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**Juliana Ferreira dos Santos**

# Influência dos sistemas de cultivo intensivo e semi-intensivo e dietas nos parâmetros zootécnicos e enzimas digestivas da tilápia do Nilo (*Oreochromis niloticus* L.)

**RECIFE**

**2013**

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal de Pernambuco, como parte dos requisitos para a obtenção do título de Doutora em Ciências Biológicas, área de concentração Biotecnologia.

**Orientador:** Prof. Dr. Ranilson de Souza Bezerra (UFPE)

**Co-orientador:** Prof. Dr. Daniel Lemos (USP)

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*Aos meus pais e aos meus filhos.*

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***Onde leva? Não perguntes, segue-o!***

***Nietzsche***

## RESUMO

A tilápia do Nilo pode ser produzida em diferentes sistemas de cultivo e escalas, dentre eles os sistemas semi-intensivo em viveiros (SSI), e o intensivo em tanques-rede (SI). Seus manejos são completamente distintos, por isso o gerenciamento se faz necessário a fim de não desestabilizar parâmetros de crescimento e afetar os custos de produção, principalmente a ração, que pode chegar a representar até 80% destes custos. Para um menor gasto, ingredientes alternativos podem ser usados na elaboração das dietas, no entanto, eles devem apresentar resultados semelhantes ou melhores, quando comparados às dietas convencionais. Sendo assim, pretende-se avaliar se a inclusão de hidrolisado proteico de camarão (HPC) na dieta pode influenciar as atividades enzimáticas da tilápia do Nilo e avaliar a influência do SSI e do SI nos parâmetros de crescimento e nas enzimas digestivas deste peixe. Para avaliar a influência do HPC nas enzimas digestivas de juvenis de tilápia do Nilo, os cultivos foram realizados em aquários. O HPC foi incluído nas dietas nas concentrações de 0, 15, 30 e 60 $g.kg^{-1}$ (HPC 0, 15, 30 e 60, respectivamente). Ao término dos ensaios (45 dias), o estômago e o intestino dos espécimes experimentais foram removidos para realização das análises. Para avaliação da influência do SSI e do SI sob os parâmetros de crescimento e as enzimas digestivas da tilápia do Nilo, os espécimes experimentais foram cultivados durante 90 dias. Foram adotados dois planos nutricionais: trifásico para SSI (ração com 35, 32 e 28% de proteína bruta – PB) e bifásico para SI (35 e 32% PB). Semanalmente foram realizadas coletas de fitoplâncton e zooplâncton, diariamente os principais parâmetros de qualidade de água foram mensurados e mensalmente foram realizadas biometrias para análise dos parâmetros de crescimento, ajustes na alimentação e remoção dos tecidos (intestino) para análises enzimáticas. A atividade de protease alcalina total foi significativamente maior nos tratamentos HPC15 e HPC60, no entanto, o efeito não foi dose-dependente. Zimograma revelou 12 bandas proteolíticas, oito delas responderam a incorporação do HPC. Já o zimograma de inibição indicou uma redução na atividade de três enzimas, uma diminuição na atividade da tripsina com o aumento da concentração de HPC, e o inverso com duas aminopeptidases. Com relação aos sistemas de criação o alimento natural foi mais abundante no SSI. Sobrevivência, eficiência proteica, eficiência alimentar e fator de condição com 31 dias de cultivo foram melhores no SSI. A relação peso-comprimento não foi alterada pelos sistemas. E quando comparado o peso relativo dos peixes do SSI e do SI com tilápias de outros ambientes ou cultivos, eles foram maiores em nove das 14 comparações. As enzimas digestivas também foram avaliadas no decorrer dos cultivos (31, 63 e 94 dias). Protease total, amilase e lipase não foram estatisticamente diferentes entre os tratamentos ao longo dos períodos analisados. No entanto, atividade de tripsina e quimotripsina foram maiores, com 31 e 63 dias de cultivo em peixes do SSI, sugerindo que o alimento natural pode ter influenciado essas atividades. Uma correlação positiva foi observada entre a concentração recomendada de aminoácidos essenciais para a tilápia do Nilo e atividade específica de aminopeptidase nos peixes do SI. Zimograma também revelou 12 bandas proteolíticas ativas em ambos os sistemas. Sendo que os valores de densidade integrada (*DI*) foram maiores nas bandas do SSI. Espécimes de ambos os sistemas exibiram cinco bandas com atividade amilolítica, com os maiores valores de *DI* aos 31 dias de cultivo. Os menores valores de *DI* foram observados com peixes do SSI, com 63 e 94 dias de cultivo. A comparação da eletroforese bidimensional do intestino de animais cultivados em SSI e SI mostraram um total de 1.233 spots, sendo 731 diferentes. Tilápia do Nilo demonstrou adaptação às dietas utilizadas e aos sistemas de cultivo empregados.



**Palavras-chave:** tilápia do Nilo, hidrolisado proteico de camarão, enzimas digestivas, sistema intensivo, sistema semi-intensivo.

## ABSTRACT

The Nile tilapia can be produced in different culture systems and scales, including the semi-intensive systems in ponds (SIS), and the intensive in cages (IS). Their managements are completely different, so it is required management to estabilize the growth parameters and production costs, mainly feed, which can represent up to 80% of these costs. For lower expenses, alternative ingredients can be used in the preparation of diets, however, they should deliver results similar or better, when compared to conventional diets. Therefore, intends to evaluate whether the inclusion of shrimp protein hydrolyzate (SPH) in the diet can influence the enzymatic activity of juvenile Nile tilapia and evaluate the influence of SIS and IS on growth parameters and digestive enzymes of this fish. To evaluate the effect of SPH on digestive enzymes of juvenile Nile tilapia, the cultures were performed in aquaria. The SPH was included in the diet at concentrations of 0, 15, 30 and 60 $g.kg^{-1}$ (SPH0, 15, 30 and 60, respectively). At the end of the assay (45 days) the stomach and intestine of experimental specimens were removed for analyzes. To assess the influence of SIS and IS on growth parameters and digestive enzymes of Nile tilapia experimental specimens were cultured for 90 days. Were adopted two nutritional plans: triphasic for SIS (diet with 35, 32 and 28% crude protein - CP) for SI and biphasic (35 and 32% CP). Weekly samples were taken for phytoplankton and zooplankton, the main daily water quality parameters were measured and biometrics were performed monthly for analysis of growth parameters, adjustments in diet and removal of tissues (intestine) for enzymatic analyzes. The activity of total alkaline protease was significantly higher in treatments SPH15 and SPH60, however, the effect was not dose-dependent. Zymogram revealed 12 proteolytic bands, eight of which responded to SPH incorporation. Inhibition zymogram indicated a decrease in the activity of three enzymes, with trypsin activity decreasing with the increase of SPH concentration, whereas occurred the opposite for two aminopeptidases. With respect to culture systems natural food was more abundant in SIS. Survival, protein efficiency, feed efficiency and condition factor with 31 days of cultivation were better in SIS. The weight-length relationship was not altered by the systems. And comparing the relative weight of the fish from SIS and SI systems with tilapia from other environments, they were higher in 9 of 14 comparisons. Digestive enzymes also were evaluated during the cultivation (31, 63 and 94 days). Total proteolytic, amylase and lipase activity were not statistically different between the treatments along of the periods analyzed. However, trypsin and chymotrypsin activity were higher with 31 and 63 days of culture in fish from SIS, suggesting that natural food may have influenced these activities. A positive correlation was observed between the recommended concentration of essential amino acids for Nile tilapia and specific aminopeptidases activity in fish IS. Zymogram also revealed 12 active proteolytic bands in both systems. Since the integrated density (*ID*) values were higher in the bands of SIS. Specimens of both systems exhibited five bands with amylase activity, with higher values of *ID* to 31 days of cultivation. The lowest values of *ID* were observed with fish from SIS with 63 and 94 days of cultivation. The comparison of two-dimensional electrophoresis of the intestine of cultured animals in SIS and IS showed a total of 1,233 spots, among them 731 different. Nile tilapia showed adaptation to the diets and employed culture systems.



**Keyword:** Nile tilapia, intensive system, semi-intensive system, digestive enzymes, shrimp protein hydrolyzate.

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CAPÍTULO III

# LISTA DE ABREVIATURAS

***a*:** Coefficient of the arithmetic weight-length relationship
Coeficiente da relação aritmética peso-comprimento

**ADG:** Average daily gain
ganho médio diário

**Ala:** Alanine
Alanina

***am:*** coefficient of the weight-length relationship (geometric mean)
Coeficiente da relação peso-comprimento (média geométrica)

**ANPU:** Apparent net protein utilization
Utilização da proteína líquida aparente

**AOAC:** Official methods of analysis
Métodos oficiais de análise

**Arg:** Arginine
Arginina

***b*:** Exponent of the arithmetic form of the weight–length relationship
Expoente da forma aritmética da relação peso-comprimento

**BA*p*NA:** Nα-benzoyl-$_{DL}$-arginine-*p*-nitroanilide
Nα-benzoil-$_{DL}$-arginina-*p*-nitroanilida

**$BCP_f$:** Final body crude protein
Proteína bruta corporal final

**$BCP_i$:** Initial body crude protein
Proteína bruta corporal inicial

**BHT:** Butylated hydroxytoluene
Hidroxitolueno butilado

***bm*:** Exponent of the weight–length relationship (geometric mean)
Expoente da relação peso-comprimento (média geométrica)

**$BW_f$:** Average final body weight$_{(g)}$ of fish
Peso corporal médio final (g) do peixe

**$BW_i$:** Average initial body weight$_{(g)}$ of fish
Peso corporal médio inicial (g) do peixe

**CONAMA:** Brazilian Council for Environment
Conselho Nacional do Meio Ambiente

**DMSO:** Dimethyl sulfoxide
Dimetil sulfóxido

**DNSA:** 3.5-Dinitro salicylic acid
3,5-Ácido dinitro salicílico

**DTT:** Dithiothreitol
Ditiotreitol

**EC:** Enzyme Commission
Comissão de enzimas

**EMPAF:** Empresa de Armazenagem Frigorífica

**FAO:** Food and Agriculture Organization
Organização para a Alimentação e Agricultura

**FB:** final biomass
Biomassa final

**FCR:** Feed conversion ratio
Taxa de conversão alimentar

**FE:** Feed efficiency
Eficiência alimentar

**GIFT:** Genetically Improved Farmed Tilapia
Tilapia cultivada melhoradas geneticamente

**Gly:** Glycine
Glicina

**Hb:** Hemoglobin
Hemoglobina

**His:** Histidine
Histidina

**IBAMA:** Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis

**Iso:** Isoleucine
Isoleucina

**IUBMB:** União Internacional de Bioquímica e Biologia Molecular

**K:** Condition factor
Fator de condição

**kDa:** Kilodaltons
Quilodaltons

**Kmean:** Mean condition factor
Fator de condição médio

**Krel:** Relative condition factor
Fator de condição relativo

**L:** Total length of fish
Comprimento total do peixe

**L:D:** Light:dark
Luz:escuro

**Leu:** Leucine
Leucina

**MM:** Mass marker
Marcador de massa

**mM:** milimolar

**µM:** micromolar

**µS:** microsiemens

**MPA:** Ministério da Pesca e Aquicultura

**Ns:** No statistical differences
Sem diferença estatística

**PBS:** Phosphate buffered saline
Tampão fosfato-salino

**P/GE:** Protein/ gross energy
Proteína / energia bruta

**PepT1:** Peptide transport
Peptídio de transporte

**PER:** Protein eficiency ratio
Taxa de eficiência proteica

**PMSF:** Phenyl-methylsulfonil-fluoride
Fluoreto de fenilmetilsulfonilo

***p*-NPP:** *p*-nitrophenyl palmitate
*p*-nitrofenil palmitato

***p*-NP:** *p*-nitrophenol
*p*-nitrofenol

**Pro:** Proline
Prolina

**S:** Survival
Sobreviência

**SApNA:** Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide
Suc-Ala-Ala-Pro-Fe-*p*-nitroanilida

**SDS-PAGE:** Dodecyl sulfate polyacrylamide gel electrophoresis
Dodecil sulfato eletroforese em gel de poliacrilamida

**Ser:** Serine
Serina

**SGR:** Specific growth rate
Taxa de crescimento específico

**SPH:** Shrimp protein hydrolysate
Hidrolisado proteico de camarão

**TCA:** Trichloroacetic acid
Ácido tricloroacético

**TLCK:** Tosyl-lysine chloromethyl ketone
Tosil-lisina clorometil cetona

**TPCK:** Tosyl phenylalanine chloromethyl ketone
Tosil fenilalanina clorometil cetona

**Tris:** Tris-hydroxymethyl aminomethane
Tris-hidróximetil aminometano

**Trp:** Tryptophan
Triptofano

**Tyr:** Ttyrosine
Tirosina

**U:** Unit of enzyme activity
Unidade de atividade enzimática

**UI:** International unit
Unidade internacional

**W:** Eeight of fish in grams
Peso do peixe em gramas

**WG:** Weight gain rate
Taxa de ganho de peso

**WLR:** Weight-length relationships
Relação peso-comprimento

**Wrm:** Relative weight
Peso relativo

**SUMÁRIO**

# 1. INTRODUÇÃO

O aumento na demanda por recursos pesqueiros para consumo humano tem gerado um crescimento na produção aquícola mundial. De acordo com a Organização das Nações Unidas para Agricultura e Alimentação (FAO, 2012) foram produzidos 154 milhões de toneladas de pescado em 2011 (90,4 milhões provenientes da pesca e 63,6 milhões da aquicultura). O extrativismo ainda é responsável por cerca de 60% do total de pescado produzido.

No entanto, as pressões antrópicas exercidas sobre os ecossistemas têm levado a uma estabilização na obtenção de recursos pesqueiros nos últimos anos, sendo improvável que ocorra um aumento substancial nas capturas totais (JABLONSKI, 2005). Desta forma, o suprimento de organismos aquáticos para atender a demanda global inevitavelmente terá que vir através da aquicultura.

Nas últimas três décadas (1980-2010), a produção mundial da aquicultura se expandiu por quase 12 vezes mais, com uma taxa média de crescimento anual de 8,8 %. Com destaque para produção em águas interiores, que além de representar cerca de 60 % do total cultivado, apresentou um crescimento de mais de 40 % no período de 2006 a 2011, passando de 31,3 para 44,3 milhões de toneladas (FAO, 2012).

Espécies exóticas têm sido amplamente introduzidas e utilizadas para a produção em massa. Bem sucedidas internacionalmente, espécies como as tilápias têm merecido destaque na aquicultura mundial, especialmente a tilápia do Nilo (*Oreochromis niloticus* L.).

No Brasil, o seu cultivo apresenta-se em expansão, principalmente nas regiões Nordeste e Sudeste (IBAMA, 2008), devido as suas características biológicas e mercadológicas relevantes (FARIA et al., 2001).

O aumento da produtividade tem trazido benefícios ao setor aquícola em todos os elos da cadeia produtiva do pescado. Entretanto, este crescimento deve ser direcionado com o intuito de dar subsídios para a prática sustentável da atividade (BUENO et al., 2011). Levando em consideração o conceito de sustentabilidade dos recursos naturais no contexto da exploração florestal e pesqueira, e de acordo com a definição da Comissão Mundial para o Desenvolvimento Econômico: *"O desenvolvimento sustentável é aquele que atende às necessidades do presente, sem comprometer a capacidade das gerações futuras em satisfazer as suas próprias"* (CASTELLO, 2007).

Corroborando com o conceito de sustentabilidade, Abery et al. (2005) e Henry-Silva et al. (2006) demonstraram que se não há um planejamento da produção, impactos negativos podem ser gerados não só sobre o sistema, mas também sobre os ecossistemas aquáticos, tornando a produção insustentável.

Uma forma de manter a sustentabilidade de um sistema de produção é a aplicação de um manejo viável tanto para os aquicultores como para os mananciais. Manejo inadequado além de promover uma produção ecologicamente insustentável pode desestabilizar parâmetros de crescimento, afetando diretamente os custos de produção. No entanto, os animais podem demonstrar algumas compensações fisiológicas, adequando-se quando necessário às mudanças ocorridas no meio.

A aquicultura brasileira têm se profissionalizado bastante nos últimos anos, e os modelos produtivos adotados estão baseados em sistemas intensivos de criação, onde são utilizadas rações como principal fonte de nutrientes para peixes (BUENO et al., 2011). Isto pode levar os produtores a optarem por dietas menos onerosas gerando uma falta de controle nutricional e um aumento nos custos e nos resíduos depositados no meio.

Saldaña e Lopez (1988) mencionam que o valor nutricional de uma dieta não se baseia simplesmente na sua composição química, mas também na capacidade fisiológica do peixe em digerir e absorver, de acordo com seus hábitos alimentares. Esta digestão é executada no trato digestório com o auxílio de enzimas (NIELSEN -SCHIMIDT, 1996).

As enzimas digestivas são estudadas como uma maneira de compreender as exigências nutricionais e o efeito dos constituintes da dieta. Sabendo que a alimentação e o manejo podem influenciar a atividade e a expressão gênica de algumas enzimas, como também em outras proteínas, refletindo no crescimento dos peixes e nos impactos ambientais, entender seu modo de regulação é importante para o uso racional do tipo e quantidade de nutrientes empregados nas dietas (MUHLIA-ALMAZÁN et al., 2003), podendo também fornecer informações sobre a forma de adaptação dos animais a diferentes sistemas de cultivo.

# 2. REVISÃO BIBLIOGRÁFICA

## 2.1 Tilápia do Nilo

A tilápia é um peixe de água doce pertencente à família Cichlidae. Elas são nativas da África, mas foram introduzidas em muitas regiões tropicais, subtropicais e temperadas do mundo durante a segunda metade do século 20 (EL-SAYED, 2006).

As espécies de importância comercial estão divididas em três gêneros de acordo com seu comportamento reprodutivo. O gênero *Tilapia* incuba seus ovos no substrato; *Sarotherodon*, tanto os machos como as fêmeas incubam os ovos na boca e *Oreochromis*, as fêmeas são responsáveis pela incubação dos ovos (TREWAVAS, 1982).

Elas habitam uma grande variedade de ecossistemas, como rios permanentes e temporários, rios com corredeiras, grandes rios equatoriais, tropicais e subtropicais, lagos pantanosos, lagos rasos e profundos, lagos artificiais, alcalinos, ácidos e salinos, fontes termais, lagos em cratera, lagos com baixo conteúdo de minerais, estuários abertos e fechados, lagos de água salobra e habitat marinho. A diversidade de *hábitats* mencionados promove uma fácil adaptação as mudanças ambientais, refletindo em sua tolerância a parâmetros físicos (variações de temperatura, fotoperíodo, correnteza, profundidade, turbidez, etc), químicos (pH, salinidade, minerais, oxigênio dissolvido, amônia, etc) e biológicos (concorrência, disponibilidade de alimentos, etc.) (EL-SAYED, 2006).

Durante os estágios larvais, a tilápia se alimenta inicialmente de fitoplâncton e zooplâncton, especialmente crustáceos (copépodos) (BOWEN, 1982). O período de transição do estágio planctófago para uma dieta especializada é geralmente curto (BOWEN, 1976). A alimentação de juvenis e adultos consiste de uma variedade considerável de vegetação aquática, zooplâncton, fitoplâncton, perifíton e detritos de origem vegetal. Por esta razão, a tilápia pode mudar de uma fonte de alimento para outro com poucas mudanças na composição da dieta (EL-SAYED, 2006).

O século 20 firmou o cultivo de tilápia. Este desenvolvimento se deu principalmente pela demanda do filé pelos Estados Unidos, o cultivo na África e América do Sul, o melhoramento genético e a expansão do cultivo em águas salinas nas Filipinas. China se tornou o maior produtor e grande fornecedor dos EUA (GUERRERO, 2008).

Dados da FAO (2009) demonstraram claramente que a produção mundial de tilápia aumentou de 398.066 t em 1991 para 2.326.413 t em 2006, promovendo um rendimento de US$

559.438 milhões para mais de US$ 2,7 bilhões. De acordo com Wing-Keong e Hanin (2007), *Oreochromis niloticus* (Figura 1) representa 80% das espécies de tilápia cultivadas no mundo, sendo considerada a mais importante.

No Brasil, sua produção em 2006 foi de aproximadamente 71.000 t, representando 37,8% da produção aquícola continental (IBAMA, 2008). Em 2010 esta produção ultrapassou 155.000 t representando 39% da aquicultura continental no país (BRASIL, 2012).

Devido as suas características biológicas e mercadológicas como rápido crescimento, rusticidade, alimentação em baixos níveis tróficos, tolerância a variações ambientais, resistência a doenças, ausência de espinhos intra-musculares, dentre outros, a tilápia do Nilo é considerada uma das principais espécies da piscicultura mundial e a principal espécie brasileira.

**Figura 1**: Exemplar de tilápia do Nilo (*Oreochromis niloticus* L.).

Fonte: http://www.terradagente.com.br/fauna/0,0,2,288;5,tilapia-do-nilo.aspx

## 2.2 Sistemas de cultivo

A tilápia é produzida em diferentes sistemas de cultivo e escalas, com maior ou menor dependência de insumos, principalmente ração (KÖPRÜCÜ e ÖZDEMIR, 2005). Sua produção em países em desenvolvimento ocorre primeiramente em sistema semi-intensivo, onde fertilizantes inorgânicos ou adubos são usados para produção do alimento natural (MATAKA e KANG´OMBE, 2007). Sistema semi-intensivo (Figura 2) constitui um meio de produção de baixo custo, sua

produtividade é geralmente menor do que a dos sistemas intensivos devido à menor densidade de estocagem e insumos de produção. Apesar disto, sua popularidade tem aumentado nas duas últimas décadas em vários países do mundo, especialmente entre os pequenos agricultores (EL-SAYED, 2006).

**Figura 2:** Cultivo semi-intensivo de tilápia do Nilo

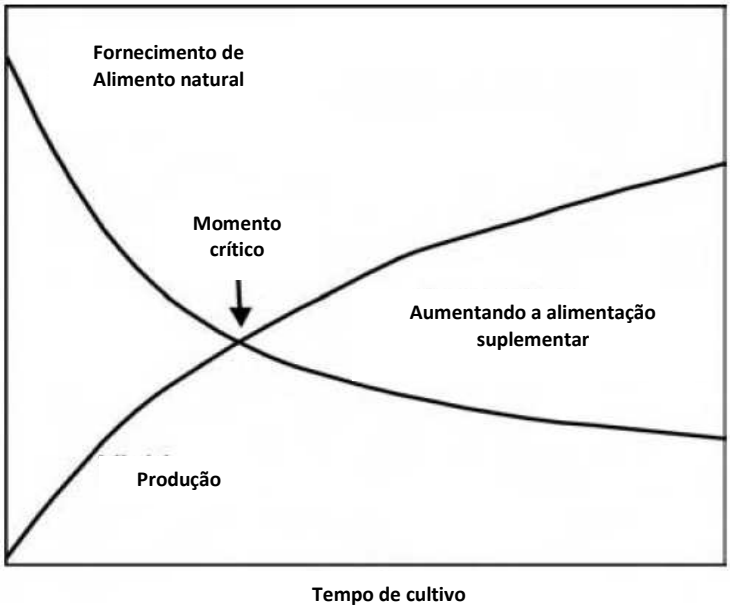
Fonte: Juliana Ferreira dos Santos

O cultivo em viveiros escavados apresenta uma menor incidência de doenças, maior sobrevivência na recria e engorda, contribuição do alimento natural e uma melhor conversão alimentar quando comparado a sistema intensivo em tanques rede (KUBITZA, 2009).

Neste sistema pode-se variar a biomassa de 0,1 a 0,5 $Kg/m^2$ dependendo do aporte de alimentação suplementar. Quando a alimentação da tilápia é suplementada com baixas concentrações de proteína em cultivo semi-intensivo, o déficit é suprido pelo alimento natural (LITI et al., 2006). No entanto, alimento natural é deficiente em energia não protéica, induzindo a utilização de parte desta proteína natural no metabolismo energético (EDWARDS et al., 2000).

Diana et al. (1994) avaliaram os efeitos da adição de alimento suplementar em viveiros fertilizados no crescimento de tilápia do Nilo, e observaram que a combinação da alimentação com fertilizantes foram mais eficientes do que um desses dois itens sozinhos. A Figura 3 demonstra a importância da alimentação suplementar em cultivos semi-intensivos. Green et al. (2002) também avaliaram o cultivo de tilápia do Nilo em viveiros fertilizados e constataram uma melhor produtividade com a alimentação suplementar.

**Figura 3**: Desenho esquemático da relação entre produção, alimento natural e alimentação suplementar em viveiros escavados.




Fonte: El-Sayed (2006).

