources.** It publishes original research on **farming** of aquatic animals and plant organisms including finfish, mollusks, crustaceans and aquatic plants for human consumption.

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Nutrition:
D.M. Gatlin

The Nutrition Section welcomes high quality research papers presenting novel data as well as original reviews on various aspects of aquatic animal nutrition relevant to aquaculture. Manuscripts addressing the following areas of investigation are encouraged:

1) determination of dietary and metabolic requirements for various nutrients by representative aquatic species. Studies may include environmental/stress effects on animal's physiological responses and requirements at different developmental stages;
2) evaluation of novel or established feedstuffs as well as feed processing and manufacturing procedures with digestibility and growth trials. Such studies should provide comprehensive specifications of the process or evaluated ingredients including nutrients, potential anti-nutrients, and contaminants;
3) comparison of nutrient bioavailability from various ingredients or product forms as well as metabolic kinetics of nutrients, food borne anti-nutrients or toxins;
4) identification of key components in natural diets that influence attractability, palatability, metabolism, growth reproduction and/or immunity of cultured organisms;
5) optimization of diet formulations and feeding practices;
6) characterization of the actions of hormones, cytokines and/or components in intracellular signaling pathway(s) that influence nutrient and/or energy utilization.
7) evaluation of diet supplementation strategies to influence animal performance, metabolism, health and/or flesh quality.

Manuscripts concerning other areas of nutrition using novel or advanced methods are also welcome. Please note that in regard to various diet additives such as probiotics, prebiotics, herbal extracts, etc., a very large number of papers have already been published. Therefore, Aquaculture will not continue to accept manuscripts that present initial and preliminary investigations of such additives. Manuscripts addressing these and other feed additives will be accepted for review only if they are of the highest scientific quality and they represent a significant

advance in our knowledge of the mechanisms involved in their metabolism. Manuscripts may also be considered if they present clinical efficacy data generated in large-scale trials and economic cost-benefit analysis of these applications.

Aquaculture Production Science:
B.Costa-Pierce

AQUACULTURE PRODUCTION SCIENCE (PS) is one of 5 sections of the international journal AQUACULTURE dedicated to research on improvements and innovations in aquatic food production.

This section supports worldwide dissemination of the results of innovative, globally important, scientific research on production methods for aquatic foods from fish, crustaceans, mollusks, amphibians, and all types of aquatic plants. Contributions are encouraged in the following areas:
1) Improvement of production systems that results in greater efficiencies of resource usage and sustainability of aquaculture; 2) Effective applications of technologies and methods of aquaculture production for improved stocking regimes; 3) The use of new species and species assemblages; and, 4) Investigations to minimize aquaculture wastes and improve water quality, including technologies for nutrient recycling in aquaculture ecosystems, and potential synergy of aquaculture and other food production systems using methods such as polyculture and integrated aquaculture. Aspects of seafood processing and technology will not be considered in this section although aquaculture techniques that may influence the nutritional value of aquatic food products may be considered in the Nutrition Section.

Physiology and Endocrinology:
Fish: A. P. (Tony) Farrell
Invertebrate: J. Benzie

The Physiology Section welcomes high quality papers that present both novel research data and original reviews, on all aspects of the physiology of cultured aquatic animals and plants. Their content must be relevant to solving aquaculture problems.

Submitted manuscripts must have a valid hypothesis or objective, clearly state the relevance to aquaculture, have proper experimental design with appropriate controls and utilize appropriate statistical analysis. Mention of trade names is limited to the main text.

Relevant physiological topics include, but are not limited to:

- Reproductive physiology, including: Endocrine and environmental controls development; Induced ovulation and spermiation; Gamete quality, storage and cryopreservation; control of sex differentiation; Physiology and endocrinology of gynogenetic, triploid and transgenic organisms
- Molecular genetic assessment of physiological processes
- Larval physiology and ontogeny in relation to aquaculture, including metamorphosis, smolting (salmonids) and molting (crustacea)
- Nutritional physiology including endocrine and environmental regulation of growth
- Performance under variable culture conditions, including temperature optima and tolerances; Altered water quality and environmental variables; Stress and disease physiology; Rearing density
- Immunology (physiological studies of probiotics must present statistically valid conclusions)
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- Physiology of harvest and handling techniques, including: Anesthesia and transport; Product and flesh quality; Pigmentation

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G. Hulata

The Genetics Section welcomes high-quality research papers presenting novel data, as well as critical reviews, on various aspects of selective breeding, genetics and genomics, so long as the content is relevant to solving aquaculture problems. Please note, however, that Aquaculture will not accept manuscripts dealing with the application of well-described techniques to yet another species, unless the application solves a biological problem important to aquaculture production. Aquaculture will not accept manuscripts dealing with gene cloning, characterizing of microsatellites, species identification using molecular markers, EST papers with small

collections, or mapping papers with a small number of markers, unless the papers also deal with solving a biological problem that is relevant to aquaculture production. Where appropriate, linkage maps should include co-dominant markers, such as microsatellite DNA and SNP markers, to enable application to other populations and facilitate comparative mapping. Aquaculture will not accept manuscripts focusing mainly on population genetics studies that are based on RAPD and AFLP markers, since the dominance and multilocus nature of the fingerprints are not suitable for making inferences about population genetic diversity and structure. There may be other journals that are more suitable for manuscripts not meeting these requirements.

Sustainability and Society:
D.C. Little

The Sustainability and Society section of the journal Aquaculture invites articles at the interface of natural and social sciences that address the broader roles of aquaculture in global food security and trade.

Aims and scope of the Sustainability and Society section are the: global dissemination of interdisciplinary knowledge regarding the management of aquatic resources and resulting impacts on people. Interconnections with other sectors of food production; resource management and implications for societal impact. Going beyond a narrow techno-centric focus, towards more holistic analyses of aquaculture within well-defined contexts. Enquiry based on understanding trajectories of change amid the global challenges of climate change and food security. Mixed methods and approaches that incorporate and integrate both social and natural sciences. Relevance for the diverse range of policy makers, practitioners and other stakeholders involved. Articles that take a value chain approach, rather than being wholly production orientated, are encouraged.

Disease
B. Austin

The Disease sections welcomes critical reviews and high quality articles containing novel data on all aspects concerning diseases of farmed aquatic species. The aims of the section are: description of new and emerging diseases including characterization of the causal agent(s), development in the understanding of fish pathogens for example including new methods of growth where this has been a problem for fastidious organisms, pathogenicity and epizootiology, developments in the diagnosis of disease going beyond the use of standard well used methods, and methods of disease control, notably new developments in vaccines, immunostimulants, dietary supplements, medicinal plant products, probiotics, prebiotics and genetically-disease resistant stock. Relevance to aquaculture must be demonstrated. Articles, which adapt well known methods without further refinement of those methods, are unlikely to be accepted.

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An International Scientific Journal Covering Research on Animal Nutrition, Feeding and Technology

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h. Lignin (pm)-Lignin determined by oxidation of lignin with permanganate.

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Reporting 'hemicellulose' values as the difference between NDF and ADF is generally only acceptable if the analyses have been sequential on the same sample. Crude fibre (CF), nitrogen-free extract (NFE) and total digestible nutrients (TDN) are not acceptable terms for describing feeds and should only be referred to in a historical context.

Results
Results should be clear and concise.

Discussion

This should explore the significance of the results of the work, not repeat them. Avoid extensive citations and discussion of published literature. Combined 'Results and Discussion' sections are only acceptable for 'Short Communications', except under compelling circumstances.

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The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

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Acknowledgements

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