Dietas convencionais podem aumentar a produtividade, no entanto também podem aumentar os custos neste tipo de sistema, tornando o cultivo mais oneroso. Desta forma, tem se focado no uso de recursos que possam tornar este cultivo mais viável (MATAKA e KANG´OMBE, 2007). Um regime alimentar adequado, baseado em taxas de alimentação, como demonstrado por Abdelghany e Ahmad (2002) pode reduzir tanto os custos como a carga de nutrientes nos viveiros.

A expansão do cultivo de tilápia no mundo juntamente com a escassez de água doce, competição com a agricultura e com as atividades urbanas, tem mudado gradualmente os tradicionais sistemas semi-intensivos de criação de tilápia para sistemas mais intensivos de produção (EL-SAYED, 2006). Sistema intensivo caracteriza-se pelas altas densidades (25 a 300 peixes/$m^3$), dietas com altos níveis proteicos e uma alta produtividade. Este tipo de cultivo vem se difundido para tilápia com o uso de tanques-rede (MOHAMMAD, 2006). O cultivo em tanques-rede (Figura 4) é visto como um caminho para aumentar a produção, proporcionar empregos e oportunidades (ABERY et al., 2005). Uma das vantagens do cultivo de tilápia em tanques-rede é que ele pode ser aplicado em vários corpos aquáticos, incluindo rios, lagos, reservatórios, lagoas, água do mar, viveiros, etc (WATANABE et al., 1990; PERSAND e BHIKAJEE, 1997; FITZSIMMONS et al., 1999; ORACHUNWONG et al., 2001; YI e LIN, 2001).

O cultivo em gaiolas apresenta como características uma tecnologia relativamente barata, quando comparado a outros sistemas intensivos de criação; pode ser aplicado na maioria dos

ambientes aquáticos; assegura maior controle do estoque e melhor observação dos peixes quando comparado com viveiros (PEIXOTO, 2007). Além disso, ele também apresenta como características positivas um tratamento mais econômico de parasitas e doenças, facilidade na despesca, manuseio dos peixes com redução na mortalidade, melhores taxas de crescimento, redução do uso de terra para construção de viveiros, controle na reprodução, boa utilização do alimento artificial, etc. (EL-SAYED, 2006). Ostrensk et al. (2008) relatam que o investimento necessário para a produção de uma tonelada de peixe em tanque rede está na ordem de 30-40% da produção em viveiros convencionais. Este fato, aliado às altas produtividades tem sido responsável pela grande expansão do sistema intensivo no país. Além disso, enquanto o sistema semi-intensivo produz cerca de 1 kg a 3 kg de peixe/$m^3$/ano, o sistema de tanques-rede pode produzir de 150 a 300 kg/$m^3$ no mesmo período. No entanto, a intensificação do cultivo requer o desenvolvimento de rações adequadas (KÖPRÜCÜ e ÖZDEMIR, 2005), já que a maior parte dos nutrientes ofertados neste sistema vem por meio das dietas artificiais, diferente do sistema semi-intesivo de criação.

**Figura 4**: Cultivo intensivo de tilápia do Nilo.

Fonte: Juliana Ferreira dos Santos

Parâmetros como qualidade de água, densidade, nutrição e alimentação são os fatores que determinam a sucesso ou fracasso de um cultivo intensivo de tilápia do Nilo. De acordo com El-Sayed (2006), o cultivo em gaiolas também apresenta algumas limitações como risco de roubo, perda devido a danos na gaiola, surtos de doenças, baixa tolerância dos peixes a má qualidade da água, completa dependência de rações de alta qualidade, perda de alimentação pelas malhas da gaiola e acúmulo de fezes e metabólitos no ambiente causando impacto negativo. Boyd (1990) enfatiza que a alimentação influencia negativamente na qualidade da água do sistema se não for

bem gerenciada. Estudos feitos por Lin et al. (1989); Mcginty (1991); Yi et al. (1996) e Liti et al. (2005) demonstraram que altas densidades de tilápia também podem influenciar negativamente o cultivo, promovendo redução no ganho de peso. De fato ambientes estressantes são adversos ao crescimento, função imune e reprodução, causando mortalidade e suscetibilidade a doenças e predadores. Diana et al. (1994) e Boyd (1990) também demonstraram que um manejo inadequado com quedas nos níveis de oxigênio dissolvido pode levar a diminuições nas taxas de crescimento e/ou eventuais mortalidades.

No entanto, os animais podem demonstrar algumas compensações fisiológicas recuperando-se da experiência estressante, além disso, a qualidade da alimentação fornecida pode influenciar nesta recuperação. Desta forma, sistemas de cultivos diferenciados, bem como manejo e alimentação utilizada, poderiam influenciar os animais promovendo alterações e adaptações na sua fisiologia.

### 2.3. Aparelho digestório X Preferência alimentar

A anatomia do trato gastrointestinal dos peixes segue o mesmo plano básico de outros vertebrados, sua compreensão também nos fornece um entendimento acerca de sua fisiologia digestiva e preferência alimentar. Os peixes podem ser classificados em quatro categorias principais com relação ao hábito alimentar: detritívoros, herbívoros, onívoros e carnívoros. Vale salientar que a versatilidade trófica envolve também a designação dos peixes como: generalistas (sem preferência acentuada por uma fonte alimentar, utilizando um amplo espectro de alimentos); especialistas (com dieta restrita a um número relativamente pequeno de itens e usualmente apresentando adaptações morfológicas tróficas) e oportunistas (que se alimentam de fonte não usual de sua dieta ou/e fazem uso de uma fonte alimentar abundante e incomum) (ABELHA et al., 2001).

Estes organismos podem ocupar todos os níveis tróficos de um ecossistema aquático, de forma a utilizar diferentes recursos alimentares, graças à grande diversificação morfológica quanto aos órgãos e estruturas relacionadas à procura, detecção, captura, ingestão e digestão do alimento (SAMPAIO e GOULART, 2011).

A compreensão da anatomia do sistema digestório nos fornece um entendimento sobre o contexto da organização espacial e sua relação com dados bioquímicos e fisiológicos, e também são essenciais para a compreensão da nutrição de peixes em aquicultura, bem como os mecanismos de adaptações fisiológicas para mudanças no ambiente (GROSELL et al., 2011).

Sampaio e Goulart (2011) observaram as relações entre os caracteres morfológicos (boca, dentes, rastros branquiais, estômago e intestino) e a alimentação dos ciclídeos, encontrando uma estreita relação entre os mesmos.

A tilápia como um peixe onívoro oportunista, é capaz de assimilar nutrientes de diferentes fontes, como as algas bentônicas, fitoplâncton, macrófitas, zooplâncton, pequenos invertebrados, detritos e cianofíceas. A escolha da alimentação é principalmente relacionada com a disponibilidade de alimentos no ambiente, indicando que a espécie possui alta plasticidade trófica (BOWEN, 1982; PHILIPPART e RUWET, 1982; FITZSIMMONS, 1997; BEVERIDGE e BAIRD, 2000; LOWE-MCCONNELL, 2000; SAMPAIO e GOULART, 2011).

Embora haja exceções, a anatomia digestiva entre espécies de uma mesma família é bastante similar. De acordo com Rust (2002), a digestão é uma ação coordenada de atividades físicas, químicas e enzimáticas que tem início quando alimento entra na boca e termina quando é excretado nas fezes.

Segundo Hyatt (1979), a forma e a posição da boca têm o potencial de influenciar na quantidade e qualidade da presa que pode ser ingerida. De acordo com Bond (1979), a boca pode ser inferior, subterminal, terminal ou superior. Sampaio e Goulart (2011) mencionam que existe profunda relação entre a morfologia da boca e o hábito alimentar. Câmara e Chellappa (1996) descrevem que a tilápia do Nilo apresenta uma boca terminal que favorece a captura de itens alimentares planctônicos.

De um modo geral a tilápia captura os alimentos por sucção (quando a presa é sugada para a cavidade bucofaringeal); captura visual (quando o peixe localiza e seleciona o alimento a ser ingerido); e pela mordida (utilizando as mandíbulas inferiores e superiores) (BEVERIDGE e BAIRD, 2000).

Os ciclídeos podem apresentar dentes na mandíbula, na maxila e na faringe, sendo estes importantes na taxonomia desta família (GOLDSTEIN, 1988). As tilápias possuem de um a cinco fileiras de dentes mandibulares, que juntamente com os dentes faringianos contribuem com a quebra dos alimentos em fragmentos menores facilitando a digestão (BOWEN, 1982; DEMPSTER et al., 1993). Câmara e Chellappa (1996) verificaram que existe estreita relação entre o desenvolvimento dos dentes e o nível trófico. A tilápia apresenta dentes rudimentares, sendo classificada como onívora-planctófaga, evidenciando que sua dieta baseia-se em organismos fitoplanctônicos e zooplanctônicos adequados ao tipo de dente.

Zayed e Mohamed (2004) demonstraram que tilápia do Nilo apresenta rastros branquiais relativamente curtos com número variável nos diferentes arcos. Eles também estabeleceram uma

relação entre o comprimento e número de rastros branquiais com a categoria trófica, caracterizando a tilápia como uma espécie onívora pelos seus rastros curtos e pouco numerosos. De acordo com Sampaio e Goulart (2011) esta relação se fundamenta no fato dos peixes planctófagos ingerirem partículas pequenas, e seus rastros numerosos e longos atuarem como uma peneira para reter os alimentos menores. Já os onívoros ingerem partículas de diversos tamanhos, seus rastros branquiais em menor quantidade e curtos favorecem a captura de presas maiores.

O esôfago de peixes é geralmente um tubo curto e reto com paredes espessas, ligado da faringe até o estômago ou intestino em peixes sem estômago. Morfologicamente o esôfago é projetado principalmente para a passagem de alimentos. A junção do esôfago e estômago não é claramente demarcada anatomicamente em teleósteos, a não ser pela mudança das fibras musculares estriadas (esôfago) e lisas (estômago) (GROSELL et al., 2011). A mudança também pode ser observada com o aparecimento de glândulas gástricas, no entanto esta transição se dá de forma gradual, revelando a zona de transição entre estes dois órgãos (KAPOOR et al., 1975).

Ao passar pelo esôfago, o alimento entra no estômago onde apresenta três regiões distintas, a região anterior denominada de cardíaca, de paredes mais finas, a região mediana ou fúndica e a região terminal ou pilórica, de paredes grossas, que é uma estrutura muscular (esfíncter) que tem a função valvular (GROSELL et al., 2011). O estômago armazena temporariamente o alimento e desempenha funções mecânicas que misturam o conteúdo estomacal auxiliando na digestão dos alimentos. A superfície interna (mucosa) contém uma variedade de células glandulares endócrinas e secretoras exócrinas, que contém células secretoras de muco e células que secretam o pepsinogênio, ácido clorídrico e enzimas digestivas (RUST, 2002; ROTTA, 2003).

O esfíncter pilórico que tem uma função valvular, mantém o alimento no estômago até ele estar fluído para passar para o intestino. Ele contribui impedindo refluxo do bolo alimentar para o estômago e controla o tempo em que o alimento fica em contato com o suco gástrico, controlando também a quantidade de material gástrico que entra no intestino (RUST, 2002; ROTTA, 2003).

A função primária do intestino é a realização dos processos digestivos (com auxílio das enzimas) que se iniciaram no estômago e a posterior absorção de nutrientes, íons e água oriundos da dieta (ROTTA, 2003; GROSELL et al., 2011). De acordo com Horn (1997) o comprimento do intestino é usado como um indicador do nível trófico em peixes. Em herbívoros seu comprimento é geralmente maior, quando comparados aos carnívoros (CLEMENTS e RAUBENHEIMER, 2005). No entanto, Kramer e Bryant (1995) alertam que a relação entre o comprimento do intestino e o hábito alimentar deve ser aplicada apenas na identificação de grandes grupos. Já Harder (1975) diz

que não há relações claras entre morfologia intestinal e o tipo de alimentação, e que esta relação não é conclusiva.

O intestino é um tubo relativamente simples, iniciando na válvula pilórica e terminando no reto (em peixes sem estômago). Ele possui glândulas digestivas e um suprimento de vasos de sangue e linfa, onde se completa a digestão iniciada no estômago (ROTTA, 2003). Uma característica básica do intestino de peixes teleósteos é a presença de pelo menos dois segmentos intestinais. O intestino superior apresenta uma quantidade mais escassa de células de Globet do que o inferior, este por sua vez apresenta uma camada muscular mais fina do que o superior. Há uma mudança de um epitélio colunar de células secretoras e de absorção para um epitélio que secreta basicamente muco no intestino inferior. De um modo geral, as porções intestinais que apresentam células mais complexas estão envolvidas com processos absortivos (SMITH, 1989).

Os cecos pilóricos são estruturas associadas ao intestino superior que apresentam formato digitiforme. Hossain e Dutta (1996) estimam que 60% das espécies de peixes conhecidas possuem cecos pilóricos. Buddington e Diamante (1987) demonstraram que os cecos pilóricos aumentam a área de superfície para a digestão e absorção, mas não apresentam um papel na fermentação ou no armazenamento. Rotta (2003) relata que não está bem definido se existe uma relação entre a presença de cecos pilóricos e o hábito alimentar dos peixes, pois eles ocorrem nos carnívoros, onívoros e herbívoros. Eles são responsáveis pela produção de enzimas digestivas e secreção de hormônios como insulina e glucagon. As tilápias não apresentam cecos pilóricos, mas em compensação possuem um intestino que pode variar de 7 a 14 vezes o comprimento do corpo, o qual pode compensar a ausência dos mesmos (POPMA e LOVSHIN, 1996).

De acordo com Rotta (2003) o pâncreas da maioria dos teleósteos é difuso e não pode ser facilmente observado durante uma dissecação total. Smith (1989) e Rotta (2003) afirmam que o pâncreas pode se apresentar em uma estrutura individualizada, ou de forma difusa, com nódulos pancreáticos espalhados no tecido adiposo, no mesentério, fígado, duto biliar, vesícula biliar, cecos pilóricos, intestino, etc. Nas lampreias, um pâncreas exócrino é inexistente e as células pancreáticas e zimogênios estão presentes no epitélio intestinal, sendo considerada uma condição primitiva (GROSELL et al., 2011).

## 2.4 Enzimas digestivas

Saldaña e Lopez (1988) mencionam que o valor nutricional de uma dieta não se baseia simplesmente na sua composição química, mas também na capacidade fisiológica do peixe em

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

As enzimas são catalizadores biológicos que aumentam de forma extraordinária as reações químicas. Sua eficiência catalítica, em geral, é bem maior que a dos catalisadores sintéticos e inorgânicos, sendo fundamentais para qualquer processo bioquímico. Com exceção de alguns RNAs catalíticos, todas as enzimas conhecidas são proteínas e podem ser classificadas de acordo com a reação específica que ela catalisa (tabela 1) (NELSON e COX, 2004).

**Tabela 1:** Classificação das enzimas segundo IUBMB.

| No. | Classe | Tipo de reação catalisada |
|---|---|---|
| 1 | Oxirredutases | Transferência de elétrons (íons hidretos ou átomos de H) |
| 2 | Transferases | Reações de transferência de grupos |
| 3 | Hidrolases | Reações de hidrólise |
| 4 | Liases | Adição de grupos as duplas ligações ou formação de duplas ligações por meio de remoção de grupos |
| 5 | Isomerases | Transferência de grupos dentro da mesma molécula para formar isômeros |
| 6 | Ligases | Formação de ligações do tipo C-C, C-S, C-O e C-N por meio de reações de condensação acopladas a quebra do ATP |

IUBMB: União Internacional de Bioquímica e Biologia Molecular. C: Carbono; N: Nitrogênio; S: Enxofre; O: Oxigênio. (Fonte: NELSON e COX, 2004).

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). A IUBMB recomenda o termo "peptidase" como sinônimo de protease, que é designado para qualquer enzima que hidrolise ligações peptídicas. A nomenclatura das peptidases é problemática, de um modo geral sua especificidade é geralmente difícil de definir, pois apresentam uma grande variedade de estruturas e ações. No entanto, uma classificação envolvendo o mecanismo catalítico é, por conseguinte utilizada.

Dois conjuntos de sub-subclasses de peptidases são conhecidas; as exopeptidases (EC 3.4.11 e EC 3.4.13-19) e as endopeptidases (EC 3.4.21-25). As exopeptidases atuam próximas as extremidades de cadeias polipeptídicas em uma extremidade N-terminal liberando um resíduo de aminoácido (aminopeptidases; EC 3.4.11), de um dipeptídeo ou de um tripeptídeo (dipeptidase e tripeptidases, EC 3.4.14). As exopeptidases que atuam no C-terminal livre libera um único resíduo (carboxipeptidases, EC 3.4.16-18), ou um dipeptídeo (peptídeo-dipeptidases; EC 3.4.15). As carboxipeptidases são divididas em três grupos com base no mecanismo catalítico: as

serinocarboxipeptidases (EC 3.4.16), os metalocarboxipeptidases (EC 3.4.17) e as cisteínocarboxipeptidases (EC 3.4.18). Outras exopeptidases são específicas para os dipeptídeos (dipeptidases, EC 3.4.13), ou para a remoção de resíduos terminais que são substituídos ou ligados por ligações isopeptídicas (omega peptidases , CE 3.4.19).

As endopeptidases são divididas em sub-subclasses, com base no mecanismo catalítico, e a especificidade é utilizada apenas para identificar enzimas individuais dentro dos grupos. As sub-subclasses são: serina endopeptidases (serinoproteases) (EC 3.4.21), cisteína endopeptidases (cisteínoprotease) (EC 3.4.22), aspartato endopeptidases (aspartatoproteases) (EC 3.4.23), metaloendopeptidases (metaloproteases) (EC 3.4.24) e treonina endopeptidases (treoninoprotease) (EC 3.4.25).

A tabela 2 baseada em Rao et al. (1998) demonstra a classificação e divisão das proteases.

**Tabela 2:** Classificação e divisão das proteases

| Local de clivagem do substrato | Sítio ativo da enzima | Número de resíduos de aminoácidos removidos |
|---|---|---|
| Exopeptidases | Aminopeptidases | Aminopeptidases<br>Aminodipeptidases<br>Aminotripeptidases |
| | | Sítio ativo da carboxipeptidase |
| | Carboxipeptidases | Serinocarboxipeptidase<br>Metalocarboxipeptidase<br>Cisteínocarboxipeptidase |
| Endopeptidases | Serinoproteases<br>Aspartatoproteases<br>Cisteinoproteases<br>Metaloproteases | |

Fonte: Rao et al. (1998).

As carboidrases e lipases também estão incluídas no grupo das hidrolases. As lipases (triacilglicerol acilhidrolases) são carboxilesterases que apresentam habilidade para hidrolisar ésteres de ácido carboxílico de cadeia longa, elas catalisam a hidrólise de ligações éster na interface orgânica-aquosa. Elas são enzimas ubíquas e são encontradas na maioria dos organismos (microbianos, vegetais e animais) (ABOUSALHAM e VERGER, 2000). Lipases são fisiologicamente importantes porque digerem gorduras em ácidos graxos para a absorção através da membrana celular, e transformam triglicerídeos em mais moléculas polares (LÓPEZ-LÓPEZ et al., 2003).

De acordo com Rotta (2003), o estômago dos peixes é capaz de secretar lipase gástrica, no entanto, se comparada à pancreática, tem pouca atuação no processo digestivo das gorduras e, em geral, hidrolisa apenas as gorduras de baixo ponto de fusão e já emulsificadas. As lipases de origem pancreática secretadas para o lúmen intestinal são as de maior importância na digestão das gorduras.

As carboidrases, também conhecidas como glicosilases, são enzimas que catalisam a degradação de carboidratos, ou seja, hidrolisam as ligações glicosídicas entre monossacarídeos formadores de oligossacarídeos ou polissacarídeos. De acordo com Kuz'mina et al. (1996) estas enzimas atuam na cavidade intestinal e na borda em escova dos enterócitos da parede intestinal em peixes, no entanto, há relatos de sua atividade no estômago de algumas espécies. De acordo com Silveira et al. (2009) as carboidrases aparecem também no suco pancreático, estômago, intestino e bile, mas não necessariamente em todos locais descritos para as espécies pesquisadas.

De um modo geral, os peixes aproveitam melhor os carboidratos complexos, como o amido, do que os açucares simples (New, 1987). Dentre as carboidrases estudadas em organismos aquáticos as amilases merecem atenção especial, já que são responsáveis pela degradação do amido e glicogênio.

As amilases são enzimas que catalisam a hidrólise de ligações glicosídicas $\alpha(1\rightarrow4)$ de polissacarídeos presentes no amido e glicogênio. Sobre o amido, atuam liberando diversos produtos, incluindo dextrinas, maltose e glicose. Elas podem ser divididas em dois grupos: as endoamilases e exoamilases. As endoamilases atuam de forma aleatória no interior da molécula do amido, causando a formação de ramos lineares de oligossacarídeos de cadeias de vários comprimentos e dessa forma quebram as ligações glicosídicas $\alpha(1\rightarrow4)$ presentes nas cadeias de amilose ou amilopectina (GUPTA, 2003). Diferentemente das β-amilases (exoamilases), que são encontradas em vegetais superiores e em algumas bactérias gram-positivas (PUJADAS et al., 1996) as α-amilases podem ser encontradas em animais, plantas, fungos e bactérias.

Em vertebrados superiores, as enzimas podem se apresentar distintamente, demonstrando claramente suas áreas funcionais. Para peixes, isto não é mostrado de forma clara, uma vez que as mesmas podem estar distribuídas ao longo do trato digestório (LUNDSTEDT et al., 2004). Rotta (2003) afirma que a maior parte da digestão dos alimentos ocorre no intestino e nos cecos pilóricos, quando presentes. Segundo o autor as três maiores classes de enzimas (proteases, lipases e carboidrases) estão presente com grande atividade nas secreções intestinais.

Geralmente, os fatores externos, como hábito alimentar e quantidade consumida desempenham um papel importante na distribuição e intensidade das atividades enzimáticas (TENGJAROENKUL et al., 2000). Elas também podem ser influenciadas pela idade e a espécie do

animal, bem como pela quantidade e composição da dieta ministrada (PEREZ et al., 1998). Jun-sheng et al. (2006) associam a distribuição e a intensidade de enzimas intestinais com o hábito alimentar e a morfologia intestinal. Seixas-Filho et al. (1999) citam que a produção da enzima α-amilase ocorre estritamente no pâncreas e no intestino, principalmente entre as espécies de peixes carnívoras e onívoras.

Rotta (2003) ainda afirma que peixes carnívoros apresentam maior atividade de lipases do que peixes onívoros e herbívoros. Seixas Filho et al. (2000) avaliaram a atividade lipolítica de três peixes de água doce com hábito alimentar diferente, encontrando uma baixa atividade para a espécie onívora piracanjuba (*Brycon orbignyanus*), e alta para as espécies piau (*Leporinus friderici*) e surubim (*Pseudoplatystoma curuscans*), onívora e carnívora, respectivamente.

Reimer (1982), estudando o matrinchã (*Brycon melanopterus*), observou que a atividade lipolítica está relacionada com a quantidade de gordura presente no conteúdo intestinal, e a atividade amilolítica aumenta quando a dieta é rica em amido. Fountoulaki et al. (2005) também demonstraram que as diferenças no perfil enzimático estão relacionados com os nutrientes presentes na dieta.

Já Nagase (1964), observou que não houve aumento na atividade de lipase quando a tilápia mossambica (*Sarotherodon mossambicus*) recebeu dieta rica em gordura. Hidalgo et al. (1999) analisaram as atividades proteolíticas e de amilase em seis espécies de peixes com diferentes hábitos alimentares, e observaram que a proporção de amilase total: atividade proteolítica total foi maior em peixes onívoros.

Kuz'mina et al. (1996) encontraram uma correlação entre a atividade amilolítica e sacarose total da mucosa intestinal, a concentração de carboidratos da dieta, e a intensidade de alimentação dos peixes. Jun-Sheng et al. (2006) observaram um aumento de atividade enzimática em tilápia com um aumento no peso corporal, sugerindo uma capacidade para utilizar alimentação de acordo com sua fase de crescimento.

Kumar et al. (2007) avaliando atividade proteolítica em três carpas (*Catla catla*, *Labeo rohita* e *Hypophthalmichthys molitrix*) observou uma atividade proteolítica total mais elevada em *L. rohita (*plânctofaga) seguido de *H. molitrix* (fitoplanctofaga) e posteriormente *C. catla* (zooplanctófaga). As atividades de tripsina e quimotripsina foram superiores em *H. molitrix*. Os autores sugerem que peixes herbívoros podem compensar sua dieta com proteína baixa, aumentando a sua atividade enzimática. Alguns peixes herbívoros apresentam atividades semelhantes de tripsina ou mesmo superiores quando comparados às espécies carnívoras, possivelmente para maximizar a eficiência de digestão de proteína.

Cara et al. (2007) avaliando as atividades de quimotripsina e tripsina em larvas de *Dicentrarchus labrax*, observaram que a atividade dos animais com restrição alimentar foram superiores a das larvas com melhor qualidade na alimentação, sugerindo que o aumento nas atividades seriam para compensar as deficiências alimentares no qual as larvas foram submetidas.

Assim, um bom entendimento do modo de regulação das enzimas digestivas se torna importante para o uso racional do tipo e quantidade de nutrientes utilizados nas dietas (MUHLIA-ALMAZÁN et al., 2003). Estudos nutricionais e enzimáticos contribuem para formulação de rações mais eficientes, reduzindo de forma extraordinária os custos na produção de peixes comerciais (CARUSO, et al., 1996). Além disto, podem-se diminuir os resíduos depositados no ambiente, que é imprescindível para tornar a aquicultura uma atividade sustentável e ecologicamente viável.

Tilápias são bem conhecidas pela sua capacidade de utilizar uma grande variedade de alimentos (LOWE-MCCONNELL 1975; BOWEN, 1982; PHILIPPART e RUWET, 1982; TREWAVAS 1983). Molina et al. (2005) e Lu et al. (2006) demonstraram que a tilápia tem a capacidade de ingerir e digerir cianobactérias em lagos eutrofizados, sugerindo o amplo espectro de digestão deste peixe. Funções digestivas capazes de hidrolisar uma variedade maior de alimentos tornam os peixes onívoros com capacidade de digestão variada, comparada com os carnívoros (ALMEIDA et al., 2006). Dentre as espécies onívoras, a tilápia tem se destacado pela elevada capacidade de utilizar nutrientes de origem vegetal e animal, o que possibilita a elaboração de rações de baixo custo e alto valor nutritivo (SILVA et al., 2006).

## 2.5 Utilização de resíduos da indústria pesqueira para fabricação de rações de organismos aquáticos

Uma boa alimentação deve satisfazer as necessidades nutricionais das espécies cultivadas, como também ser de fácil digestão e com uma boa perspectiva custo-benefício (LEMOS et al., 2004).

A determinação de fontes proteicas de menor custo e que promovam bom crescimento, é vantajoso tanto para a indústria de rações, como também para os aquicultores (COYLE et al., 2004). Sendo assim, vários estudos têm sido realizados para avaliar novas fontes proteicas que poderiam ser usadas na fabricação de rações (JACKSON et al., 1982; GABER, 1996; OLVERA-NOVOA et al., 1997; OLVERA-NOVOA et al., 2002; PLASCENCIA-JATOMEA et al., 2002; EL - SAIDY e GABER, 2003; GABER, 2006, LEAL et al., 2009). Tais recursos devem garantir os mesmos ou melhores resultados de produção, sanidade do animal, crescimento e qualidade do produto.

O crescimento da indústria de pescado tem gerado uma grande quantidade de resíduos e subprodutos que representam um desafio para os empresários do setor e para a comunidade científica especializada, em buscar estratégias para que essa atividade seja sustentável e ecologicamente viável. Segundo Arruda (2004), cerca de 50 % do pescado mundial produzido em 2000 transformou-se em resíduo. Supondo-se que esse percentual tenha-se mantido, dos 154 milhões de toneladas de pescado produzidos em 2011 (FAO, 2012), 77 milhões teriam sido descartados ou subutilizados, constituindo-se em uma fonte significativa de desperdício de recursos e de contaminação ambiental.

Ao longo dos anos, diversos têm sido os esforços empreendidos por pesquisadores em todo o mundo para desenvolver métodos que possibilitem a transformação desses resíduos em produtos passíveis de utilização tanto na nutrição humana quanto na animal (ARMENTA e GUERRERO-LEGARRETA, 2009; CHEN et al., 2011; CAHÚ et al., 2012).

A produção de farinha, hidrolisado proteico ou silagem de camarão a partir de subprodutos das indústrias pesqueiras (Figura 5) representa uma excelente alternativa para o incremento da oferta de proteína animal (MACKIE, 1982; HAARD, 1992; KENT, 1997; CAVALHEIRO et al., 2007), já que estes subprodutos são usualmente descartados.

**Figura 5:** Resíduos da indústria de camarão.

Fonte: Juliana Ferreira dos Santos

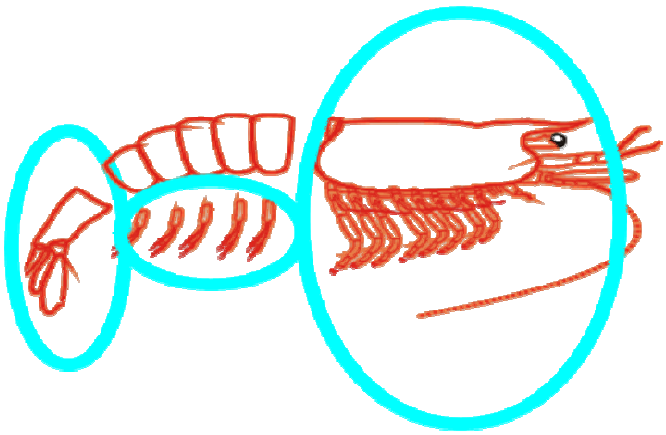
No entanto, o uso de farinha de cabeça de camarão na formulação de alimentos para organismos aquáticos não é recomendado devido a sua alta concentração de fibras e cinzas, o que resulta na formação de péletes fracos com uma baixa estabilidade em água (CAVALHEIRO et al., 2007).

Uma alternativa seria a produção de proteína hidrolisada. Vários são os métodos empregados para obtenção do hidrolisado proteico dos produtos e subprodutos pesqueiros (GILDBERG, 1993). A presença de enzimas proteolíticas no trato digestório de animais aquáticos tem uma influência significante na produção de hidrolisados, que podem ser obtidos empregando o processo de autólise ou um método de hidrólise, através da adição de enzimas (SHAHIDI et al., 1995). Gilberg e Stenberg (2001) demonstraram que a proteína dos subprodutos do camarão pode ser hidrolisada por proteases comerciais e recuperada como hidrolisado proteico com alto conteúdo de aminoácidos essenciais. A hidrólise utilizando substâncias exógenas permite um melhor controle das propriedades dos produtos resultantes. No entanto, os custos das substâncias (enzimas, ácidos e solventes) são um obstáculo para a viabilidade do processo. Plascencia-Jatomea et al. (2002) relataram que as condições ácidas em que o hidrolisado de silagem de camarão é produzido faz com que haja perda de nutrientes lábeis, como o triptofano.

A autólise é um processo enzimático que pode ser usado como uma alternativa aos processos que empregam substâncias exógenas, além de haver a possibilidade de recuperar moléculas bioativas, tais como quitina (para a produção de quitosana) e carotenóides (ARMENTA e GUERRERO-LEGARRETA, 2009; CHEN et al., 2011;. CAHÚ et al., 2012).

No processamento do camarão, geralmente são removidas a cabeça, o exoesqueleto e a porção posterior (Figura 6). Estes subprodutos correspondem a aproximadamente 50% do seu peso total, o que torna importante seu aproveitamento do ponto de vista econômico, industrial e ambiental (HEU et al., 2003).

**Figura 6**: Desenho esquemático de camarão identificando os resíduos da indústria pesqueira passíveis de utilização na produção de proteína hidrolisada.



Designer: Lin Diniz

De acordo com Leal et al., (2009) a composição de hidrolisado proteico de camarão mostrou ser uma fonte adequada de triptofano, lisina, leucina, arginina, fenilalanina, valina e metionina.

De fato, a qualidade nutricional da proteína hidrolisada pode estar relacionada com a elevada concentração de pequenos peptídeos e aminoácidos essenciais, como também de compostos que estimulam a resposta imune nos peixes, promovendo assim crescimento e resistência a doença (GILDBERG e STENBERG, 2001). Segundo Silvestre et al. (1994), é preferível utilizar hidrolisado em dietas do que misturas de aminoácidos, uma vez que di e tripeptídeos são absorvidos pelo intestino mais rapidamente do que os aminoácidos livres. Estes peptídeos são também aceitos como atrativo alimentar devido à estimulação química que eles podem produzir em larvas e juvenis, contribuindo para o consumo de dietas artificiais nas fases iniciais de cultivo.

De acordo com Muhlia-Almazán et al. (2003) e Lundstedt et al. (2004), mudanças na origem e quantidade de nutrientes podem afetar as concentrações ou as expressões gênicas das enzimas digestivas. No entanto, Ezequerra et al. (1997); Moraes e Bidinoto (2000) sugerem que os animais aquáticos podem adaptar os níveis enzimáticos de acordo com mudanças na alimentação.

A inclusão de hidrolisado proteico na dieta de animais aquáticos tem sido amplamente estudada, principalmente avaliando seus efeitos sobre o crescimento, sobrevivência e atratividade (KOLKOVISK et al., 2000; PLASCENCIA-JATOMEA et al., 2002; SAVOIE et al., 2006 ). No entanto, existe pouco conhecimento sobre o efeito de hidrolisados proteicos sobre as enzimas digestivas de organismos aquáticos. Autores como Córdova-Murueta e García-Carreño (2002); Cahu et al. (2004); Zambonino-Infante e Cahu (2007) e Kotzamanis et al. (2007) realizaram estes estudos, contudo ainda são necessários mais pesquisas para fornecer a comunidade científica e aos produtores respostas mais conclusivas.

Kotzamanis et al. (2007) avaliaram o efeito da inclusão de hidrolisado proteico (dietas contendo 10 e 19% de proteína hidrolisada) sobre as atividades de enzimas digestivas em larvas de *Dicentrarchus labrax* (L.). Não foram encontradas diferenças estatísticas entre os tratamentos nas atividades de amilase e tripsina. No entanto, a atividade de aminopeptidase foi maior na concentração de 10% de hidrolisado proteico. Cahu et al. (2004) verificaram que a secreção de tripsina foi maior em larvas de *D. labrax* alimentadas com dietas contendo 14% de proteína hidrolisada, sendo reduzida a concentrações crescentes (46%).

O efeito da inclusão de proteína hidrolisada de camarão sobre as enzimas digestivas de tilápia do Nilo não estão descritos na literatura. Esta informação pode contribuir para um melhor

entendimento do modo de regulação destas enzimas e de possíveis adaptações da tilápia do Nilo na digestão de ingredientes alternativos.

## 3. OBJETIVOS

### 3.1 Objetivo geral:

Avaliar se mudanças na alimentação (inclusão de hidrolisado proteico de camarão) pode afetar as atividades enzimáticas do extrato bruto dos tecidos do trato digestório da tilápia do Nilo (*Oreochromis niloticus* L.) e avaliar a influência dos cultivos semi-intensivo e intensivo nos parâmetros zootécnicos e nas enzimas digestivas deste peixe.

### 3.2 Objetivos específicos:

1. Avaliar se a inclusão do hidrolisado proteico de camarão, em diferentes concentrações, pode promover mudanças nas principais atividades enzimáticas do extrato bruto dos tecidos do trato digestório da tilápia do Nilo;
2. Obter o perfil enzimático através de zimograma, de proteases dos extratos brutos do intestino dos espécimes experimentais, alimentados com diferentes concentrações de hidrolisado proteico de camarão;
3. Avaliar a diferença entre os sistemas intensivo e semi-intensivo através dos parâmetros de crescimento da tilápia do Nilo;
4. Obter o peso relativo dos peixes produzidos em sistema intensivo e semi-intensivo em comparação com tilápias provenientes de outros cultivos e de ambiente natural;
5. Determinar as atividades de enzimas digestivas nos extratos brutos dos tecidos do intestino de tilápia do Nilo nos diferentes sistemas de cultivo utilizando substratos específicos e inespecíficos;
6. Obter o perfil enzimático através de zimograma de protease e de amilase dos extratos brutos do intestino de tilápia do Nilo nos diferentes sistemas de cultivo;
7. Obter a eletroforese bidimensional dos extratos brutos do intestino de tilápia do Nilo em ambos os cultivos.

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## 5. CAPÍTULO I

ARTIGO CIENTÍFICO:

**Digestive enzyme activity in juvenile Nile tilapia (*Oreochromis niloticus*, L) submitted to different dietary levels of shrimp protein hydrolysate**

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# Digestive enzyme activity in juvenile Nile tilapia (*Oreochromis niloticus*, L) submitted to different dietary levels of shrimp protein hydrolysate

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**Abstract** The effect of different dietary concentrations of shrimp protein hydrolysate (SPH) on digestive enzyme activity of Nile tilapia juveniles was evaluated. SPH concentrations in diets were 0, 15, 30 and 60 g $kg^{-1}$ (treatments SPH0, SPH15, SPH30 and SPH60, respectively). Hemoglobin, azocasein, BApNA (*N*α-benzoyl-DL-arginine-*p*-nitroanilide), SApNA (Suc-Ala-Ala-Pro-Phe *p*-nitroanilide), aminoacyl of β-naphthylamide and starch were used as substrates for enzyme activity determinations. The activity of total alkaline protease was significantly higher ($P < 0.05$) in fish under SPH15 and SPH60 treatments than in the control (SPH0). However, the effect was not dose-dependent. Substrate-SDS-PAGE was also performed to evaluate changes in the profile

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of Nile tilapia digestive proteases caused by SPH. Substrate-SDS-PAGE revealed 12 active proteolytic bands, eight of which responded to SPH dietary incorporation. Inhibition substrate-SDS-PAGE indicated a decrease in the activity of three enzymes, with trypsin activity decreasing with the increase of SPH concentration, whereas the opposite occurred for two aminopeptidases. Distinct protease profiles were also found for each treatment, suggesting adaptability of digestive proteases from Nile tilapia to the different diets.

**Keywords** Digestive proteases and amylases · Nile tilapia · SDS-PAGE zymograms · Shrimp protein hydrolysate

## Introduction

Tilapia production has increased significantly on a global scale in the last decade. This development has been followed by an increase in feed consumption, stimulating the search for new ingredients in diet formulations (Schulz et al. 2007). The growth of the aquaculture industry has generated large amounts of waste and by-products, which represent a challenge to the sustainability of the activity (Bezerra et al. 2001). Shrimp processing waste, for instance, may be disposed of in the environment, where it may potentially pollute water and land. By-products of terrestrial and aquatic farming systems may be processed into suitable ingredients and used as components in animal feeds, following biosecurity principles for feed applications. Shrimp processing waste has been identified as an animal protein source of great potential (Fanimo et al. 2000). A simple protocol for producing protein hydrolysate from white shrimp *Litopenaeus vannamei* (Boone) processing waste through autolysis has recently been developed (Cahú et al. 2012). This method renders a protein concentrate that is considered to be an excellent source of amino acids, with high levels of glutamate, aspartate, leucine, lysine, tyrosine and arginine (Leal et al. 2010). In fact, crustacean protein silage and hydrolysate have been used in fish feeds both as a new protein source (Plascência-Jatomea et al. 2002) and, in small amounts, as flavoring to enhance the attractiveness of feeds (Kolkovski et al. 2000).

Variations in the quality and quantity of nutrients used in diet formulations may modify enzymatic profile and activity in the digestive tract of animals (Lundstedt et al. 2004). Thus, feed composition could induce biological adaptations, including an increase in nutrient absorption (Moraes and Bidinotto 2000). Digestive enzymes have been investigated as a way of understanding nutritional requirements, and the effects of diet composition on enzyme activity in order to reduce feeding costs in fish farms (Caruso et al. 1996). Most studies thus far have evaluated the effect of different concentrations of protein, carbohydrates and lipids in feed formulations, correlating these results with enzyme activity, and differences in enzyme quality profile may be related to nutrient levels in the diet (Fountoulaki et al. 2005).

The present survey focuses on the following aspects: (a) the detectable changes in the activity of the main digestive enzymes caused by different concentrations of shrimp protein hydrolysate in tilapia feeds and (b) the use of substrate-SDS-PAGE zymograms as an effective tool to improve the analysis of these changes.

## Materials and methods

### Materials

All reagents were of analytical grade and purchased from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany). The diets were prepared in Poytara LTDA (Araraquara—São Paulo—Brazil).

### Shrimp protein hydrolysate production

Shrimp head processing waste, comprising the cephalothorax without the body carapace, from *L. vannamei* juveniles (total body weight about 10–12 g) was provided by a local fishery processing plant (EMPAF Ltd.). SPH used in experimental diets was prepared according to Cahú et al. (2012). In total, approximately 20 kg of shrimp cephalothoraxes was obtained. The shrimp heads were washed and stored at −20 °C in plastic bags containing 1 kg of the material. After that, the raw material was crushed in distilled water (1:1 ratio; particle size approximately 5 mm). The enzymatic autolysis (proteases from the shrimp midgut gland) occurred in a vessel placed in a water bath at 45 ± 2 °C for 180 min with constant stirring (700 rpm). The solution was then heated to 100 °C for 10 min in order to deactivate the enzymes, and the solid portion was strained through a 1.0-mm sieve (Cahú et al. 2012). The resulting material was centrifuged at 10,000 × *g* for 10 min, and the supernatants (SPH) was stored in plastic bottles at −20 °C until the preparation of the diets (Leal et al. 2010). The production method was similar to the industrial processes. Previously frozen-dried samples of SPH ($n = 3$) were used for proximate composition and AA analyses (Tables 1, 2) in Protein Chemical Center of the Faculdade de Medicina de Ribeirão Preto, São Paulo, Brazil (AOAC 1984).

### Hydrolysis profile of SPH

Enzymatic hydrolysis of SPH (45 °C) was followed by sampling at the incubation times of 0, 30, 60, 90, 120, 150 and 180 min. The hydrolysis profile of shrimp protein hydrolysate (Aliquots of 100 µg of protein, $n = 3$) was evaluated in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), using stacking gel at 4 % (w/v) and separation gel at 17 % (Laemmli 1970). The gels were stained for protein overnight in 0.01 % (w/v) Coomassie Brilliant Blue. The background of the gel was destained by washing in a solution containing 10 % (v/v) acetic acid and 25 % (v/v) methanol. The molecular mass of the protein bands was estimated using the 220–10 kDa molecular mass protein standards (Sigma).

**Table 1** Proximate composition (%) and energy of lyophilized shrimp protein hydrolisate

| Composition | Mean |
|---|---|
| Moisture (%) | 9.7 |
| Crude protein (%) | 43.6 |
| Ether extract (%) | 6.2 |
| Ash (%) | 7.3 |
| Carbohydrate (%) | 33.1 |
| Energy (Kcal 100 $g^{-1}$) | 363.3 |

SPH, from *Litopenaeus vannmei* (Leal et al. 2010)

**Table 2** Amino acid composition of the shrimp protein hydrolisate

| Amino acids | SPH | |
|---|---|---|
| | mg 100 $g^{-1}$ | % |
| *Essential* | | |
| Arginine | 3400 ± 0.043 | 7.3 |
| Histidine | 1060 ± 0.005 | 2.3 |
| Isoleucine | 2000 ± 0.021 | 4.3 |
| Leucine | 3490 ± 0.021 | 7.4 |
| Lysine | 3350 ± 0.000 | 7.2 |
| Methionine | 1290 ± 0.005 | 2.7 |
| Phenylalanine | 2370 ± 0.002 | 5.1 |
| Threonine | 2120 ± 0.031 | 4.5 |
| Thryptophan | 670 ± 0.016 | 1.4 |
| Valine | 2250 ± 0.012 | 4.8 |
| *Non-essential* | | |
| Tyrosine | 3370 ± 0.004 | 7.2 |
| Aspartic acid | 4270 ± 0.031 | 9.1 |
| Glutamic acid | 5780 ± 0.003 | 12.4 |
| Glycine | 2890 ± 0.005 | 6.2 |
| Serine | 2030 ± 0.001 | 4.3 |
| Alanine | 3070 ± 0.017 | 6.6 |
| Proline | 2970 ± 0.024 | 6.3 |
| Cystine | 410 ± 0.015 | 0.9 |
| Total | 46790 | 100 |

SPH from *Litopenaeus vannamei* (Leal et al. 2010)

## Diets preparation

According to Leal et al. (2010), four isonitrogenous (37 % crude protein), isocaloric (total energy: 440 kcal 100 $g^{-1}$) experimental diets were formulated to feed *Oreochromis niloticus* juveniles (Table 3). Shrimp protein hydrolysate (SPH) was included in the diets at concentrations of 0 (control—SPH0), 15 (SPH15), 30 (SPH30) and 60 g $kg^{-1}$ (SPH60), as a fed basis. A 1:2 animal/plant protein ratio (fishmeal and soybean meal, respectively) in the diets was established. SPH (liquid) was mixed with soybean meal, and the dough was dried at 65 °C for 24 h. The ingredients were mixed and the diets prepared by extrusion under industrial conditions to obtain 1-mm diameter pellets.

## Animals and culture conditions

Juvenile sex-reversed Nile tilapias were obtained from the Aquaculture Station of the Universidade Federal Rural de Pernambuco. The fish were stocked in fifteen 40-L glass aquaria (8 ind. per aquarium) equipped with a biological filter and continuous aeration and then submitted to a 7-day acclimatization period both for diets and experimental conditions in a completely randomized design, with four treatments and three replicates. The animals were kept in a photoperiod of 12:12 L:D. Prior to the feeding trial, fish were weighed (1.7 ± 0.4 g) and measured (4.7 ± 0.4 cm). Individuals were fed a ratio of 15, 14, 12, 10, 8 and 6 % of biomass per day, adjusted every 9 days for a period of 45 days. Feed was

**Table 3** Composition and approximate analysis of the experimental diets

| Ingredients (%) | Diets | | | |
|---|---|---|---|---|
| | SPH 0 | SPH 15 | SPH 30 | SPH 60 |
| Fish meal (57 % CP) | 23.0 | 22.0 | 21.0 | 18.0 |
| Shrimp protein hydrolysate (SPH) | 0.0 | 1.5 | 3.0 | 6.0 |
| Soybean meal (40.4 % CP) | 47.0 | 47.5 | 47.5 | 47.5 |
| Wheat meal | 16.0 | 13.5 | 13.5 | 15.5 |
| Corn starch | 10.5 | 12.0 | 11.5 | 9.5 |
| Soybean oil | 1.0 | 1.0 | 1.0 | 1.0 |
| Dicalcium phosphate | 1.0 | 1.0 | 1.0 | 1.0 |
| Mineral and vitamin mix[a] | 1.0 | 1.0 | 1.0 | 1.0 |
| Salt | 0.5 | 0.5 | 0.5 | 0.5 |
| Antioxidant BHT | 0.02 | 0.02 | 0.02 | 0.02 |
| Proximate analysis (as-fed basis, g $kg^{-1}$) | | | | |
| Dry matter | 944.8 | 935.9 | 936.5 | 946.7 |
| Crude protein | 371.9 | 374.3 | 376.2 | 380.6 |
| Ether extract | 48.1 | 56.2 | 52.1 | 55.9 |
| Crude fiber | 39.7 | 38.8 | 41.1 | 46.6 |
| Ash | 105.7 | 102.9 | 101.6 | 101.9 |
| Nitrogen-free extract | 434.6 | 427.8 | 429.0 | 435.0 |
| Calcium | 22.2 | 21.7 | 20.0 | 17.2 |
| Phosphorus | 12.4 | 12.5 | 12.6 | 12.8 |
| Gross energy (kcal 100 $g^{-1}$)[b] | 438.3 | 444.5 | 442.2 | 431.8 |
| P/GE ratio (mg $kcal^{-1}$) | 84.9 | 84.1 | 84.6 | 88.0 |

Leal et al. 2010

*CP* crude protein, *BHT* butylated hydroxytoluene

[a] Mineral and vitamin mix (quantity kg-1 premix): vitamin A (20,000 UI), vitamin $D_3$ (5,000UI), vitamin E (250 mg), vitamin $K_3$ (25 mg), vitamin $B_1$ (37.5 mg), vitamin $B_2$ (37.5 mg), vitamin $B_6$ (25 mg), vitamin $B_{12}$ (0.053 mg), vitamin C (250 mg), niacin (200 mg), pantothenic acid (100 mg), biotin (1,25 mg), choline (1000 mg), inositol (250 mg), Fe (100 mg), Cu (12 mg), Zn (125 mg), Mn (37.5 mg), Se (0.25 mg), I (1.25 mg), Co (0.25 mg)

[b] Estimate based on 5.65, 4.2 and 9.5 kcal $g^{-1}$ for proteins, carbohydrates and lipids, respectively

offered 4 times per day. The fish accepted and fed on all diets over the experimental period (Leal et al. 2010).

The aquaria were siphoned twice a day to remove solids, with 66 % water being replaced with clean water every day. Temperature (28.7 ± 0.59 °C), dissolved oxygen (3.5 ± 0.92 mg $L^{-1}$) and pH (8.1 ± 0.19) were measured twice a day ($n = 90$). Ammonia (0.14 ± 0.22 mg $L^{-1}$) and nitrite (0.08 ± 0.02 mg $L^{-1}$) were monitored once a week ($n = 6$). Values are expressed as mean ± SE

## Growth and nutrient utilization

The effects of SPH on the growth performance and nutrient utilization are shown in Table 4. Growth performance was evaluated through weight gain rate (WG), average daily gain (ADG), specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency

**Table 4** Growth performance and nutrient utilization in Nile tilapia fed on diets containing shrimp protein hydrolysate instead of fish meal replacement

| Diets | SPH 0 | SPH 15 | SPH 30 | SPH 60 |
|---|---|---|---|---|
| Initial weight (g) | 1.68 ± 0.14 | 1.72 ± 0.07 | 1.75 ± 0.09 | 1.81 ± 0.06 |
| Final weight (g) | 27.18 ± 2.43$^{ns}$ | 29.46 ± 1.05$^{ns}$ | 26.02 ± 3.07$^{ns}$ | 25.19 ± 2.49$^{ns}$ |
| Survival (%) | 100.0$^{ns}$ | 100.0$^{ns}$ | 100.0$^{ns}$ | 100.0$^{ns}$ |
| WG (g)[a] | 25.51 ± 2.57$^{ns}$ | 27.73 ± 1.11$^{ns}$ | 24.29 ± 3.04$^{ns}$ | 23.39 ± 2.49$^{ns}$ |
| ADG (g day$^{-1}$)[b] | 0.57 ± 0.10$^{ns}$ | 0.62 ± 0.04$^{ns}$ | 0.54 ± 0.12$^{ns}$ | 0.52 ± 0.10$^{ns}$ |
| SGR (% day$^{-1}$)[c] | 7.15 ± 0.58$^{ns}$ | 7.38 ± 0.10$^{ns}$ | 6.85 ± 0.68$^{ns}$ | 6.73 ± 0.57$^{ns}$ |
| FCR[d] | 1.15 ± 0.22$^{ns}$ | 1.09 ± 0.16$^{ns}$ | 1.13 ± 0.10$^{ns}$ | 1.17 ± 0.12$^{ns}$ |
| PER[e] | 2.26 ± 0.39$^{ns}$ | 2.33 ± 0.36$^{ns}$ | 2.20 ± 0.18$^{ns}$ | 2.14 ± 0.22$^{ns}$ |
| ANPU[f] | 39.31 ± 3.90$^{ns}$ | 40.40 ± 3.64$^{ns}$ | 38.59 ± 1.84$^{ns}$ | 34.72 ± 2.08$^{ns}$ |

Leal et al. 2010

Different superscripts in the same column denote statistical differences ($P < 0.05$), and "ns" denotes no statistical differences. Values are mean ± SE of five replicates

[a] Weight gain rate, [b] Average daily gain, [c] Specific growth rate, [d] Feed conversion ratio, [e] Protein efficiency ratio, [f] Apparent net protein utilization

ratio (PER) and apparent net protein utilization (ANPU) based on the following formulae: WG (g) = $BW_f - BW_i$; ADG = WG (g)/time (days); SGR = 100 (Ln $BW_f$ − Ln $BW_i$)/time (days); FCR = dry feed offered (g)/wet weight gain (g); PER = wet weight gain (g)/protein fed (g); and ANPU = 100 [($BW_f \times BCP_f$) − ($BW_i \times BCP_i$)]/(TF × CP), in which $BW_i$ and $BW_f$ = average initial and final body weight (g) of fish, respectively; $BCP_i$ and $BCP_f$ = initial and final body crude protein (g 100 g$^{-1}$), respectively; TF = total amount of diet fed (g) and CP = crude protein of diet (g 100 g$^{-1}$) (Leal et al. 2010).

## Extraction of digestive enzymes

At the end of trial, fish were left to fast for 24 h, and six individuals from each replicate aquarium (divided into two replicates per aquarium) were removed and killed in an ice bath for biometric measurements and tissue removal, according to standard methodology (Bezerra et al. 2001). Stomach and intestines were immediately collected and homogenized (40 mg tissue mL$^{-1}$) in 0.01 M Glycine–HCl pH 3.0 and 0.01 M Tris–HCl pH 8.0 buffers, respectively, containing 0.15 M NaCl, using a tissue homogenizer. The resulting preparations were centrifuged at 10,000 × *g* for 10 min at 4° C to remove cell debris and nuclei. The supernatants (crude enzyme extracts) were frozen at −20 °C and used in further assays (Bezerra et al. 2005). Protein concentration was determined according to Bradford (1976) using bovine serum albumin (BSA) as the standard and reported as mg of protein equivalent to BSA.

## Total acid proteolytic activity

Acid protease activity was evaluated using hemoglobin (Hb) as the substrate as follows: in microcentrifuge tubes, 100 µL of 2 % Hb in 0.06 M Glycine–HCl buffer pH 3.0 was mixed with 50 µL stomach crude extract and 350 µL 0.5 M Glycine–HCl buffer pH 3.0 for 60 min at 25 °C. Five hundred microliters of 10 % trichloroacetic acid (TCA) was then added to stop the reaction. After 15 min, centrifugation was carried out at 8,000 × *g* for

10 min. The absorbance of supernatant (70 µL) was measured at 280 nm (Bio-Rad SmartSpec 3000, USA) versus a similarly prepared blank in which 0.01 M Glycine–HCl buffer pH 3.0 replaced the crude extract sample, based on a methodology adapted from Díaz-López et al. (1998). Previous experiments showed that, for the first 60 min, the reaction carried out under the conditions described above followed first-order kinetics. One unit (U) of enzyme activity was defined as the amount of enzyme capable of hydrolyzing hemoglobin to produce a 0.001 change in absorbance per minute per milligram of protein.

### Total alkaline proteolytic activity

In microcentrifuge tubes, 1 % azocasein prepared in 0.1 M Tris–HCl buffer pH 8.0 was incubated with intestine crude enzyme extract (30 µL) for 60 min at 25 °C. Two hundred and forty µL of 10 % trichloroacetic acid (TCA) was then added to stop the reaction. After 15 min, centrifugation was carried out at 8,000×*g* for 5 min. The supernatant (70 µL) was added to 1 M NaOH (130 µL) in a 96-well microtiter plate, and the absorbance of this mixture was measured in a microtiter plate reader (Bio-Rad 680, Japan) at 450 nm versus a similar prepared blank in which 0.01 M Tris–HCl pH 8.0 replaced the crude extract sample. One unit (U) of enzyme activity was defined as the amount of enzyme capable of hydrolyzing azocasein to produce a 0.001 change in absorbance per minute per milligram of protein (Bezerra et al. 2005).

### Trypsin and chymotrypsin activity

The activity of trypsin and chymotrypsin was determined using 8.0 mM BApNA (*N*α-benzoyl-DL-arginine-*p*-nitroanilide) and 8.0 mM SApNA (Suc-Ala-Ala-Pro-Phe *p*-nitroanilide) in DMSO (dimethyl sulfoxide), respectively. Intestine crude enzyme extract (30 µL) was incubated with 0.1 M Tris–HCl buffer pH 8.0 (140 µL) and respective substrates (30 µL) in a microtiter plate reader (Bio-Rad 680, Japan). Absorbance was measured at 405 nm versus a similar prepared blank in which 0.1 M Tris–HCl pH 8.0 replaced the crude extract sample. Enzyme activity was determined in triplicate. Trypsin and chymotrypsin units of activity were expressed as a change in absorbance per minute per milligram of protein (Bezerra et al. 2005).

### Aminopeptidase activity

Aminopeptidase activity was evaluated using aminoacyl of β-naphthylamide (AA of arginine) as substrate. The procedure adapted from Oliveira et al. (1999) was carried out in triplicate, by incubating 4.2 mM substrate (50 µL), 50.0 mM sodium phosphate buffer pH 7.0 (600 µL) and deionised $H_2O$ (50 µL) at 37 °C. After temperature equilibration, intestine crude extract (50 µL) was added, and 30 min later, the reaction was stopped by adding 1 mg $mL^{-1}$ fresh Garnet reagent (250 µL) in 0.2 M sodium acetate buffer pH 4.2 containing 10 % Tween 20 (v/v). After 10 min, absorbance was measured at 525 nm (Bio-Rad SmartSpec 3000, USA), and the amount of β-naphthylamine was determined using a standard β-naphthylamine curve. Activity was expressed as protease mU $mg^{-1}$ of protein. One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze one µmol of *p*-nitroaniline per minute per milligram of protein.

### Amylase activity

Amylase activity was evaluated according to Bernfeld (1955) using 2 % starch as unspecific substrate: 60 µL intestine crude extract were incubated with 375 µL starch solution and 375 µL 10 mM phosphate buffer pH 8.0 containing 15 mM NaCl at 25 °C. After 20 min, 3.5-dinitro salicylic acid (DNSA) was added and the solution was heated to 100 °C for 10 min. After temperature equilibration, absorbance was measured at 570 nm (Bio-Rad SmartSpec 3000, USA) versus a similarly prepared blank in which 10 mM phosphate buffer replaced the crude extract sample. Enzyme activity was determined in triplicate. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 mg of maltose per milligram of protein per min.

### Enzyme characterization in substrate-SDS-PAGE

Proteases from intestine crude extract of *O. niloticus* (Aliquots of 50 µg of protein, $n = 3$) were studied in substrate sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using stacking gel at 4 % (w/v) and separation gel at 12.5 % (Laemmli 1970).

Zymograms of protease activity were carried out based on Garcia-Carreño et al. (1993). After electrophoresis, the gels were immersed in 2.5 % Triton X-100 dissolved in 0.1 M Tris–HCl buffer pH 8.0 to remove the SDS and incubated with 3 % casein (w/v) in 0.1 M Tris–HCl buffer pH 8.0 for 30 min at 4 °C. The temperature was raised to 37 °C and maintained at this value for 90 min to allow the digestion of casein by the active fractions. Finally, the gel was stained overnight for protein in 0.18 % (w/v) Coomassie Brilliant Blue R250 prepared in acetic acid and methanol (10:25 % v/v), and the background of the gel was distained in acetic acid and methanol (10:25 % v/v). Clear bands in blue background denoted protease bands by digestion of casein substrate.

### Inhibition assays using substrate-SDS-PAGE

The following inhibitors prepared in DMSO at a final concentration of 2 mM were used: Tosyl phenylalanine chlorometyl ketone (TPCK—chymotrypsin inhibitor); Phenyl-methyl-sulfonil-fluoride (PMSF—serine proteases inhibitor); benzamidine and tosyl-lysine chloromethyl ketone (TLCK), both trypsin inhibitors; and bestatin (leucine aminopeptidase inhibitor). For the inhibition study, only the crude extracts from animals under SPH0 treatment ($n = 3$) were used, since all caseinolytic bands were found in this treatment. Samples of enzyme extract (100 µL) and inhibitors (100 µL) were incubated at 25 °C for 30 min. An aliquot of 50 µg of protein was collected and applied on each respective lane. After that, the zymogram was performed as described above. The 100 % values (control) were established using DMSO without inhibitors (Lemos et al. 2002, 2004).

### Statistical analysis

All data were tested for normal distribution (Shapiro–Wilk test) and homogeneity of variances (Bartlett's test). One-way analysis of variance (ANOVA) by Tukey test was used for normally distributed data, whereas Kruskal–Wallis test was used for non-normally distributed data. Differences were reported as statistically significant when $P < 0.05$, using the SysEapro software (beta version).

## Results

Figure 1 shows the hydrolysis profile of SPH. Enzymatic autolysis (proteases from the shrimp midgut gland) visibly promoted the digestion of proteins with the greatest molecular mass over time. At time 0, most proteins had molecular mass between 15 and 70 kDa, at time 150 min, most proteins were smaller than 15 kDa.

Table 5 shows the enzyme activities in the crude extracts. Statistical differences ($P < 0.05$) between animals fed the experimental diets (SPH0, SPH15, SPH30 and SPH60) were only observed for total alkaline proteases.

Substrate-SDS-PAGE zymograms revealed caseinolytic activity in crude extracts of fish from all treatments (Fig. 2). Twelve caseinolytic bands were found in animals fed on SPH0 and were named P1 to P12, showing the highest number of active bands among treatments. Proteases P1 to P4 were not significantly affected by the addition of the protein hydrolysate, but P5 to P12 underwent alterations in the caseinolytic pattern. As the concentration of hydrolysate increased, there was a decrease in the activity of proteases P6, P7 and P8. The opposite occurred for P5, P9, P10, P11 and P12, in which the caseinolytic bands proved more intense.

The effect of specific inhibitors on the caseinolytic bands from fish crude enzyme extract is shown in Fig. 3. As observed in Fig. 2, the zymogram of the crude extract (SPH0) without inhibitors (control) revealed 12 caseinolytic bands. TPCK did not significantly affect the caseinolytic activity of *O. niloticus* enzymes, and P11 was the only

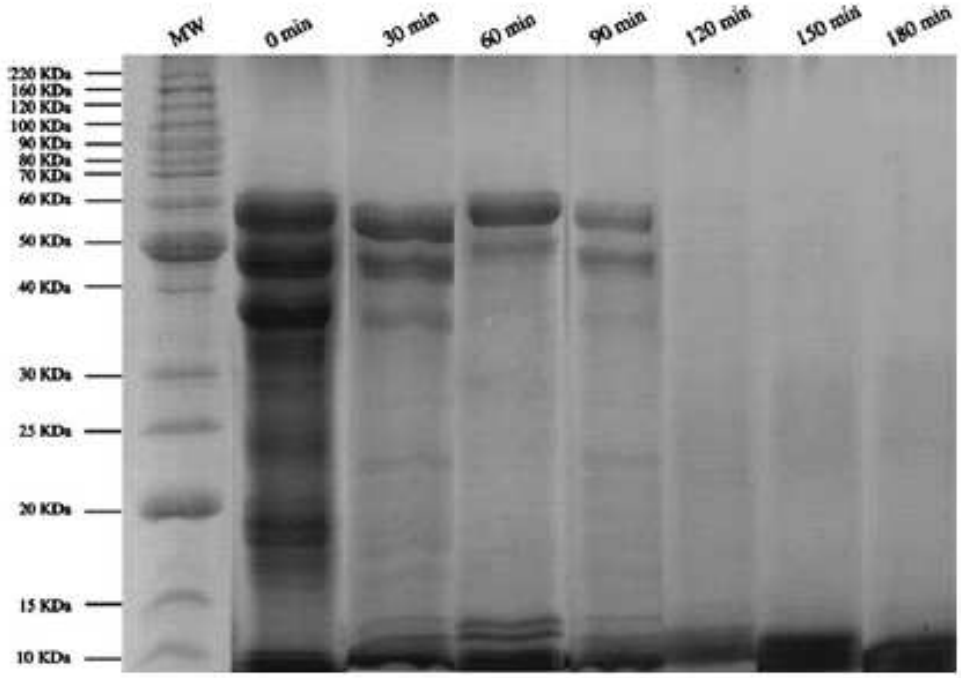


**Fig. 1** Hydrolysis profile of shrimp protein hydrolysate (using autolysis) in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using stacking gel at 4 % (w/v) and separation gel at 17 %. Lanes correspond to molecular mass marker (MM) and incubation times of 0, 30, 60, 90, 120, 150 and 180 min of enzymatic hydrolysis. SPH was produced through autolysis of *Litopenaeus vannamei* cephalothorax (heads)

**Table 5** Digestive enzyme activity of crude extracts recovered from stomach (acid) and intestine of juvenile Nile tilapia *O. niloticus* fed on experimental diets containing increasing shrimp protein hydrolysate levels (0–60 g $kg^{-1}$)

| Diets | Acid proteolytic activity | Alkaline proteolytic activity | Trypsin | Chymotrypsin | Aminopeptidase | Amylase |
|---|---|---|---|---|---|---|
| SPH 0 | $155.84 \pm 14.22^{ns}$ | $11.80 \pm 0.55^{b}$ | $2.58 \pm 0.09^{a,b}$ | $22.70 \pm 1.26^{ns}$ | $41.68 \pm 3.32^{ns}$ | $45.47 \pm 3.92^{a,b}$ |
| SPH 15 | $111.38 \pm 22.83^{ns}$ | $15.35 \pm 0.76^{a}$ | $3.38 \pm 0.17^{a,b}$ | $39.74 \pm 3.94^{ns}$ | $45.34 \pm 1.63^{ns}$ | $53.50 \pm 4.65^{a,b}$ |
| SPH 30 | $162.49 \pm 16.69^{ns}$ | $13.60 \pm 0.73^{a,b}$ | $2.82 \pm 0.20^{a,b}$ | $30.89 \pm 2.42^{ns}$ | $47.66 \pm 2.08^{ns}$ | $48.79 \pm 4.82^{a,b}$ |
| SPH 60 | $132.19 \pm 13.14^{ns}$ | $15.23 \pm 0.88^{a}$ | $2.62 \pm 0.15^{b}$ | $36.66 \pm 3.60^{ns}$ | $39.85 \pm 2.61^{ns}$ | $56.77 \pm 4.31^{a,b}$ |

Acid and alkaline proteolytic activity expressed as U mg of Protein (P) $^{-1}$; trypsin, chymotrypsin and aminopeptidase activity expressed as mU $mg^{-1}$ of P; and amylase activity expressed as mg of maltose $min^{-1}$ mg of $P^{-1}$. Different superscripts in the same column denote statistical differences ($P < 0.05$), and "ns" denotes no statistical differences. Values are mean ± SE of five replicates

enzyme inhibited by TPCK. PMSF revealed high inhibition of P9 and P11 caseinolytic bands. Benzamidine completely inhibited P6 and P8 bands. One band between P5 and P6 enzymes (white arrow) revealed activity in the presence of benzamidine, but it did not appear in the control. TLCK strongly inhibited P3, P4, P6, P7, P8 and P11. Bestatin totally inhibited seven digestive enzyme bands of *O. niloticus* (P3, P4, P5, P6, P7, P8 and P9). Inhibitors had no effect on P1, P2, P10 and P12 caseinolytic bands.

## Discussion

As can be observed in Fig. 1, autolysis efficiently solubilized and hydrolyzed protein from shrimp processing waste (cephalothorax section) and produced protein hydrolysate, which was shown to be a promising protein feedstuff for tilapia.

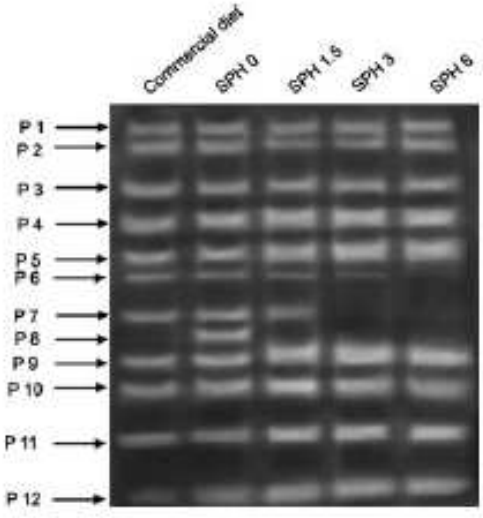


**Fig. 2** Zymogram of digestive proteases (3 % casein as substrate) of intestine enzyme extracts from juvenile Nile tilapia *O. niloticus* fed on diets containing different concentrations of shrimp protein hydrolysate (SPH). Lanes correspond to dietary SPH inclusion (%): SPH 0, SPH 1.5, SPH 3 and SPH 6. Further details in "Materials and methods"

Hydrolysis using exogenous substances allows better control of the properties of the resulting products. However, the cost of the substances (enzymes, acids and solvents) is an obstacle to the viability of the process. Autolysis is an enzymatic process that can be used as an alternative to processes that employ exogenous substances to recover bioactive molecules, such as chitin (for chitosan production) and carotenoids (Armenta and Guerrero-Legarreta 2009; Chen et al. 2011; Cahú et al. 2012). Moreover, autolysis is a common method used to treat fish and shrimp waste, since it does not use any exogenous chemical or biological additive, for example silage. The degree of hydrolysis affects certain product properties, like viscosity, solubility and the partition of proteins. These, in turn, influence the absorption capacity and rate of passage of the diet through the gastrointestinal tract. The solubility of the hydrolysates depends on the nature of the raw material, method, temperature and hydrolysis time (Kotzamanis et al. 2007). Plascência-Jatomea et al. (2002) reported that the acidic conditions in which fermentative shrimp silage hydrolysate is produced cause the loss of labile nutrients, such as tryptophan. Although the two products have a very similar essential amino acid composition, the SPH produced here proved to be an adequate source of tryptophan, as well as lysine, leucine, arginine, phenylalanine and valine (Leal et al. 2010).

The use of hydrolyzed protein in diets for aquatic animals involves some particularities that should be taken into account. For example, diets with high content of hydrolyzed protein can lead to a loss of nutrients by lixiviation, because it contains high levels of small peptides that are usually more soluble (Leal et al. 2010). The extent of hydrolysis can result in protein hydrolysates with different contents of amino acids, di- and tripeptides, oligopeptides, polypeptides and proteins, which will ultimately result in differences in the quality of the diet.

The nutritional value of a diet is not based simply on its chemical composition, but also on the ability physiological fish to digest and absorb, according to these animals' eating habits. During digestion, these proteins are broken down into simpler compounds (peptides and free amino acids) and then are absorbed by specific membrane proteins specialized in peptide transport (e.g., PepT1) and utilized by the body (Sangaletti et al. 2009; Terova et al. 2009; Verri et al. 2011). This degradation in the digestive tract is performed with the assistance of enzymes. A number of authors have analyzed digestive enzyme activity in aquatic organisms and dietary composition, reporting divergent results. Nagase (1964) and Kohla et al. (1992) found enhanced trypsin activity, corresponding to an increase in

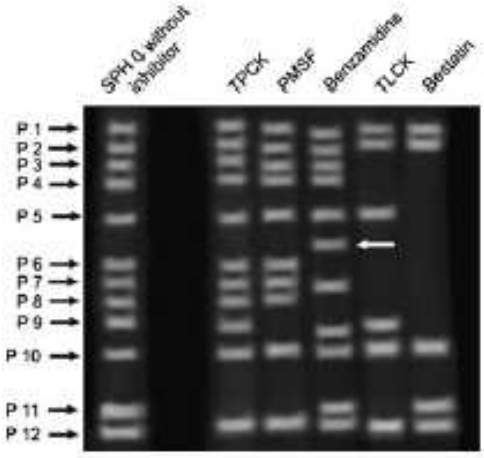


**Fig. 3** Inhibition zymogram of digestive proteases (3 % casein as substrate) of intestine enzyme extracts from juvenile Nile tilapia *O. niloticus* fed the SPH 0 diet. Lanes correspond to treatments: control without inhibitors, TPCK, PMSF, Benzamidine, TLCK and Bestatin. Further details in "Materials and methods" and Fig. 2

feeding rates for the species Mozambique tilapia, *Oreochromis mossambicus* (Peters) and *Colossoma macropomum* (Cuvier), respectively. Papoutsoglou and Lyndon (2006) found an increase in chymotrypsin activity in Spotted wolfish, *Anarhichas minor* (Olafsen), when the protein concentration was reduced, indicating the adaptation of this species to low protein concentrations as a way to better absorb nutrients from the diet. Studies about the effect of protein hydrolysates on the digestive enzymes from aquatic organisms are available in the literature (Córdova-Murueta and García-Carreño 2002; Cahu et al. 2004; Zambonino Infante and Cahu 2007). However, there is little information regarding the specific effects of dietary supplementation with shrimp protein hydrolysate on the digestive enzymes in Nile tilapia.

In the present paper, no statistical differences in proteases activities were observed among the SPH 0 (control) and treatments with higher concentrations of SPH (SPH15, SPH30 and SPH60) (Table 5), or in growth parameters (final weight, survival, weight gain, average daily gain, specific growth rate, feed conversion ratio, protein efficiency ratio, or apparent net protein utilization) (Table 4) (Leal et al. 2010). Only the activity of total alkaline protease was significantly higher ($P < 0.05$) in the fish under SPH15 and SPH 60 treatments, when compared to SPH0 (control) (Table 5). No correlation was observed between different concentrations of hydrolysate in the diets and the activity of any of the enzymes studied.

European seabass, *Dicentrarchus labrax* (L) were fed diets containing enzymatic hydrolyzed replacement for fish meal (native protein) in the proportions of 0, 20 and 40 %. Zambonino Infante et al. (1997) demonstrated that fish to the trypsin activity was enhanced by the native protein, whereas chymotrypsin activity was enhanced by the diets containing di- and tripeptides. On the other hand, Kotzamanis et al. (2007) also evaluated the inclusion effect of protein hydrolysate on the digestive enzyme activities in European seabass, *D. labrax* larvae fed on diets with 10 and 19 % protein hydrolysate. They found no statistical differences in amylase and trypsin activity between treatments. However, aminopeptidase activity was higher when 10 % protein hydrolysate was administered. Thus, the absence of statistical difference found for most enzymes, and growth parameters can be related to the loss of SPH by lixiviation. In addition, commercial substrates used in this study showed no sensitivity to detect differences in most of the enzymatic activities; in other words, it was observed that the effects of SPH on the digestive enzymes were comparably small and not dose-dependent.

The different results observed in the literature and those obtained here led us to consider other methods. The zymograms afforded to observe the dose-dependent effect of SPH on some proteases (caseinolytic bands). In fact, zymograms appear to be a more sensitive biochemical tool in comparison with other methods for detecting the proteinase composition of crude extracts from tissues and to allow the determination of enzyme activity zones (Garcia-Carreño et al. 1993).

Through analyses of inhibition zymograms, it was possible to determine the following: one caseinolytic band with aminopeptidase activity (inhibited only by Bestatin, P5) and probably another aminopeptidase (P9) inhibited by Bestatin and by PMSF; some other active bands, P3, P4, P6, P7, P8 and P11, seemed to be proteases of low specificity, with trypsin/aminopeptidase activity (P3, P4, P6, P7 and P8 which were inhibited by trypsin inhibitors and by Bestatin) and chymotrypsin/trypsin activity (P11 which was inhibited by PMSF, TPCK and TLCK). It was not possible to identify P1, P2, P10 and P12, as none of the inhibitors had any effect on these proteases. Comparing the zymogram of enzyme activity according to dietary treatment to the inhibition gel, at increased concentration of shrimp protein hydrolysate, there was an overall slowdown of proteases with trypsin

activity (P6, P7 and P8). On the other hand, it was identified that the caseinolytic bands P5 and P9 (identified as aminopeptidase activity, Fig. 3) grew in intensity with increasing SPH contents. The caseinolytic bands P10 and P12 (not identified) and P11 (with chymotrypsin-like/trypsin-like activity) also exhibited an increase in intensity with the inclusion of SPH in the diet. Cahu et al. (2004) found that trypsin secretion was higher in larvae of the *D. labrax* fed on diets with 14 % protein hydrolysate, being reduced with increasing concentrations (46 %).

Zymograms in Figs. 2 and 3 show that the classical protease inhibitors herein employed were not effective upon some caseinolytic bands (P1, P2, P10 and P12), and in some cases, the inhibitory effect was not conclusive (P3, P4, P6, P7, P8, P9 and P11), suggesting a low compatibility of these enzymes with mammalian proteases. In fact, these commercial inhibitors are generally synthesized based on the mammalian enzyme mechanism, and as the fish digestive system may be considerably different from that of mammals, differences in the mechanisms of their enzymes may also occur. In spite of the results of the present work, tilapia digestive enzymes were not considerably affected by classical trypsin inhibitors. Bezerra et al. (2005) showed that purified Nile tilapia trypsin could be strongly inhibited by TLCK and Benzamidine. PMSF was shown able to inhibit about 50 % of trypsin activity.

Although there were differences in digestive enzyme activity (total alkaline proteases) between fish fed different experimental diets, there was no clear relationship between enzyme activity and different concentrations of shrimp protein hydrolysate in these diets. Substrate-SDS-PAGE zymogram proved to be efficient in detecting changes in enzyme activity in fish submitted to different diets. Through this method, different protease profiles could be detected according to experimental diet. These data underscore the suggested ability of tilapia to adapt to different food sources. Actually, as an omnivorous opportunistic fish, tilapia is able to assimilate nutrients from different sources, like benthic algae, phytoplankton, macrophytes, zooplankton, small invertebrates, detritus and cyanobacteria. The feed choice is mainly related to the availability of food items in the environment (Bowen 1982; Fitzsimmons 1997; Stickney 1997; Beveridge and Baird 2000; Lowe-McConnell 2000). To benefit from all these sources, the enzyme arsenal of tilapia should be diverse enough to digest the wide range of ingested food.

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## 6. CAPÍTULO II

**ARTIGO CIENTÍFICO:**

# Use of condition factor and its derivatives to evaluate the effect of different culture systems on the growth of Nile tilapia (*Oreochromis niloticus* L.)

**ARTIGO A SER SUBMETIDO A REVISTA AQUACULTURE ISSN: 0044-8486**

# Use of condition factor and its derivatives to evaluate the effect of different culture systems on the growth of Nile tilapia (*Oreochromis niloticus* L.)

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Running title: Influence of culture systems on the growth parameters of tilapia

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Highlights

- Even though no statistical differences in growth parameters have not been observed between fish reared in semi-intensive and intensive systems, protein and feed efficiencies and condition factor were better in semi-intensive system in 31 days of culture.
- In this work, the relative weight of fish grown in semi-intensive and intensive systems was higher in 9 out of 14 comparisons with natural conditions and other cultures.
- The equation of condition factor and its derivatives (Kmean and Krel) proved to be useful tools when assessing growth.

**Abstract**

Nile tilapia is a fish species that can be produced in different culture systems and scales, including the semi-intensive systems in ponds and the intensive system in cages. Their handlings are completely different, so it is required management to stabilize the growth parameters and production costs. Therefore, the present work intends to evaluate the influence of semi-intensive system and intensive system on growth parameters of Nile tilapia and compare the relative weight with tilapia in natural environment and farming by the method of Froese. Juvenile Nile tilapia (87.61 ±1.52g) were cultured in semi-intensive and intensive system during 90 days. Two nutritional plans were adopted: triphasic for semi-intensive systems (diet with 35, 32 and 28% crude protein - CP) and biphasic for intensive system (35 and 32% CP). Biometrics measurements were performed monthly for analysis of growth parameters and adjustments in diet. The water quality indicators were monitored continuously and were in accordance with the requirements for animal welfare. The presence of natural food was evaluated in the two systems, being more abundant in semi-intensive systems. Survival, protein efficiency, feed efficiency and condition factor with 31 days of cultivation were better in semi-intensive system. The weight-length relationship of tilapia was not altered by the systems. Comparing the fish relative weight from semi-intensive systems and intensive systems with tilapia from other environments, they were higher in 9 of 14 comparisons. The calculations of Fulton's condition factor (K), $K_{mean}$, $K_{rel}$ and relative weight ($W_{rm}$) demonstrated by Froese, proved to be useful tools when assessing growth. Furthermore, the relative weight equation allowed a comparison of tilapia from the natural environment and other cultures, expanding the growth studies comparisons. However, one can realize the importance of continuous monitoring of these parameters, since differences were observed in the relative weight of the studied species of tilapia when compared to other environments or cultures, leading us to reflect on how to improve the cultivation conditions of Nile tilapia.

K**eywords:** Nile tilapia, culture systems, semi-intensive and intensive systems, growth parameters.

## 1.Introduction

The increase in demand for fisheries resources for human consumption has generated a growth in aquaculture production worldwide. In the last three decades (1980-2010), world aquaculture production has expanded by almost 12 times, with an average annual growth of 8.8%, with emphasis on production in freshwater, which represents about 60% of the total cultivated (FAO, 2012).

Among cultured fishes of the world, tilapia is ranked third in terms of production, only after carps and salmonids (El-Sayed, 2006). According to Wing-Keong and Hanin (2007), *Oreochromis niloticus* represents 80% of the species of tilapia cultured in the world and is considered the most important. The demand for tilapia has been increasing in recent years and this could only be met by increasing supply and expansion of aquaculture production (Fasakin et al., 2005). However, this growth must be sustainable and in accordance with Abery et al. (2005) if there is no production planning, negative impacts can be generated not only on the system, but also on aquatic ecosystems.

Tilapia can be produced in different culture systems and scales, with higher or lower dependence on inputs, especially feed. According to El- Sayed (2006), semi-intensive culture is the production of fish using natural food, through pond fertilization, or fertilization and supplemental feeding, It is a low-cost means of production, which contributes to hunger alleviation especially in rural areas of developing countries. However this system has some barriers, such as the limitation and shortage of supplies of fresh water besides the competition for this resource with agricultural, industrial and other urban requirements, especially in arid regions.

Intensive system is characterized by high densities (25-300 fish.$m^{-3}$), diets with high protein levels and high productivity (Mohammad, 2006), furthermore, their technology is relatively inexpensive when compared to other intensive farming systems. Nevertheless, El-Sayed (2006) cites some limitations in cage culture: risk of theft, disease outbreaks, loss due to cage damage

caused by predators or storms; low tolerance of fish to poor water quality; dependence on high-quality feeds; the accumulation of faeces and metabolites underneath the cage has a negative environmental impact, among others.

The present survey focuses on the following aspects: influence of culture systems / management on the growth parameters of Nile tilapia; different concentrations of protein along with the presence of natural food can contribute to the growth of tilapia in different systems; condition factor of the fish may make changes in cultivation in cages and ponds; condition factor, length-weight relationship and relative weight can be useful tools in growth compared with other populations.

## 2.Materials and methods

### 2.1 Obtaining experimental specimens / Animals and culture conditions

The experiment was conducted at the Aquaculture Station of Unidade Acadêmica de Serra Talhada (UAST) / Instituto Agronômico de Pernambuco (IPA) located in the city of Serra Talhada, semi-arid region of Pernambuco State, Brazil, during the period January-May 2011.

Juvenile sex-reversed Nile tilapias (variety QAAT1/Arizona-USA) were obtained from the fish farming at the city of Paulo Afonso - BA - Brazil. The fish were stocked in concrete tanks (40 fish.$m^{-2}$) and then submitted to a 20-day acclimatization period for both diets and experimental conditions. Before acclimation, fish were weighed (38.4 ± 11.9g) and measured (12.7 ± 1.1cm). Individuals were fed a ratio of 8% of biomass per day, adjusted every seven days, offered 2 twice a day. The experiment consisted of two treatments (intensive and semi-intensive cultures) and three replicates. In the intensive treatment fish were cultured in cages, while in the semi-intensive they were grown in ponds.

For the intensive culture, the dimensions of the cages were 2m x 2m x 1.5m, capacity of 5 m³ and 30 mm mesh nylon net. The animals were stocked at a density of 110 fish.$m^{-3}$ (three replicates) and 30 days after the density was decreased to 70 fish.$m^{-3}$ (four replicates). The cages were mounted in reservoir Saco I - Serra Talhada – PE.

For the semi-intensive culture were used three ponds with an area of 338 $m^2$ and a maximum depth of 1.5 m, supplied by water from the reservoir Saco I. The animals were stocked at a density of 1 fish.$m^{-2}$ (0.67 fish.$m^{-3}$) and remained so until the end of the experiment. Before stocking the ponds were dried in the sun and went through the process of liming ($CaCO_3$ - 15 kg per 1000 $m^{-2}$) and fertilization (bovine manure - 1 ton.$ha^{-1}$) according to Woynarovich and Horváth (1983) for the growth of natural food such as phytoplankton and zooplankton.

The ponds and cages were stocked with juvenile Nile tilapia with an initial average weight of 88.6 ± 1.5g and an average total length of 16.3 ± 0.12cm (defined as zero time). 10% of the population were sampled monthly (defined as time 1, 2 and 3) to monitor growth and make feeding adjustments. For this purpose, the fish were anaesthetized using AQUI-S® solution (0.5mL. $L^{-1}$) for easy handling and return to the ponds/cages after each sampling. Both cultures lasted 94 days.

**2.2 Feed management**

Commercial diets containing 35, 32 and 28% crude protein (CP) were used. Composition described in Table 1. Intensive culture used nutritional biphasic plan (35 and 32% CP) and semi-intensive triphasic plan (35, 32 and 28% CP), as described in Table 2. The fish were fed 2 twice a day (8 a.m. and 3 p.m) with feeding rates of 5, 4 and 3% biomass, adjusted monthly after biometrics.

## 2.3 Water quality

During both experiments, temperature (ºC), dissolved oxygen ($mg.L^{-1}$), pH, salinity (‰) and electric conductivity ($\mu S.cm^{-1}$) were monitored daily, twice a day *in situ* (n = 188) using a multiparameter analyzer Hanna®.

Water samples were taken monthly from each pond and cage and transported to the laboratory of Limnology in the Universidade Federal Rural de Pernambuco to perform the analyzes (n = 4). Ammonia ($mg.L^{-1}$) was measured according Koroleff (1976); nitrite and nitrate ($mg.L^{-1}$) in accordance with Mackereth et al. (1978); orthophosphate ($mg.L^{-1}$) under Strickland and Parsons method (1965); alkalinity and hardness (mg $CaCO_3.L^{-1}$) according to Golterman et al. (1978), wherever total hardness was determined from the concentrations of $Ca^{+2}$ and $Mg^{+2}$.

To estimate plankton (phytoplankton and zooplankton) abundance in experimental tanks, 50 L of water were collected in three points of each pond and cage and filtered through 50 mm plankton net to obtain a concentrated sample and stored in plastic bottles of 500 mL. Samples were collected weekly and preserved immediately with 5% formalin. Samples of 2 mL were placed on the counting chamber of the Sedgewick Rafter cell, and then organisms on 10 randomly selected fields of the chamber were identified and counted (Keshavanath, et al., 2002). Phytoplankton ($cel.mL^{-1}$) and zooplankton ($ind.L^{-1}$) were identified in large groups or families, according to Bicudo and Menezes (2006), Brusca and Brusca (2007) and Hickman et al. (2001). Phytoplankton biomass in terms of pheophytin and chlorophyll *a* concentration ($\mu g.L^{-1}$) of water tanks were determined monthly according to Nusch (1980).

198 **2.4 Growth, survival and feed utilization**

199

200 At the end of the experiment, growth was assessed by determination of weight gain rate
201 (WG), average daily gain (ADG), specific growth rate (SGR), final biomass (FB), Survival (S),
202 feed conversion ratio (FCR), protein eficiency ratio (PER) and feed efficiency (FE) as follows:

203 Weight gain rate ($WG_{(g)}$) = $BW_f$ - $BW_i$

204 Average daily gain (ADG) = $WG_{(g)}$/time (days)

205 Specific growth rate (SGR) = 100 (ln $BW_f$ - ln $BW_i$)/time (days)

206 Final biomass ($FB_{(Kg)}$) = Final mean $weight_{(Kg)}$ x Final number of fish

207 Survival ($S_{(\%)}$) = (Final number of fish/ fish stocked) x 100

208 Feed conversion ratio (FCR) = dry feed $offered_{(g)}$/wet $weight\ gain_{(g)}$

209 Protein efficiency ratio (PER) = wet $weight\ gain_{(g)}$/ $protein\ fed_{(g)}$

210 Feed efficiency (FE) = wet $weight\ gain_{(g)}$/dry feed $offered_{(g),}$

211 in which $BW_i$ and $BW_f$ = average initial and final body $weight_{(g)}$ of fish.

212

213 **2.5 Weight-length relationships (WLR) / Condition factor (K)**

214

215 Weight-length relationships were estimated at the end of experiment using the equation:

216 $W = aL^b$,

217 in which W = weight of fish in grams, L = total length of fish in centimeters, *a* is the intercept and *b*
218 is the allometric coefficient (Froese, 2006).

219 Fulton's condition factor (K) was determined in time 0 (zero), 31, 63 and 94 for each culture
220 using the equation:

221 $K = 100\ W / L^3$,

222 where K = condition factor, W = total weight and L = total length (Froese, 2006).

223 Clark's mean condition factor ($K_{mean}$) was determined at times 0 (zero), 31, 63 and 96 days,
224 which relates Fulton's condition factor (K) with *a* and *b* and which represents the mean condition
225 factor for a given length derived from the respective WLR:
226 $K_{mean} = 100\ aL^{b-3}$ ,
227 where $K_{mean}$ = mean condition factor for a given length, where *a* is the intercept and *b* is the
228 allometric coefficient, both estimated by method of least squares (Clark, 1928).
229 Le Cren's relative condition factor ($K_{rel}$) was calculated for both systems at times described
230 above, according to Le Cren (1951), which compensates for changes in form or condition with
231 increase in length, and thus measures the deviation of an individual from the average weight for
232 length in the respective sample (Froese, 2006):
233 $K_{rel} = (W / aL^{b})*100$ ,
234 relative condition factor comparing the observed weight of an individual with the mean weight for
235 that length, the factor 100 is used to bring the value for percentage.
236 This same equation (relative condition factor) was used to compare different populations of
237 Nile tilapia in natural environment (using the parameters *a* and *b* from **FISHBASE** for *Oreochromis*
238 *niloticus* and the geometric mean of them) and cultivation (with weight-length relationships in
239 published papers). However it appears as relative weight ($W_{rm}$):
240 $W_{rm} = (W / aL^{b})*100$ or $W_{rm} = (W / a_m L^{bm})*100$,
241 where $a_m$ and $b_m$ are the geometric mean of WLR.
242
243 **2.6 Statistical analysis**
244
245 All data was tested for normal distribution (Shapiro-Wilk test) and homogeneity of variances
246 (Bartlett´s test). Student's *t*-test was used for normally distributed data, whereas Wilcoxon-Mann-
247 Whitney test was used for non-normally distributed data. To assess whether weight-length

relationships were affected by culture systems was used General Linear Model. Differences were reported as statistically significant when $P < 0.05$, using Origin™ version 8.0, Assistat 7.6, GraphPad Prisma 6.0, IBM SPSS Statistics (Version 19) and Excel 2007.

### 2.6.1 Descriptive analysis

For descriptive analyzes of the $K_{rel}$, total length and weight of Nile tilapia in the two culture systems were used box-plot graphs. The degree of asymmetry between the samples was evaluated according Bisquerra et al. (2004), considering for symmetric data: Mean = Median = Moda; for asymmetric data positive: Mean ≥ Median ≥ Moda and for asymmetric data negative Mean ≤ Median ≤ Moda.

## 3. Results

The composition of diet and nutritional plan are shown in Tables 1 and 2, respectively. Table 3 shows the parameters of water quality in the intensive and semi-intensive systems. Most of the analyzes showed a significant difference, except ammonia, nitrite, nitrate and orthophosphate. The values of temperature, hardness, Chlorophyll *a* and pheophytin were higher in semi-intensive system; while pH, dissolved oxygen and conductivity were higher in the intensive system.

In Table 4 has shown the analyzes of phytoplankton (Chloroficeae, Cyanophyceae and Bacillariophycea) performed in the two farming systems in time "0" (zero), 31, 63 and 94 days. At time "0" the chlorophytes were higher in ponds and at 31, 63 and 94 days of culture, the chlorophytes and bacillariophytes also had their highest densities in semi-intensive system, but the cyanophytes showed higher density in the intensive system at the end of cultivation.

Figure 1 shows the development of phytoplanktonic groups during the cultivation. Can be observed a decrease in the total concentration of algae (cel.mL$^{-1}$) in the two farming systems with the ponds always presenting higher concentrations. The densities of chlorophytes and cyanophytes in intensive system, demonstrated an inverse relationship in time "0", 31 and 63 days.

The concentration of zooplankton (rotifers, copepods and cladocera) in different cultures can be seen in Table 5 (Ind.L$^{-1}$). As for phytoplankton, statistical differences were evaluated by comparison within the times during breeding systems. The concentration of rotifers was higher in semi-intensive system at the times "0", 63 and 94 days. The Cladocera also showed higher density in ponds in the days 31, 63 and 94 days. While copepods were higher in all periods analyzed in semi-intensive system. The zooplankton also decreased their concentrations during the two cultures, but was always higher in semi-intensive farming (Figure 2).

The growth parameters of Nile tilapia are shown in Table 6. Only survival, protein efficiency and feed efficiency were better in the semi-intensive system, the remainder of the data showed no significant difference.

The parameters of the mathematical models for the evaluation of weight-length relationships of fish under different cultures are displayed in Figure 3. An analysis of the models revealed that the total weight-length relationships were not affected by culture system ($P<0.05$).

The Fulton's condition factors (K) of the experimental specimens are shown in Table 7. The only statistical difference was noted with 31 days of culture when the fish of the semi-intensive system showed better conditions when compared to intensive system.

Figure 4 shows a comparison between Fulton's condition factor (K) of the population in the present study with the respective $K_{mean}$. At the beginning of the culture, condition factor of fish in intensive system was similar to the $K_{mean}$ of the population, however this factor decreased in 31 days, then increased and stabilized with $K_{mean.}$ The condition factor of fish in the semi-intensive

296 system also showed similarity to the respective $K_{mean}$ in the times "0", 63 and 94 days, however, this
297 condition factor was greater than its corresponding $K_{mean}$ with 31 days of cultivation.

298 The $K_{rel}$ of cultured fish under intensive and semi-intensive system were analyzed by Box-
299 plot graphics (Figure 5). At time "0" the intensive system showed 25% of the fish with a $K_{rel}$
300 between 82 and 97% (between the minimum and the first quartile), 50% exhibited $K_{rel}$ between 97
301 and 107% (1st quartile to 3rd quartile) and the remainder of the animals (25%) showed a $K_{rel}$
302 between 107 and 119% (3rd quartile to the maximum value). The mean and median are presented
303 overlapping (approximately 102%) and most fish exhibited a $K_{rel}$ of 95% for intensive culture. The
304 fish in the semi-intensive the mode show overlapping the mean and median, and minor fluctuations
305 that time.

306 Evaluating fish $K_{rel}$ with 31 days of cultivation were observed changes in condition. The
307 semi-intensive system showed 75% of its population between 96 and 119% of relative condition,
308 mean, mode and median higher than in intensive system. Seventy-five percent of the fish in the
309 intensive system exhibited $K_{rel}$ between 93 and 113%.

310 This distribution pattern remained until at least day 63 of cultivation in the semi-intensive
311 system. However, an improvement was observed in the intensive system with 94 days of
312 cultivation. The same showed 75% of its population with a $K_{rel}$ between 96 and 120% compared
313 with 93 to 114% of semi-intensive system. At the end of the experiment, most fish have had a $K_{rel}$
314 of 93% for the intensive system and 88% for semi-intensive system presenting mean and median
315 similar in the two farming systems (approximately 100%).

316 The total length and weight of fish in intensive and semi-intensive farming systems can be
317 seen in Figure 6A and 6B, respectively, in Box-plot format.

318 A similar increase was observed in both systems up to 63 days of culture (Figure 6A).
319 However, the minimum and maximum growth in the intensive showed a higher variation when
320 compared to semi-intensive system. Was observed a symmetry (mean = median = mode) at the

times "0" and 31 days for both systems. From 63 days of cultivation occurred changes in the consistency of growth of the animals in intensive system. They began to show positive asymmetric growth (mean ≥ median ≥ mode) whereas animals in semi-intensive remained in symmetric pattern until the end of the experiment. However, both systems exhibited outliers in the course of cultivation.

Evaluating increase in weight of fish in different farming systems (Figure 6B) was observed in the time "0" and 31 days similarity in growth. However, a larger weight variation was noticed in intensive system from 63 days culture. Evaluating the symmetry in the two culture systems, up to 63 days, the animals exhibited positive asymmetry and the fish of the semi-intensive cultivation exhibited up to 94 days. Fish intensive system exhibited negative asymmetry with 94 days culture.

The relative weight of fish with 94 days of cultivation in the intensive and semi-intensive was compared with total weight-length relationships of fish obtained in other environments (natural and cultivated) (Table 8).

The relative weight of fish in the present study was higher in 9 of the 14 cases analyzed, and 3 cases with similar relative weight. Our relative weight found for tilapia in comparison with the tilapia from natural environment was higher in 4 of the 6 cases analyzed, and one situation similar to the relative weight.

Comparing studies with tilapia cultured in cages with the two systems described here, the relative weights obtained were higher in the present work in 1 of the 2 situations analyzed. This comparison was also made with tilapia cultured in ponds. The tilapia cultured in the present study had higher relative weights in 2 of the 4 studied cases and two similar situations.

Nile tilapia cultured in aquaria subjected to different diets were also compared with the present study and the relative weight found here was also greater than those of fish from aquaria.

## 4. Discussion

According to the Brazilian Council for Environment – CONAMA, resolution N$^{o}$ 357 of March 17, 2005, which provides on the classification and environmental guidelines of surface waters, the water farming systems analyzed were defined as brackish (waters with salinity higher than 0.5 ‰ and lower than 30 ‰), corroborating with the value found, the electrical conductivities of the water in intensive and semi-intensive systems were 1,356 ± 468.1 and 1,285 ± 249.1 mS. cm$^{-1}$ respectively, being considered a brackish water (Bouvy et al., 1998). Tilapia species and hybrids tolerate a wide range of salinity, they can grow and reproduce normally in brackish water and was selected for this type of cultivation (El-Sayed, 2006). Alfredo and Hector (2002) define a limit of salinity for optimal growth of Nile tilapia of 15 ‰ and can tolerate 36 ‰ with acclimation (Al-Amoudi, 1987).

According to El-Sayed (2006), temperature is one of the most important factors affecting the physiology, growth, reproduction and metabolism of tilapia. This fish is thermophilic and known to tolerate a wide range of water temperatures. Balarin and Haller, (1982); Chervinski, (1982); Philippart and Ruwet, (1982) state that the optimum temperature for development of the species is about 25-30°C, however, the temperature range for the normal development, reproduction and growth of tilapia is about 20 to 35°C, values found in the present study.

Nile tilapia can survive at a pH range of 4 – 11, however, this variation was not found in this study, where the values of pH were in accordance to CONAMA (357/2005) resolution.

Only a minimum value of dissolved oxygen in the semi-intensive system (3.27 mg . L$^{-1}$), was out of the requirements of CONAMA, which advocates a value equal or higher than 5 mg . L$^{-1}$. However, according to Coche (1982), Boyd and Tucker (1998), the minimum condition of dissolved oxygen for cultivation of tilapia is 3.0 mg . L$^{-1}$. El-Sayed (2006) mentions that tilapia can

370 tolerate levels between 0.1 - 0.5 mg. $L^{-1}$ dissolved oxygen, however these values can cause decrease
371 in growth.

372 Ammonia levels in the two farming systems were compatible with a good growth performance
373 of Nile tilapia. El-Shafai et al. (2004) evaluated the effect of prolonged exposure to sublethal
374 Ammonia nitrogen on the growth performance of Nile tilapia. They found that the toxic level of
375 $NH_3$-N and its negative effect on the growth performance ranges from 0.07 to 0.14 mg . $L^{-1}$. They
376 further recommended that the $NH_3$-N concentration should be maintained below 0.1 mg . $L^{-1}$.

377 The amounts of nitrite and nitrate in the present study are at acceptable levels according to
378 CONAMA. Nitrite is highly toxic to fish, because it disturbs the physiological functions of the fish
379 and leads to growth retardation. Nitrate is relatively non-toxic to tilapia, however their exposure to
380 high levels for a long time, can decrease the immunity of fish (El-Sayed, 2005).

381 The orthophosphates are directly available for biological metabolism without conversion to
382 simpler forms. Nevertheless, to characterize a system as eutrophic this must have total P values
383 higher than 0.05 mg.$L^{-1}$. According to the CONAMA polyphosphate recommendations (presenting
384 structure more complex than the orthophosphate) should be less than 0.02 mg. $L^{-1}$, value found in
385 this study.

386 The CONAMA do not provide optimal values of alkalinity and hardness, however, Boyd and
387 Tucker (1998) suggest that these parameters should be above 20mg.$L^{-1}$. In the present study, the
388 intensive system had lower alkalinity (13.95 ± 1.86 mg.$L^{-1}$ $CaCO_3$) vs. 161.28 ± 106.97 mg.$L^{-1}$
389 $CaCO_3$ in semi-intensive, thus demonstrating that the addition of $CaCO_3$ in the semi-intensive
390 promoted effect "buffer" necessary to minimize variations in pH (7.15 ± 0.4).

391 Water hardness reflects mainly the concentration of $Ca^{+}$ and $Mg^{+}$ which are combined with
392 carbonate and/or bicarbonate, may also be associated with sulphate and chloride. The hardness
393 values found in intensive system (20.06 ± 1.09 mg. $L^{-1}$ $CaCO_3$) were reduced significantly from the
394 values found in the semi-intensive (28.50 ± 4.37 mg. $L^{-1}$ $CaCO_3$), furthermore they were at the limit

395 defined by Boyd and Tucker (1998). The hardness values presented in this study define water
396 culture like soft, though these values are constantly changing due to the characteristics of the semi-
397 arid environment (relative humidity around 40%, long droughts and occasional rains concentrated
398 in a few months, with precipitation between 400 and 800 mm annually). This study was conducted
399 between January and May 2010, months where the occasional rains are more concentrated.

400 Levels of chlorophyll *a* indicate the degree of eutrophication of an environment, and provide
401 the phytoplankton biomass. According to CONAMA recommended levels of chlorophyll *a* (for
402 freshwater aquaculture) are up to 30 μg. $L^{-1}$, presenting no reference to brackish water. Addition of
403 feed and manure to culture ponds resulted in different biomass of phytoplankton in the ponds. The
404 levels found in chlorophyll *a* for intensive and semi-intensive system (17.75 ± 5.92 and 88.49 ±
405 31.16 μg. $L^{-1}$, respectively) show clearly the effect of fertilization performed in ponds, which
406 favored the development of natural food. Uddin et al., (2007) assessed the concentration of plankton
407 in monoculture (tilapia) and polyculture (tilapia and shrimp), and observed values of chlorophyll
408 "*a*" ranging from 50 to 500 μg.$L^{-1}$ (minimum and maximum between treatments) but these values
409 were not statistically different, however, he noted a decrease in values over time.

410 According to Wetzel (1981) and Lewis (1995), pheophytin is a result of degradation of
411 chlorophyll *a* after the loss of magnesium atom. Thus, the determination of chlorophyll *a*
412 concentrations provides an estimate of chlorophyll whereas pheophytin determination indicates
413 their physiological status, i.e. high values of pheophytin, indicates a larger degradation of
414 chlorophyll *a*, suggesting death of phytoplankton and high decomposition. Phaeophytin values were
415 higher in semi-intensive system (274.97 ± 151.95 μg. $L^{-1}$) (which also had higher chlorophyll *a*),
416 suggesting a high renewal of phytoplankton.

417 With 14 weeks of cultivation in ponds Keppeler (2005) found average values of phaeophytin
418 ranging between 585 ± 789 and 624 ± 707 μg. $L^{-1}$, in the morning and afternoon respectively,
419 however no statistical difference. Keppeler (2005) also found a positive correlation between

chlorophyll *a* and pheophytin, demonstrating a renewal of plankton during cultivation. Lima (2010) found phaeophytin values in nursery ponds with *Astyanax lacustris* ranging from 8.585 to 73.238 $\mu g.L^{-1}$, however time the experiment was lower than in the present work.

The microalgae contain high concentration of soluble fiber and fatty acids of the omega-3 series and can contribute positively in the feeding of aquatic organisms (Azaza et al., 2007). Generally was observed a greater abundance of the three groups analyzed during cultivation in semi-intensive system. Analyses of chlorophyll *a* confirmed this observation. This shows the importance of fertilization in ponds, as well as maintenance of the main biotic and abiotic parameters that promote equilibrium in the system.

The largest groups of algae found were chlorophyceae and cyanophyceae. However, the intensive system showed a particularity. Analyzing Figure 1A, the cyanophyceae and Chlorophyceae showed an inverse relationship development with 31 and 63 days of cultivation. This may be related to changes in the environment that promoted an ecological succession of these algae in the months analyzed.

In ponds this characteristic was not observed since the occurrence of Chlorophyceae was predominant. This fact is important, since cyanophyceae can produce biotoxins that are significant hazards to animals (Molica et al., 2002) and in addition, is an indicative of eutrophication of an environment. The CONAMA does not define the maximum amount of this algae in brackish water, but freshwater for aquaculture its maximum allowed is 50,000 $cel.mL^{-1}$. The greatest value of this alga in the studies was in intensive system with 31 days of cultivation (46,000 ± 13,600 $cel.mL^{-1}$) (maximum value above recommended). However, Deblois et al. (2008) cite the tilapia as an excellent filtrating of cyanophyceae, demonstrating a preference for this group compared to bacilariofíceas (Uddin et al., 2007). Furthermore, Uddin et al. (2009) reported that tilapia can exert a very effective biological control over plankton in semi-intensive system.

444 The abundance of zooplankton was also observed during the cultivation of Nile tilapia. These
445 organisms play an important role in the trophic web, serving as a link between primary producers
446 and higher consumers. According Roche and Rocha (2005), the availability of these organisms in
447 fish farming becomes in most cases essential for fish feeding.

448 The densities of rotifer, copepod and cladocera were significantly higher in semi-intensive
449 system in the majority of times analyzed and the copepods reached higher values in both systems.
450 Despite the diversity of feed from primary producers, consumers also depend on the primary
451 continuous supply of organic detritus as a source of energy. Thus, the predominance of copepods in
452 the analyzed systems can be linked to the eating habits of cyclopoid - the main order of Copepoda
453 found during this study (data not shown). The cyclopoid are raptoriais predators who thrive in
454 eutrophic environments rich in decomposing organic matter (Landa et al., 2007).

455 Actually, as an omnivorous opportunistic fish, tilapia is able to assimilate nutrients from
456 different sources, like benthic algae, phytoplankton, macrophytes, zooplankton, small invertebrates,
457 detritus and cyanophyceae. The feed choice is mainly related to the availability of food items in the
458 environment (Bowen, 1982; Fitzsimmons, 1997; Stickney, 1997; Beveridge and Baird, 2000; Lowe-
459 McConnell, 2000).

460 When *O. niloticus* in semi-intensive ponds are supplemented with low-protein feeds, dietary
461 protein is largely provided by the natural food. Liti et al. (2006) mentions that the protein content of
462 natural food ranges between 550 and 700 g. $kg^{-1}$ on a dry matter basis. This is far above the range
463 (270 - 350 $g.kg^{-1}$) recommended for intensive culture of Nile tilapia (El-Sayed, 2006).

464 Complementing this observation there were no statistical differences in final weight of Nile
465 tilapia grown in the intensive and semi-intensive, i.e. the triphasic nutrition plan (35, 32 and 28%
466 CP) applied to fish in the pond (Table 2), together with the natural food available, was sufficient to
467 obtain the same weight gain of intensive cultivation (biphasic plan with 35 and 32% CP). Liti et al.

(2006) mentions that natural food contributed between 300 and 500 $g.kg^{-1}$ of growth when tilapia were supplemented with artificial feeds in fertilized ponds.

The effectiveness of natural food was also observed in protein efficiency ratio (PER) and feed efficiency factor (FE). Even the two systems with the same feed conversion rates, evaluating the FE and PER of fish the semi-intensive system showed better utilization of provided food. This means that they had the same weight gain of fish in intensive system with less protein in the provided diet. In addition, both systems exhibited growth rates, feed conversion and survival in agreement with commercial scale production.

However, the intensive system showed advantages with respect to productivity, precisely because it is a system that enables higher stocking densities, compensating the greatest amount of protein supplied in the diet. In fact, the constant movement of the cages by water changes, high fish densities and low densities of phytoplankton and zooplankton, hinder consumption of natural food in this system. So diets provided for intensive systems must meet all the nutritional requirements of the produced species.

Corroborating data growth, weight-length relationship ($W = aL^b$) of Nile tilapia in the two cultures was not affected by the systems. The weight-length relationship showed the exponent b> 3.0 for both systems. According to Froese (2006), an exponent less than 3 indicates a "decrease in condition or form" or elongated shape and fish presenting b> 3 means an increase in the condition or form, i.e., the specimens analyzed further increased in height/width than length. Evaluating the exponent *b* of the weight-length relationship of Nile tilapia available in the literature (natural and cultivated), were found values ranging between 2.7 and 3.3.

In fact Nile tilapia appears to exhibit allometric growth (growth which does not comply to cube law). For weight-length relationship, there are cases in which the slope was significantly below or above the expected value of three for fish growth in general to be assumed as isometric.

Different exponents could also suggest that the fish we are dealing with are from different stocks (Njiru et al., 2006).

In zootechnical interest of obtaining the condition of live animals, non-destructive techniques have been applied enabling individuals to be cultivated at lower stress levels. This can be done by the use of structure and weight, which combined as indices provide indirect estimates of energy storage for animals (Camara et al., 2011). Condition factor studies take into account the health and general well-being of a fish in relation to its environment (Olurin and Aderibigbe, 2006). Morphometric indices which assume that heavier fish of a given length are in better condition are simple indicators of energy storage (Lloret et al., 2000). This is a common practice in studies with fish, where the conditions are estimated from its weight and length (Camara et al., 2011).

Table 7 shows the Fulton's condition factor found in fish submitted to different cultures, where was observed statistical difference in the factor of fish with 31 days of cultivation. The best conditions found at this time (semi-intensive system) may be associated with the consumption of natural food, as currently feeding rate and CP concentration in the diet were the same for both systems. Diana et al. (1994) evaluated the effects of adding supplemental food in ponds fertilized on the growth of Nile tilapia, and observed that the combination of feeding and fertilizers were more efficient than the growth based on these two items alone.

Assessing the condition factor found with population $K_{mean}$ (Figure 4) with 31 days of cultivation, condition factor of fish in semi-intensive system increased according to its corresponding $K_{mean}$ and the opposite occurred for fish in intensive system. This reinforces the importance of natural food in the early stages of cultivation. However, with decreasing concentrations of crude protein in diets for fish of the semi-intensive system along with decreased availability of natural food, the condition factor of the fish in the ponds decreased (not decaying $K_{mean}$ below) whereas in the intensive increased, making the two systems not statistically different

516 in the course of cultivation. The condition factor in both cultures remained close to $K_{mean}$ of the
517 population by the end of cultivation, demonstrating the efficiency of management.

518 Offem et al. (2007) assessed the condition factor in male Nile tilapia in a tropical floodplain
519 River and observed values ranging from (0.554-1.376). They considered these values as efficient for
520 tilapia and ascribed this condition to the abundance of natural food in the area. Huchette and
521 Beveridge (2003) also observed an improvement in the condition of Nile tilapia cultured in cages
522 with presence of natural food and diet. Olurin and Aderibigbe (2006) found a mean condition factor
523 of 1.14 for males of tilapia cultured in ponds, considering the value acceptable.

524 Condition factor can influence the reproduction and survival of populations (Lambert and
525 Dutil, 2000) reflecting interactions between biotic and abiotic factors and the physiological
526 condition of the fish. It shows the well-being of the population during the various stages of the life
527 cycle (Lizama and Ambrósio, 2002) and since it is a simple calculation can be used to manage fish
528 farms.

529 The relative condition factor ($K_{rel}$) represents the ratio of the observed weight and the weight
530 estimated by the weight-length relationship (Le Cren, 1951; Froese, 2006). The use of Box-plot
531 graphs in an evaluation of $K_{rel}$ provided interesting information about the two populations under
532 study (Figure 5). With these tools together is possible to identify the lowest and highest value of $K_{rel}$
533 in farming systems, as well as variations of $K_{rel}$ between the 1st and 3rd quartile, identifying in
534 more detail the possible changes in population. For the same distribution, measures of central
535 tendency (mode, mean and median) of $K_{rel}$ should be overlapped, and minimum and maximum
536 values near the 1st and 3rd quartile, respectively.

537 Since the beginning of the cultivation the values of $K_{rel}$ ranged between 80 and 120%,
538 indicating a large variation in the condition of farmed fish. However, the mean and median showed
539 up near 100%. The mode was not viewed in a standardized manner, in three cases it was below
540 average/median (in intensive system, with zero and 94 days of cultivation in both systems).

However, these values were close to 95%. The variations found in $K_{rel}$ of populations (± 20%) may be related to uneven growth of the Nile tilapia, since after 31 days of culture values outside the box plot (between the 1st and 3rd quartile) began to be more evident.

In Figure 6 is more evident the variation in length and weight of Nile tilapia during the cultivation and the measures of central tendency were still closer. However, positive and negative asymmetries were observed in both systems. McGinty (1986) evaluated the effect of density on weight gain of Nile tilapia and noted that the growth of small individuals was being depressed by the larger ones because of competition.

A comparative study of the relative weight of Nile tilapia was done with specimens of natural environment and culture. In general, we observed a higher relative weight of experimental specimens (9 of 14 cases). Better growth is expected in the cultivations, as well as a good carcass yield, feed conversion and homogeneity, since it works with improved strains of Nile tilapia. However, a decrease in genetic variability can reverse the situations described above, since a substantial loss of this variability is expected, due to the characteristics of the reproduction of tilapia and the appearance of a small size population when there is no proper genetic management of breeding stock (Moreira et al., 2007). The relative weight of the fish in this experiment was greater than the weight of tilapia from Lake Tana (Ethiopia), Ouémé River Basin (Benin), Hippopotamus Pond (Burkina Faso) and Lake Victoria (Kenya).

In Lake Tana, Tadesse (1997) observed a seasonal variation in condition factor, assigning lower values to energy expenditure after spawning. This variation in the condition found by the author may have generated differences in the WLR, decreasing their relative weight. A similar fact may have occurred in Ouémé River Basin (Benin), (Laleye, 2006). The same author also mentions that the WLR can change according to fish, sex, maturity, season and time (because of gastric fullness). The relative condition factor was also lower in fish from Hippopotamus Pond (Béarez, 2003). This lake is characterized by having a high fishing effort, with total absence of large fish.

566 The fishing activity during the dry season has affected spawning, as a consequence dwarfism
567 becomes more evident in populations.

568 According to Njiru et al. (2006) Nile tilapia from Lake Victoria showed good adaptation to the
569 site including little seasonal variation in their condition, becoming the most commercially important
570 fish in the lake and the third most captured. In this case, fish farmed presented here also showed
571 improved relative weight (approximately 15% more). The authors report that there was an increase
572 in fertility, suggesting a tactic to maximize reproductive success in Lake Victoria. This tactic may
573 be a response to overfishing of the population whose effects probably led to stunting.

574 However, Ahmed et al. (2003) indicate that in the Kaptai Reservoir (Bangladesh), fishing
575 pressure is below the capability of the stock to be increased by about 16%. They also mention that
576 the introduction of *O. niloticus* was accidental, however it has successfully established itself in the
577 reservoir. In this case the relative weight was found to be similar to the present study.

578 In Kainji Lake (Nigeria), Feu and Abiodun (1998) mentioned that anthropogenic activity was
579 great in that lake and the fish were captured below the ideal size, but with actions such as the
580 eradication of purse seine and adjustments in the size of meshes allowed the growth of the fish.
581 Moreover, according FISHBASE, today there are native species of Nile tilapia that can reach up to 74
582 cm in total length. These factors may have contributed to a poor relative weight of tilapia in the
583 present work compared with the tilapias from Kainji Lake.

584 One can see that action/awareness conducted by the authorities towards the population
585 behavior, contributed to the growth of the fish in Lake Kainji. From the ecological point of view
586 this fact is essential to maintaining the population status of the species. However, this
587 greater/similar relative weight in fish from natural environment must be observed with caution
588 (when compared with strains of aquaculture), since the species of tilapia used in fish farming went
589 through genetic improvements aiming to improve the quality of the breeding.

590 Comparing the tilapia described in the present work with those cultured in cages was observed
591 one relative weight major and other minor. Saraiva (2004) evaluated the effect of stocking density
592 on growth of juvenile Nile tilapia. He noted that there was no significant difference in the growth of
593 his specimens. However, when comparing with the present study the relative weight of tilapia
594 cultured in this work was higher than Saraiva (2004). He mentions that he worked with the strain of
595 tilapia chitralada or Thai and the various squads probably have their genetic variability decreased,
596 causing reduction in growth.
597 However, cultivation performed by Medri et al. (1999) showed better relative weight in
598 comparison with the work described here. Unlike Saraiva (2004), Medri et al. (1999) evaluated the
599 growth of tilapia with the inclusion of an alternative ingredient in feed (distillers yeast -
600 *Saccharomyces cerevisiae*), and noted that the rates used showed no statistical difference. This
601 change in diet may have influenced for a better relative weight.
602 Olurin and Aderibigbe (2006) analyzed the condition factor and WLR of Nile tilapia grown in
603 nurseries in Ijebu-Ode, Nigeria. When compared with the present study it was observed that the
604 tilapia produced here showed better relative weight. Also greater than the tilapia grown by Carmo et
605 al. (2008) in a Station of aquaculture in Brazil. Olurin and Aderibigbe (2006) did not mention if
606 they used tilapia with genetic modification, whereas Carmo et al. (2008) worked with a common
607 strain. This fact may have contributed to better growth observed in this study.
608 Carmo et al. (2008) also evaluated the growth of chitralada and red strain in ponds. The two
609 cultures performed by him had a relative weight similar to that observed in the present study,
610 indicating that there was not much difference in growth patterns of the three strains.
611 Leal et al. (2009) evaluated the growth of juvenile Nile tilapia in aquaria, fed with different
612 concentrations of hydrolyzed protein and a commercial feed. It was observed that the tilapia of this
613 study had a higher relative weight of tilapia fed commercial feed, however, when compared with
614 tilapia fed with 5% hydrolyzed protein diet (better WLR according to the author) observed a similar

615 growth. Leal et al. (2009) reported no strain used, however it can be seen that the feeding influence
616 the results.

617 El-Sayed (2006) cites that variations in body size of tilapia in natural habitat may be related to
618 the different environmental factors rather than genetic differences. Mair et al. (2002) evaluated the
619 performance of tilapia farms in the Philippines (mainly Nile tilapia) and noted that growth has
620 declined after the newly introduced pure strains, probably due to loss of genetic variation through
621 founder effect (*bottleneck effect*) and introgression with Mozambican tilapia (*Oreochromis*
622 *mossambicus*). In fact genetic and environmental variations may cause changes in growth. Dey et
623 al. (2000) analyzed the performance of the GIFT strain (Genetically Improved Farmed Tilapia)
624 compared to non-GIFT on farms in Bangladesh, China, Philippines, Thailand and Vietnam, and
625 observed a better growth of the GIFT strain. Santos et al. (2008) evaluated the growth of Nile tilapia
626 in Brazil (GIFT and Thai strains) and also noted the improved performance of GIFT. Tenório et al.
627 (2012) compared the performance of three strains of Nile tilapia defined as common chitralada and
628 mestizo (crossroads of common with chitralada) and found better growth with chitralada and
629 mestizo.

630

631 **5. Conclusion**

632

633 At the end of cultivation, the two farming systems showed the same trend of growth, i. e., the
634 adopted management promoted the same growth performance for both systems.

635 The calculations of Fulton's condition factor (K), Clark's mean condition factor ($K_{mean}$), Le
636 Cren's relative condition factor ($K_{rel}$) and relative weight ($W_{rm}$) proved to be useful tools when
637 assessing growth. Furthermore, the relative weight equation allowed a comparison of tilapia from
638 the natural environment and other cultures, expanding the growth studies comparisons. However,
639 one can realize the importance of continuous monitoring of these parameters, since differences were

observed in the relative weight of the studied species of tilapia when compared to other environments or cultures, leading us to reflect on how to improve the cultivation conditions of Nile tilapia.

The intensification of cultivation generates a greater need for skilled workers and therefore implies in an increase in the risks of production, especially with regard to the genetic variability of these animals. The lack of proper selection and genetic information from families can lead to an inbreeding framework reflecting in decreasing productivities.

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## 7. Tables

Table 1: Proximate analysis of the commercial diets used in the experiment

| Proximate analysis (as-fed basis, g . $kg^{-1}$)* | Crude Protein | | |
|---|---|---|---|
| | 35% | 32% | 28% |
| Pellet diameter (mm) | 3 - 4 | 4 - 6 | 6 - 8 |
| Moisture | 130 | 130 | 130 |
| Crude protein | 350 | 320 | 280 |
| Ether extract | 65 | 50 | 50 |
| Crude fiber | 50 | 50 | 50 |
| Ash | 120 | 120 | 120 |
| Calcium | 15 | 15 | 15 |
| Phosphorus | 6 | 6 | 6 |
| Vitamin C | 0.5 | 0.5 | 0.5 |

*Data from private company

Table 2: Nutritional plan used as feed for Nile tilapia (*O. niloticus*) subjected to different culture systems

| Time (days) | Nutrition plan | |
|---|---|---|
| | Intensive culture | Semi-intensive culture |
| 0 – 31 | 35% CP | 35% CP |
| 32 – 63 | 35% CP | 32% CP |
| 64 – 94 | 32% CP | 28% CP |

Table 3: Quality indicators of water in intensive and semi-intensive cultured Nile tilapia (*O. niloticus*)

| Parameters | Intensive culture | Semi-intensive culture |
|---|---|---|
| Salinity (‰) | $0.62 \pm 0.14^{a}$ | $0.64 \pm 0.1^{a}$ |
| Eletric conductivity ($\mu S \cdot cm^{-1}$) | $1{,}356 \pm 468.1^{b}$ | $1{,}285 \pm 249.1^{a}$ |
| Temperature ($^{o}C$) | $29.40 \pm 1.4^{b}$ | $30.15 \pm 2.6^{a}$ |
| pH | $8.07 \pm 0.2^{b}$ | $7.15 \pm 0.4^{a}$ |
| Dissolved oxygen (DO) ($mg \cdot L^{-1}$) | $8.19 \pm 1.9^{b}$ | $6.67 \pm 3.4^{a}$ |
| Ammonia nitrogen ($mg \cdot L^{-1}$) | $0.065 \pm 0.032^{a}$ | $0.126 \pm 0.061^{a}$ |
| Nitrite ($mg \cdot L^{-1}$) | $0.053 \pm 0.050^{a}$ | $0.017 \pm 0.013^{a}$ |
| Nitrate ($mg \cdot L^{-1}$) | $0.001 \pm 0.000^{a}$ | $0.002 \pm 0.001^{a}$ |
| Orthophosphate ($mg \cdot L^{-1}$) | $0.016 \pm 0.016^{a}$ | $0.001 \pm 0.000^{a}$ |
| Alkalinity ($mg\ CaCO3 \cdot L^{-1}$) | $13.95 \pm 1.86^{b}$ | $161.28 \pm 106.97^{a}$ |
| Hardness ($mg\ CaCO3 \cdot L^{-1}$) | $20.06 \pm 1.09^{b}$ | $28.50 \pm 4.37^{a}$ |
| Chlorophyll *a* ($\mu g \cdot L^{-1}$) | $17.75 \pm 5.92^{b}$ | $88.49 \pm 31.16^{a}$ |
| Pheophytin ($\mu g \cdot L^{-1}$) | $19.53 \pm 1.86^{b}$ | $274.97 \pm 151.95^{a}$ |

Values are mean ± SE of mean. Within a row, means with different superscript letters differ significantly ($P < 0.05$) by Wilcoxon-Mann-Whitney test and Student's *t* test.

Table 4: Abundance of phytoplankton (cel.ml$^{-1}$) in water of intensive and semi-intensive systems Nile tilapia (*O. niloticus*) in time "0" (zero), 31, 63 and 94 days.

| Groups | Time (days) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 day | | 31 days | | 63 days | | 94 days | |
| | Intensive culture | Semi-intensive culture | Intensive culture | Semi-intensive culture | Intensive culture | Semi-intensive culture | Intensive culture | Semi-intensive culture |
| Chlorophyceae | 32,500 ± 12,200$^{b}$ | 73,300 ± 22,300$^{a}$ | 5,000 ± 2,300$^{b}$ | 55,000 ± 12800$^{a}$ | 36,300 ± 3,000$^{b}$ | 48,000 ± 53$^{a}$ | 8,300 ± 1,200$^{b}$ | 26,000 ± 5,700$^{a}$ |
| Cyanophyceae | 24,000 ± 15,700$^{a}$ | 12,600 ± 9,600$^{a}$ | 46,000 ± 13,600$^{a}$ | 26,000 ± 5700$^{a}$ | 2,000 ± 700.5$^{a}$ | 2,200 ± 600$^{a}$ | 7,400 ± 2,200$^{a}$ | 1,200 ± 200$^{b}$ |
| Bacillariophyceae | 600 ± 500$^{a}$ | 2,200 ± 1,200$^{a}$ | 300 ± 200$^{b}$ | 1,300 ± 173$^{a}$ | 200 ± 150$^{b}$ | 1,100 ± 300$^{a}$ | 700 ± 600$^{b}$ | 800 ± 200$^{a}$ |

Values are mean ± SE of mean. Within a row, at the same times, means with different superscript letters differ significantly ($P < 0.05$) by Wilcoxon-Mann-Whitney test and Student's *t* test (n=4).

Table 5: Abundance of zooplankton (Ind.L$^{-1}$) in water of intensive and semi-intensive systems Nile tilapia (*O. niloticus*) in time "0" (zero), 31, 63 and 94 days.

| Groups | Time (days) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 day | | 31 days | | 63 days | | 94 days | |
| | Intensive culture | Semi-intensive culture | Intensive culture | Semi-intensive culture | Intensive culture | Semi-intensive culture | Intensive culture | Semi-intensive culture |
| Rotifera | 9,503 ± 2,370$^{b}$ | 33,490 ± 8,478$^{a}$ | 16,020 ± 3,703$^{a}$ | 18,521 ± 4,718$^{a}$ | 16 ± 29$^{b}$ | 714 ± 198$^{a}$ | 123 ± 32$^{b}$ | 753 ± 276$^{a}$ |
| Cladocera | 12,521 ± 2,401$^{a}$ | 15,512 ± 3,902$^{a}$ | 7,500 ± 2,302$^{b}$ | 20,036 ± 552$^{a}$ | 3.5 ± 3.0$^{b}$ | 80 ± 55$^{a}$ | 3.0 ± 2.0$^{b}$ | 220 ± 92$^{a}$ |
| Copepoda | 88,029 ± 12,200$^{b}$ | 147,496 ± 33,201$^{a}$ | 86,498 ± 2,622$^{b}$ | 113,527 ± 2,237$^{a}$ | 182 ± 25$^{b}$ | 1,423 ± 740$^{a}$ | 172 ± 7.0$^{b}$ | 767 ± 326$^{a}$ |

Values are mean ± SE of mean. Within a row, at the same times, means with different superscript letters differ significantly ($P < 0.05$) by Wilcoxon-Mann-Whitney test and Student's *t* test (n=4).

968 Table 6: Growth performance and nutrient utilization of Nile tilapia (*O. niloticus*) cultivated in
969 intensive and semi-intensive system in 94 days.

| Parameters | Intensive culture | Semi-intensive culture |
|---|---|---|
| Density ($m^3$) | 110 | 0.67 |
| Initial weight (g) | $88.33 \pm 1.54^a$ | $86.90 \pm 1.50^a$ |
| Final weight (g) | $543.58 \pm 48.11^a$ | $501.74 \pm 4.37^a$ |
| WG (g)[1] | $455.24 \pm 41.68^a$ | $414.84 \pm 3.48^a$ |
| ADG (g $day^{-1}$)[2] | $4.84 \pm 0.44^a$ | $4.41 \pm 0.04^a$ |
| SGR (% $day^{-1}$)[3] | $1.96 \pm 0.08^a$ | $1.87 \pm 0.01^a$ |
| Final biomassa (kg) | $228.46 \pm 16.18^a$ | $143.99 \pm 2.74^b$ |
| Survival (%) | $75.0^b$ | $85.0^a$ |
| FCR[4] | $1.59 \pm 0.12^a$ | $1.57 \pm 0.11^a$ |
| PER[5] | $4.23 \pm 0.37^b$ | $6.51 \pm 0.30^a$ |
| FE[6] | $1.6 \pm 0.02^b$ | $2.30 \pm 0.11^a$ |

970 Values are mean ± SE of mean. Within a row, means with different superscript letters differ
971 significantly ($P < 0.05$) by Wilcoxon-Mann-Whitney test and Student's *t* test

972 [1] Weight gain, [2] Average daily gain, [3] Specific growth rate, [4] Feed conversion ratio, [5] Protein
973 efficiency ratio, [6] Feed efficiency.
974

975 Table 7: Fulton's condition factor of Nile tilapia cultivated in intensive and semi-intensive systems
976 at times "0" (zero), 31, 63 and 94 days

| Time (days) | Intensive culture | Semi-intensive culture |
|---|---|---|
| 0 | $1.97 \pm 0.06^a$ | $2.00 \pm 0.01^a$ |
| 31 | $1.95 \pm 0.02^b$ | $2.17 \pm 0.07^a$ |
| 63 | $2.03 \pm 0.06^a$ | $2.11 \pm 0.04^a$ |
| 94 | $2.10 \pm 0.08^a$ | $2.08 \pm 0.01^a$ |

977 Values are mean ± SE of mean. Within a row, means with different superscript letters differ
978 significantly ($P < 0.05$) by Student's *t* test
979

980

Table 8: Relative weight ($W_{rm}$) of fish with 94 days of cultivation in the intensive and semi-intensive systems compared with total weight-length relationships of fish obtained in other environments (natural and cultivated) of the experimental specimens in the analyzed environments.

| Country | Environment | Locatity | Length class (cm) | Sex | *a* | *B* | $R^2$ | References | $W_{rm}$ Intensive system | $W_{rm}$ Semi-intensive system |
|---|---|---|---|---|---|---|---|---|---|---|
| Brazil | Cage culture | Aquaculture Station UAST/IPA | 16.0 - 30.0 | Male[a] | 0.0139 | 3.117 | 0.985 | Present work | 101.6 ± 3.5 | 101.2 ± 0.8 |
| Brazil | Ponds culture | Aquaculture Station UAST/IPA | 16.0 - 30.1 | Male[a] | 0.0160 | 3.081 | 0.987 | Present work | 99.79 ± 3.5 | 99.3 ± 0.8 |
| Ethiopia | Natural | Lake Tana | 13.0 - 32.0 | Unsexd | 0.0424 | 2.740 | 0.941 | Tadesse (1997) | 118 ± 5.1 | 117 ± 0.7 |
| Benin | Natural | Ouémé River Basin | 7.5 - 26.5 | Unsexd | 0.0330 | 2.790 | 0.961 | Lalèyè (2006) | 126 ± 5.2 | 124 ± 0.8 |
| urkina Faso | Natural | Hippopotamus Pond | 9.7 – 22.1 | Unsexd | 0.0377 | 2.800 | 0.986 | Béarez (2003) | 110 ± 4.5 | 108 ± 0.7 |
| Kenia | Natural | Lake Victoria | 10.0 - 60.0 | Male | 0.0118[d] | 3.130[d] | 0.987[d] | Njiru et al. (2006) | 115 ± 3.9 | 114 ± 0.9 |
| Bangladesh | Natural | Kaptai Lake | 15.0 - 53.0 | Unsexd | 0.0366 | 2.840 | 0.980 | Ahmed et al.(2003) | 97 ± 3.9 | 96 ± 0.7 |
| Nigeria | Natural | Kainji Lake | 8.3 - 19.5 | Unsexd | 0.0169 | 3.130 | 0.958 | Feu and Abiodun (1998) | 79 ± 2.7 | 79 ± 0.6 |
| Brazil | Cage culture | Fish Culture Association /Xingó | 3.0 - 17.3 | Male[b] | 0.0272[d] | 2.840[d] | 0.096[d] | Saraiva (2004) | 132.7 ± 5.4 | 131.4 ± 0.9 |
| Brazil | Cage culture | Fish Culture Station / UEL | 3.6 - 18.8 | Male | 0.0195[d] | 3.040[d] | 0.984[d] | Medri et al. (1999) | 94.2 ±3.4 | 93.7 ± 0.7 |
| Nigeria | Ponds culture | Ijebu-Ode | 5.5 - 11.4 | Male | 0.0093 | 3.140 | uninformed | Olurin and Aderibigbe (2006) | 140 ± 4.7 | 140 ± 1.1 |
| Brazil | Ponds culture | Aquaculture Station / UFRPE | 13.0 - 28.2 | Male | 0.0342 | 2.801 | 0.998 | Carmo et al. (2008) | 120.3 ± 5.0 | 119.0 ± 0.8 |
| Brazil | Ponds culture | Aquaculture Station / UFRPE | 13.0 - 28.0 | Male[b] | 0.0230 | 2.968 | 0.998 | Carmo et al. (2008) | 101.4 ± 3.8 | 100.7 ± 0.8 |
| Brazil | Ponds culture | Aquaculture Station / UFRPE | 13.0 - 28.1 | Male[c] | 0.0171 | 3.053 | 0.997 | Carmo et al. (2008) | 102.4 ± 3.7 | 101.9 ± 0.8 |
| Brazil | Aquarium | Laboratory of Aquaculture/UFPE | uninformed | Male | 0.0163[e] | 2.990[e] | 0.988[e] | Leal et al. (2009) | 129.0 ± 4.8 | 128.2 ± 1.0 |
| Brazil | Aquarium | Laboratory of Aquaculture/UFPE | uninformed | Male | 0.0132[f] | 3.116[f] | 0.995[f] | Leal et al. (2009) | 107.4 ± 3.7 | 107.0 ± 0.9 |

[a] Male Nile tilapia strain GAAT-1, [b]Male Nile tilapia strain Chitralada, [c] Male Nile tilapia red strain, [d] Geometric mean of *a*, *b* and $R^2$, [e] Values of *a*, *b* and $R^2$ from Nile tilapia fed commercial diet, [f] Values of *a*, *b* and $R^2$ from Nile tilapia fed diet containing 5% shrimp protein hydrolysate.

## 8. Figures

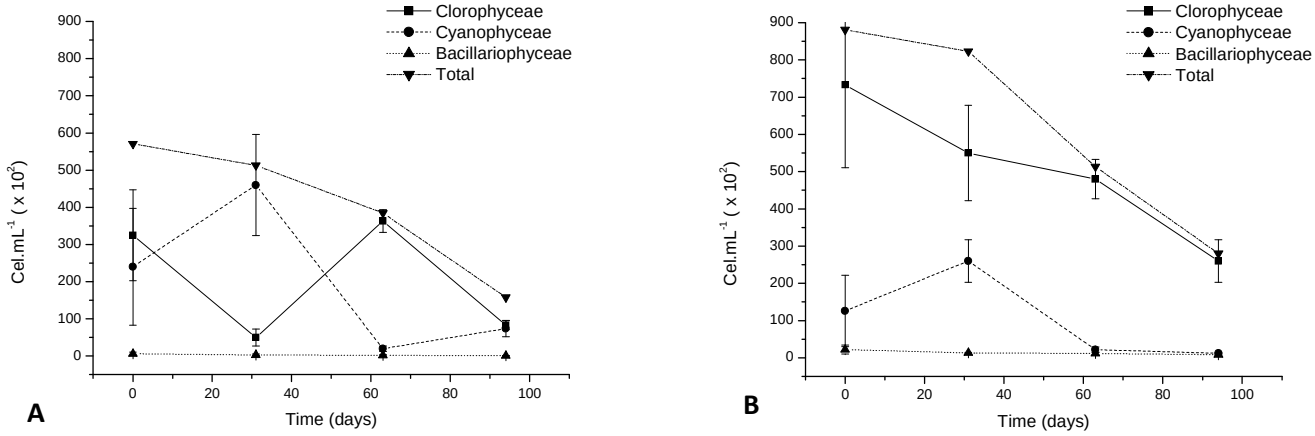



Figure 1: Mean ± SE of mean of the evolution of the abundance of phytoplankton (cel.mL$^{-1}$ x 10$^{2}$) intensive (Figure 1A) and semi-intensive cultivation (Figure 1B) of Nile tilapia (*O. niloticus*).

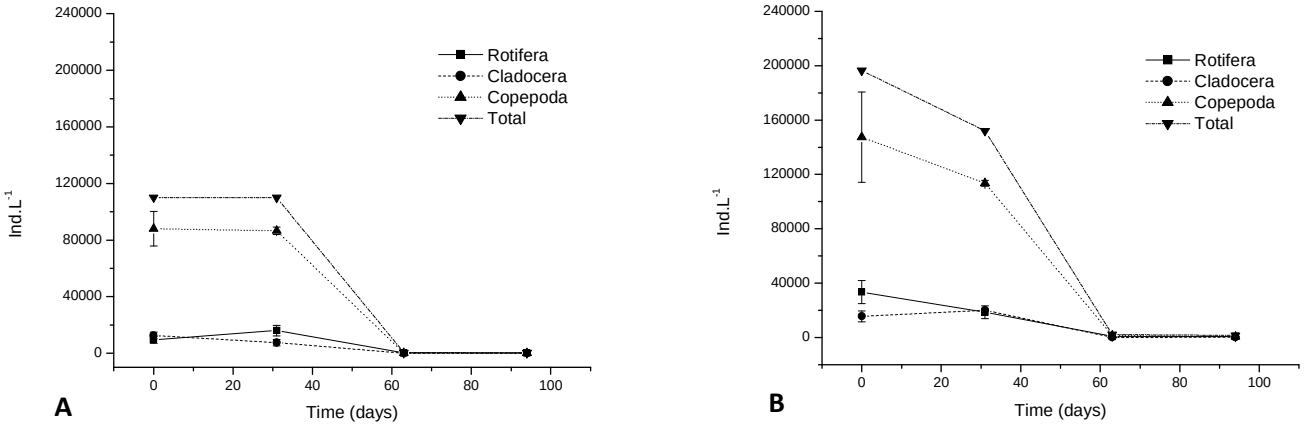
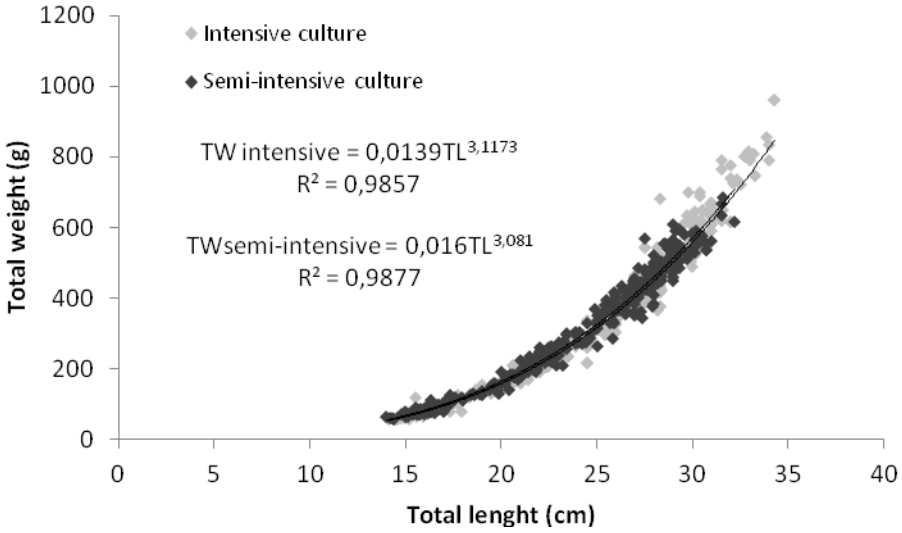


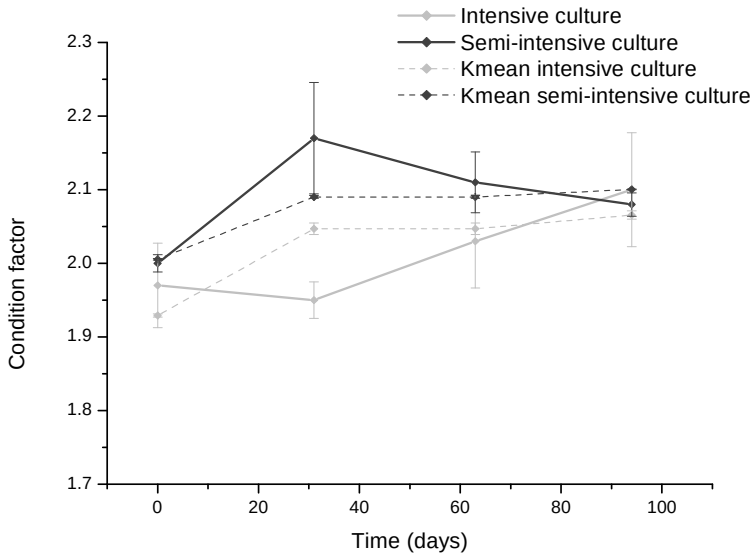

Figure 2: Mean ± SE of mean of the evolution of the abundance of zootoplankton (Ind.L$^{-1}$) intensive (Figure 2A) and semi-intensive cultivation (Figure 2B) of Nile tilapia (*O. niloticus*).




1009

1010 Figure 3: Parameters of the mathematical models of Nile tilapia (Wt = *a* Lt $^{b}$) adjusted to total
1011 weight-length data from fish under different culture systems.

1012




1013

1014 Figure 4: Mean of the evolution of Fulton's condition factor (K) of fish subjected to different
1015 cultures in comparison with the corresponding population $K_{mean.}$

1016

1017

1018

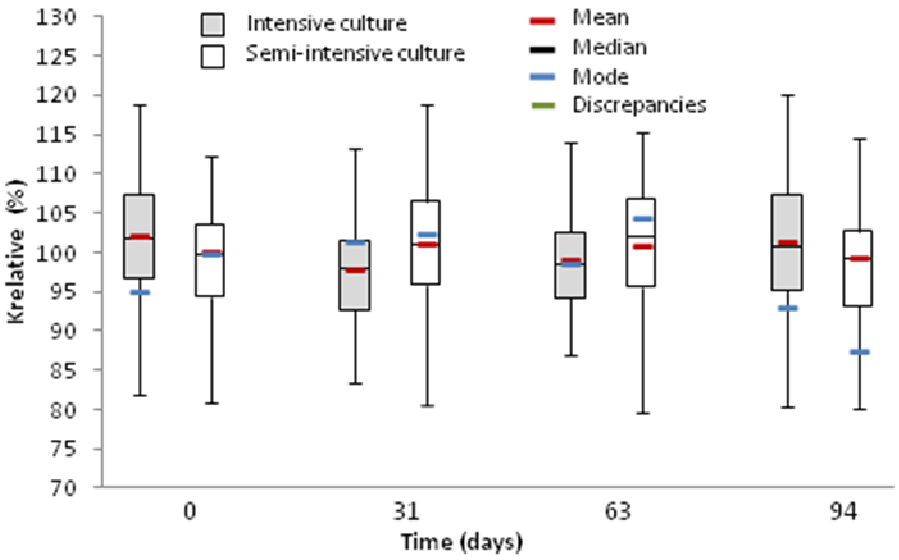



Figure 5: Boxplot of the evolution in the values of $K_{rel}$ (%) from fish under different cultures.

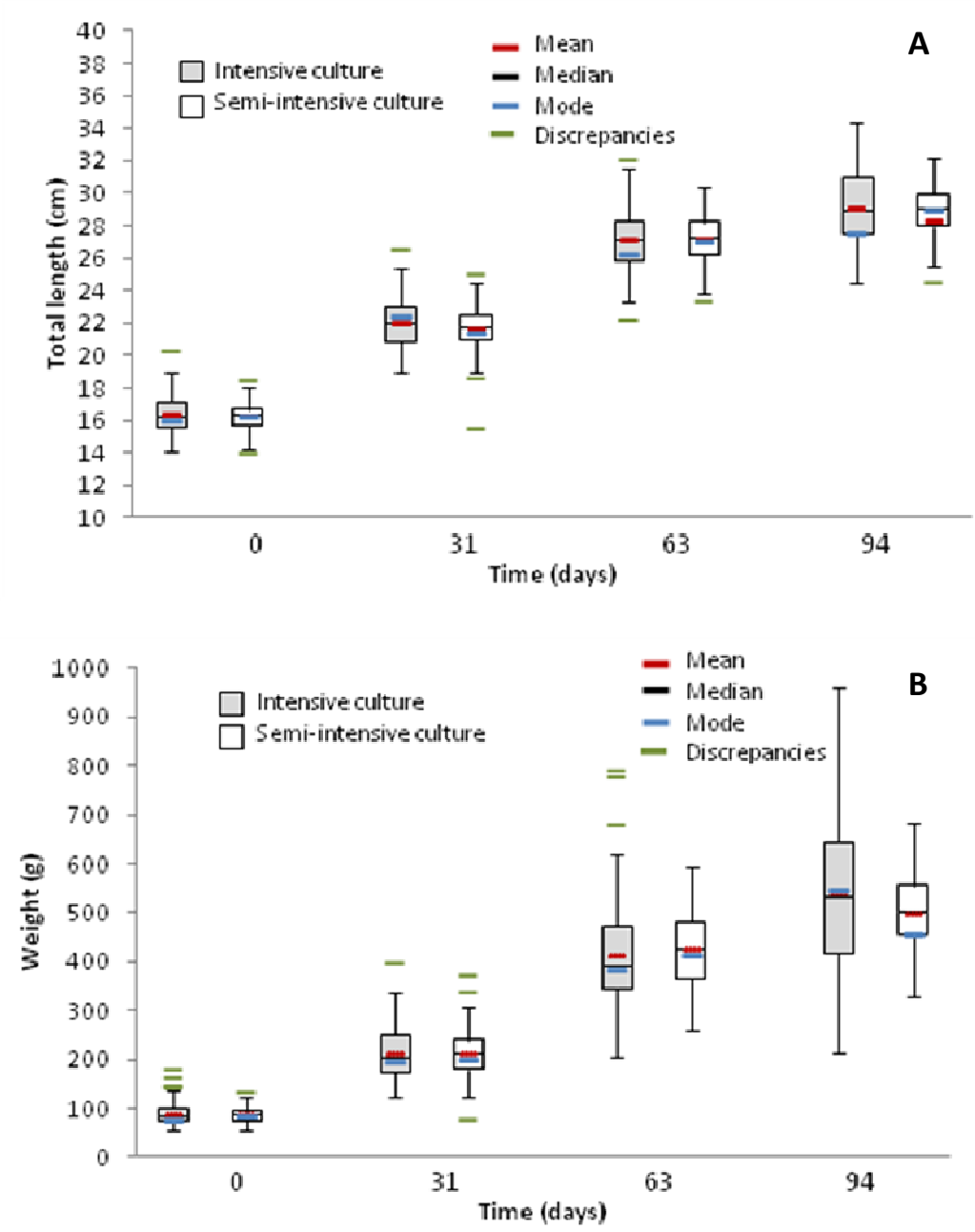



Figure 6: Boxplot of the evolution of Total length (cm) (Figure 6A) and Weigth (g) (Figure 6B) of fish under different cultures.

## 7. CAPÍTULO III

## ARTIGO CIENTÍFICO:

# Enzymatic changes in the intestine of Nile tilapia (*Oreochromis niloticus* L.) on intensive and semi-intensive culture systems

**ARTIGO A SER SUBMETIDO A REVISTA AQUACULTURE ISSN: 0044-8486**

# Enzymatic changes in the intestine of Nile tilapia (*Oreochromis niloticus* L.) under intensive and semi-intensive culture systems

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Running title: Influence of culture systems on the digestive enzymes of tilapia

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## Highlights

- Total proteolytic, amylase and lipase activity did not statistically differ between treatments whereas trypsin and chymotrypsin activity increased with 31 and 63 days of culture.
- A positive correlation was observed between the recommended concentration of essential amino acids for Nile tilapia and specific aminopeptidases activity.
- PAGE revealed 12 active proteolytic bands in both systems, however integrated density values were higher in the bands of semi-intensive.
- Specimens of the intensive and semi-intensive exhibited five bands of amylolytic activity, with higher integrated density at 31 days of cultivation for both systems.
- The two-dimensional electrophoresis of the intestine of cultured animals in intensive and semi-intensive systems showed a total of 1,233 spots, among them 731 different.

**Abstract**

The effect of different culture systems on digestive enzyme activities of Nile tilapia was evaluated. Juvenile Nile tilapia (87.61 ± 1.52g) were cultured in semi-intensive and intensive system during 90 days. Two nutritional plans were adopted: triphasic for semi-intensive systems (diet with 35, 32 and 28% crude protein - CP) and biphasic for intensive system (35 and 32% CP). Biometric measurements were monthly performed for adjustments in diet and removal of intestine tissues to evaluate the performance of enzyme activities. Total proteolytic, amylase and lipase activity were not statistically different between the treatments along of the periods analyzed (31, 63 and 94 days of culture). However, trypsin and chymotrypsin activity were higher with 31 and 63 days of culture in fish from semi-intensive system, suggesting that natural food may have influenced these activities. A positive correlation was observed between the recommended concentration of essential amino acids for Nile tilapia and specific aminopeptidases activity in fish intensive system. Substrate-SDS-PAGE revealed 12 active proteolytic bands in both systems. However, integrated density (*ID*) values were higher in the bands of semi-intensive. Specimens of the intensive and semi-intensive exhibited five bands of amylolytic activity. Fish from intensive and semi-intensive systems showed the highest values of *ID* with 31 days of cultivation. The lowest values of *ID* were observed with fish from semi-intensive system with 63 and 94 days of cultivation. The comparison of the proteomic profile of the intestine of cultured animals in intensive and semi-intensive system showed a total of 1,233 spots, among them 731 different. It can be noticed that the natural food and diets used greatly influenced the culture systems. The expression/activity of these proteins appears to have a form of performance according to environmental and feeding conditions, so that the digestive process can be as efficient as possible in accordance with the conditions of cultured animals.

K**eywords:** Nile tilapia, culture systems, semi-intensive and intensive systems, digestive enzymes.

## 1. Introduction

In aquaculture, good rates of growth are essential to ensure the success of the production and for such achievement, an efficient management becomes necessary. Inadequate management in addition to ecologically unsustainable production can destabilize growth parameters directly affecting production costs (El-Sayed, 2006).

The growth depends mainly on the food provided. However, the nutritional value of a diet is not based simply on their chemical composition, but also the physiological ability of the fish to digest and absorb, according to its feeding habits and nutritional requirements (Santos et al., *in press*). According to Tengjaroenkul et al. (2000), the ability of fish to utilize ingested nutrients depends on the presence of appropriate enzymes along of the digestive tract. The study of the digestive enzymes in fish may clarify some aspects of their physiology and, therefore, can also afford to solve some nutritional problems in fish feeding (Ribeiro et al., 1999). Ferron and Leggett (1994) suggested that digestive enzymes can be interesting as condition indices due to their specificity and sensitivity. Furthermore, according to Cara et al. (2007) its value as an indicator can be justified if its activity clearly shows genetic variations (between different populations) or shows a response to environmental changes.

Enzymatic activities appear to be related to age, feeding habits of fish and other factors (Falcón-Hidalgo et al., 2011). Santos et al. (*in press*) suggest that Nile tilapia has the ability to adapt to different food sources. For optimum utilization of all these sources, the enzymatic arsenal of tilapia should be sufficiently diversified to digest the wide range of ingested food.

Enzymes have been studied over the last decades for their applications in nutritional physiology and biochemistry for aquaculture (Tengjaroenkul et al. 2000; Muhlia-Almazan et al., 2003; Buarque et al., 2009a; Buarque et al., 2009b; Santos et al., *in press*). However, changes in enzyme activity resulting from management are not well elucidated. A better knowledge of the

enzymatic activity during growing is relevant for the understanding of fish nutritional needs (Ribeiro et al., 1999). Since the growth is directly dependent on the absorption of the nutrients from the diet, it is very important to attempt that the digestive enzymes may play a key role on the nutrient incorporation. Therefore, investigations conducted during the cultivation, could provide valuable information for a better understanding of the physiological processes associated with the culture system employed.

Nile tilapia is often commercially cultivated in intensive or semi-intensive system, under different stocking densities, management strategies and environmental conditions. Furthermore, many features make them an ideal candidate for aquaculture, especially in developing countries (El-Sayed, 2006). Thus, the aim of this work is to detect enzymatic changes in the intestine of Nile tilapia cultured in the intensive and semi-intensive culture systems, relating these activities to the production system adopted.

## 2. Materials and methods

### 2.1 Culture conditions

The animals were cultivated at the Aquaculture Station of Unidade Acadêmica de Serra Talhada (UAST) / Instituto Agronômico de Pernambuco (IPA) located in Serra Talhada, semi-arid region of Pernambuco State, Brazil.

The experiment consisted of two treatments (intensive and semi-intensive cultures) and three replicates. In the intensive treatment fish were cultured in cages, while in the semi-intensive they were grown in ponds.

For the intensive culture, the animals were stocked at a density of 110 fish.m$^{-3}$ (three replicates), the cages were located in the reservoir Saco I - Serra Talhada – PE.

The semi-intensive culture was carried out using three ponds with an area of 338 $m^2$ and a maximum depth of 1.5 m, supplied by water from the reservoir Saco I. The animals were stocked at a density of 1 $fish.m^{-2}$ (0.67 $fish.m^{-3}$). Before stocking, the ponds were dried in the sun light and went through the process of liming ($CaCO_3$ - 15 kg per 1000 $m^2$) and fertilization (bovine manure - 1 $ton.ha^{-1}$) according to Woynarovich and Horváth (1983) for the growth of natural food such as phytoplankton and zooplankton.

The ponds and cages were stocked with juvenile sex-reversed Nile tilapia (variety QAAT1/Arizona-USA) with an initial average weight of 88.6 ± 1.5g and an average total length of 16.3 ± 0.12cm. An aliquot of 10% of the populations were sampled monthly to monitor growth and make feeding adjustments. Both cultures lasted 94 days.

Commercial diets containing 35, 32 and 28% crude protein (CP) were used (composition described in Table 1). Intensive culture used nutritional biphasic plan (35 and 32% CP) and semi-intensive triphasic plan (35, 32 and 28% CP), as described in Table 2. The fish were fed twice a day (8 a.m. and 3 p.m.) with feeding rates of 5, 4 and 3% biomass, adjusted monthly after biometrics.

Water temperature, dissolved oxygen, pH, ammonia nitrogen, nitrite, nitrate, orthophosphate, alkalinity and hardness were monitored and the average for intensive and semi-intensive system were 29.40 ± 1.4 and 30.15 ± 2.6 $^oC$ (mean ± SD); 8.19 ± 1.9 and 6.67 ± 3.4 mg $L^{-1}$; 8.07 ± 0.2 and 7.15 ± 0.4; 0.065 ± 0.032 and 0.126 ± 0.061 mg $L^{-1}$; 0.053 ± 0.050 and 0.017 ± 0.013 mg $L^{-1}$; 0.001 ± 0.000 and 0.002 ± 0.001 mg $L^{-1}$; 0.016 ± 0.016 and 0.001 ± 0.000 mg $L^{-1}$; 13.95 ± 1.86 and 161.28 ± 106.97 mg $L^{-1}$; 20.06 ± 1.09 and 28.50± 4.37 mg $L^{-1}$, respectively. Data described in Santos (2013).

At the end of the experiment, the main growth parameters were calculated from the following equations:

Weight gain rate ($WG_{(g)}$) = $BW_f$ - $BW_i$

Survival ($S_{(\%)}$) = (Final number of fish/ fish stocked) x 100

Feed conversion ratio (FCR) = dry feed offered$_{(g)}$/wet weight gain$_{(g)}$

Protein efficiency ratio (PER) = wet weight gain$_{(g)}$/ protein fed$_{(g)}$

Feed efficiency (FE) = wet weight gain$_{(g)}$/dry feed offered$_{(g),}$

Fulton's condition factor (K): $K = 100\ W / L^3$,

in which $BW_i$ and $BW_f$ = average initial and final body weight$_{(g)}$ of fish; W = total weight in the times 0, 31, 63 and 94 days and L = total length at the same times. Data described in Santos (2013).

**2.2 Extraction of digestive enzymes**

In the times 31, 63 and 94 days of cultivation, fish remained under fasting for 24 h and five individuals from each replicate were sacrificed according to Pedrazzani et al. (2007) by sectioning of medulla and bleeding by cutting the gills. Intestines were immediately collected and homogenized (40 mg tissue.mL$^{-1}$) in 0.15 M NaCl, using a tissue homogenizer. The resulting preparations were centrifuged at 10,000 x *g* for 10 min at 4 $^{o}$C to remove cell debris and nuclei. The supernatants (crude enzyme extracts) were frozen at -20 $^{o}$C and used in further assays (Bezerra et al., 2005).

**2.3 Protein concentration**

Protein concentration was determined according to Bradford (1976) using bovine serum albumin (BSA) as the standard.

## 2.4 Enzyme assays

### 2.4.1 Total proteolytic activity

For total proteolytic activity 1% (w/v) azocasein was used as substrate according Bezerra et al. (2005). One unit (U) of enzyme activity was defined as the amount of enzyme capable of hydrolyzing azocasein to produce a 0.001 change in absorbance per minute.

### 2.4.2 Trypsin and chymotrypsin activity

Trypsin and chymotrypsin activity were determined using 8.0 mM BApNA (Nα-benzoyl-DL-arginine-p-nitroanilide) and 8.0 mM SApNA (Suc-Ala-Ala-Pro-Phe p-nitroanilide) as substrate, respectively, according Bezerra et al. (2005). One unit (U) of activity was defined as the amount of enzyme required to produce 1 µmol *p*-nitroaniline.min$^{-1}$. Specific activity was expressed as U.mg$^{-1}$ of protein.

### 2.4.3 Aminopeptidase activity

Aminopeptidase activity was evaluated using 4.2 mM aminoacyl-β-naphthylamide as substrate. The aminoacyl moieties of substrates used were Leu, Arg, Ala, His, Tyr, Gly, Pro, Iso and Ser, as described in Buarque et al. (2009a). One unit of enzyme activity was defined as the amount of enzyme required to hydrolyse 1 µmol β-naphthylamine.min$^{-1}$. Specific activity was expressed as mU.mg$^{-1}$ of protein.

### 2.4.4 Amylase activity

Amylase activity was evaluated according to Bernfeld (1955) using 2% (w/v) starch as substrate. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 µg of maltose per milligram of protein per min (specific activity).

### 2.4.5 Lipase activity

Lipase activity was assayed according to Aryee et al. (2007) using 8mM *p*-nitrophenyl palmitate (*p*-NPP), as substrate. One unit of activity (U) was defined as the amount of enzyme that catalyzed the release of 1µmol of *p*-nitrophenol (*p*-NP) per min under the assay conditions. Specific activity was expressed as $U.mg^{-1}$ of protein.

## 2.5 Enzyme characterization in substrate-SDS-PAGE (zymograms)

Zymogram of protease activity (aliquots of 50 µg of protein, $n = 3$) were carried out based on Garcia-Carreño et al. (1993) after electrophoresis. The gels were immersed in 2.5 % Triton X-100 dissolved in 0.1 M Tris–HCl buffer pH 8.0 to remove the SDS and incubated with 4 % casein (w/v) in 0.1 M Tris–HCl buffer pH 8.0 for 30 min at 4 $^{o}$C. The temperature was raised to 25 $^{o}$C and maintained for 90 min to allow the digestion of casein by the active fractions. Finally, the gels were stained overnight for protein in 0.18 % (w/v) Coomassie Brilliant Blue R250 prepared in acetic acid and methanol (10:25 % v/v), and the background of the gel was distained in acetic acid and methanol (10:25 % v/v). Clear bands in blue background denoted protease bands by digestion of casein substrate.

Zymogram of amylase activity ($n$ = 3) were carried out according to the modified methodology described by Castro et al. (2012). 30 μg of protein was applied to a 7.5% (w/v) separating gel. Electrophoresis was performed at a constant current of 15 mA per gel at 4 $^{o}$C for 150 minutes, for better visualization of the bands. The gels were immersed in 2.5 % Triton X-100 dissolved in 0.1 M Tris–HCl buffer pH 8.0 to remove the SDS and incubated with 2 % starch (w/v) in 0.1 M Tris–HCl buffer pH 8.0, containing 1 mM $CaCl_2$ for 30 min at 37°C, to allow the digestion of starch by the active fractions. Finally, the gels were washed with distilled water and stained with an iodine/KI solution (15 and 12 mM) for 5 minutes. The gels were washed with distilled water to stop the reaction and then with an acetic acid and methanol solution (10:25 % v/v). Clear bands in black background denoted amylase bands by digestion of starch substrate.

### 2.6 Densitometry of the bands

Zymograms images ($n$ = 3) were obtained and the grayscale intensity of the bands were evaluated by densitometry using ImageJ software (Image Processing and Analysis in Java, USA). Values were analyzed in integrated density (*ID*) which is the sum of the values of the pixels in the image or selection. This is equivalent to the product of a given area and the respective mean gray value (Rasband, 2012; Schneider et al., 2012).

### 2.7 Two-dimensional electrophoresis

#### 2.7.1 Extraction and protein quantification

Samples of intestine tissues of Nile tilapia cultivated in the intensive and semi-intensive systems were macerated in liquid nitrogen. 100 mg of tissue was added to 1 mL of Trizol

(Invitrogen®) and this material was incubated for five minutes. Then, after the addition of 200 μL of 100% chloroform, the material was incubated for 3 minutes. The samples were centrifuged at 12,000 x *g* for 20 minutes at 4 °C. 300 μL of cold 100% ethanol was added to the precipitate and then, incubated for 3 minutes. The material was centrifuged again under the same conditions and the supernatant was collected and added to 1 ml of cold 100% isopropanol, waiting 10 minutes at 27°C. The samples were centrifuged and the supernatant discarded. The pellet was washed three times, at each wash was added 1 ml of hypochlorite solution of guanidine and 95% ethanol. The pellet was left in the solution under stirring for 20 minutes at 27°C and then centrifuged at 8,000 x *g* for five minutes at 4 °C. After the three washes 1 ml of ethanol was added to the pellet and a stirring was performed and allowed to incubate for 20 minutes at room temperature. The material was again centrifuged at 8,000 x *g* for 10 minutes at 4 °C. The supernatant was discarded and the precipitate was dried for 10 minutes at room temperature. Then, the pellet was dissolved with 200μL of 1% SDS and 1X PBS (phosphate buffered saline) and incubated for two hours at 50 °C in a water bath (Kirkland et al., 2006).

The protein quantification was performed using the 2D Quant Kit (GE Healthcare) according to manufacturer's instructions.

### 2.7.2 Precipitation of proteins

The extracted proteins were precipitated using 20% trichloroacetic acid (TCA). An aliquot containing 600 mg of protein (according to the previous quantification) was separated and added to a volume three-fold higher of 20% TCA. This material remained at 4 °C for two hours and subsequently centrifuged at 14,000 x *g* for 30 minutes at 4 ° C. The supernatant was discarded and the centrifugate was washed twice with 1 ml of 100% cold acetone. Between the washes (after the

addition of acetone) the samples were incubated for 10 minutes and then centrifuged at 14,000 x *g* for five minutes.

### 2.7.3 Two-dimensional electrophoresis in polyacrylamide gels

For two-dimensional electrophoresis was used methodology Kirkland et al. (2006), adapted to our conditions.

#### 2.7.3.1 First Dimension - Isoelectric focusing

After precipitation of proteins, 250 µl of sample buffer Dithiothreitol (DTT) and 1% ampholyte (GE Healthcare) were added. 13 cm Strips (Immobiline DryStrip pH 3-10 - GE Healthcare) were hydrated for 16 hours. The isoelectric focusing was performed in the Ettan IPGphor III (GE Amersham Biosciences) for about 6 hours.

#### 2.7.3.2 Second Dimension - SDS-PAGE

Before electrophoresis (SDS-PAGE) strips were equilibrated in equilibration buffer according to Kirkland et al. (2006), in two phases: the first with equilibration buffer with addition of 1% DTT (Dithiothreitol) and the second with the addition of 2.5% iodoacetamide, each phase with 20 minutes of incubation under stirring at room temperature. The strips were applied over a 12.5% polyacrylamide concentration gel (16 x 18 cm height-width and 1 mm thick). The electrophoresis was performed by applying two voltages: 15 mA for 30 minutes and after 45 mA until the end of the run.

For staining, the gels were incubated with staining solution (20% methanol, 5% acetic acid and 0.2% Coomassie Blue) for two hours under stirring and at room temperature and destained with solution (20 % methanol and 5% acetic acid) for 16 hours under stirring and also at room temperature.

### 2.8 Statistical analysis

All data was tested for normal distribution (Shapiro-Wilk test) and homogeneity of variances (Bartlett´s test). Student's *t*-test and One-way analysis of variance (ANOVA) followed Tukey tests were used for normally distributed data, whereas Wilcoxon-Mann-Whitney and Kruskal-Wallis test were used for non-normally distributed data. Correlations were tested using Pearson's Product Moment Correlation following regression analysis. Densitometry to quantify the bands in gels was done with the software ImageJ from imagej.nih.gov/ij/. Differences were reported as statistically significant when $P < 0.05$, using Origin™ version 8.0, Assistat 7.6 and GraphPad Prisma 6.0.

The two-dimensional gels were subjected to analysis with Platinum 2D ImageMaster software (Amersham Biosciences) for the detection of quantitative and qualitative differences of proteins between treatments ($n = 3$).

## 3. Results

The proximate analysis of the commercial diets used in the experiment and nutritional plan is shown in Tables 1 and 2, respectively. The main growth parameters calculated for Nile tilapia can be seen in Table 3. There was no statistical difference between the final weight, weight gain and feed conversion ratio of experimental specimens. However, the best values of survival, protein efficiency ratio and feed efficiency were observed for fish in semi-intensive system. The condition

factor found for Nile tilapia systems in semi-intensive and intensive during cultivation can be seen in Table 4. At 31 days of cultivation statistical difference was observed between the systems, reaching the best value for semi-intensive system (Santos, 2013).

Figure 1 shows the alkaline proteolytic activity of Nile tilapia cultivated under intensive and semi-intensive systems. Total proteolytic activity (Figure 1A) was not statistically different between the treatments ($P \geq 0.05$) along of the periods analyzed (31, 63 and 94 days of culture). However, trypsin and chymotrypsin activity (Figures 1B and 1C) were higher with 31 and 63 days of culture ($P < 0.05$) in fish from semi-intensive system, but no statistical difference between the treatments was observed for these enzymes at 94 days of cultivation. Specific aminopeptidase activities (Figure 2) were measured using the following substrates: Leu-, Arg-, Tyr-, Gly-, Ser-, Ala-, His-, Iso- and Pro- β-naphthylamide (Figure 2A-I, respectively). After 31 days of culture, most activities of aminopeptidases showed no statistical difference except for Pro- and Iso- that showed higher activity in fish of semi-intensive and intensive systems, respectively. After 63 days of cultivation Iso- showed no statistic difference between treatments, the other enzymes showed higher values in intensive system. At 94 days of culture Leu-, Arg-, Tyr-, Gly-, Ser- and Pro- aminopeptidases remained higher in the intensive system, while Ala-, His-, and Iso- showed no statistical difference between the systems. There were no significant differences in amylase (Figure 3) and lipase activity (Figure 4) in the crude extract of the intestine of Nile tilapia cultured in intensive and semi-intensive systems. A correlation was observed between the recommended concentration of essential amino acids for Nile tilapia (Fagbenro, 2000; Celik, 2012) and specific aminopeptidases activity in fish intensive system, using aminoacyl-β-naphthylamide as substrates (Figure 5).

Proteolytic activities in fish from the intensive and semi-intensive system were detected in substrate (casein) gel electrophoresis (Figure 6A). Intensive and semi-intensive system specimens exhibited twelve intense activity bands (P1 to P12) with 31, 63 and 94 days of culture. Evaluating the integrated density (*ID*) of the caseinolytic bands of proteases during the cultivations, the semi-

intensive system showed constant values for the times analyzed, while in intensive system the value decreased at 63 days, increasing again at 94 days of cultivation (Figure 6B). The Figure 6C can be seen the intensity of pixels values of the bands along the lanes of zymogram of farmed fish in the intensive (subfigure 1, 2 and 3) and semi-intensive (subfigure 4, 5 and 6) system. Images can confirm, with increasing intensity of the pixels values, the twelve bands observed in Figure 6A. In their respective subfigures are integrated density values (*ID*) of the bands in cultivation systems at the time periods analyzed (corresponding lanes of Figure 6A). Statistical differences were observed between the cultures. Fish from the semi-intensive system showed the highest values of *ID* at the three times (31, 63 and 94 days of cultivation) corresponding to 228.9±6.96, 229.27±7.12 and 231.10±4.93 respectively. The lowest value of *ID* was observed for fish from the intensive system with 63 days of cultivation (183.17±1.72).

Table 5 shows *ID* related to the caseinolytic bands from intestine of Nile tilapia cultured in the intensive and semi-intensive systems. The bands followed the profile found by Santos et al. (*in press*). Therefore, the previously results were employed to identify the caseinolytic bands found in this study. The bands with trypsin / aminopeptidase activity (P3, P4, P6, P7 and P8) showed the following results: P3 showed the highest *ID* values in semi-intensive system at 63 and 94 culture days (19.84±0.57 and 19.37±0.43, respectively). However, no statistically significant difference was observed between *ID* values in the semi-intensive with 31 days and in the intensive with 31 and 94 days of culture. P4 also showed a higher value in the semi-intensive system with 63 days of culture (16.51±0.51). Nevertheless, this value did not statistically differ with 94 days of culture (15.29±0.60). P6 and P7 bands presented a higher *ID* in semi-intensive system in the three times studied. P8 showed a higher value in this system at 94 days of culture (13.80±0.20), but this value did not differ statistically from the times 31 and 63 days on this same system, and was also equal to the intensive system with 31 days of culture. The P5 band (aminopeptidase activity) showed higher values in fish from semi-intensive system at 94 days of culture (28.36±1.40), but this value did not

statistically differ from 63 days of culture (26.91±1.12). P9 showed higher aminopeptidase activity in intensive system with 94 days of culture (11.23±0.21), but was not statistically different from the semi-intensive system in the three times studied. The P11 band with trypsin/chymotrypsin activity presented a higher value in fish from the semi-intensive system with 31 days of culture (17.03±0.89). The bands P1 and P10 demonstrated higher values of *ID* with 31 days of culture in semi-intensive system (4.60 ± 0.37 and 18.32±0.78, respectively). The P2 band showed no statistical difference in most times analyzed. Finally, the band P12 showed a higher value of *ID* at 63 days of culture in semi-intensive system (12.80 ± 0.74), but showed no significant difference compared to days 31 and 94 and neither differed from intensive system at 94 days.

Amylase activities in fish from the intensive and semi-intensive systems were detected in substrate (starch) gel electrophoresis (Figure 7A). Intensive and semi-intensive system specimens exhibited five activity bands (A1 to A5) related to 31, 63 and 94 days of culture. The integrated density (*ID*) of the amylase bands during the cultivations (Figure 7B), showed a similar trend at the times analyzed. At 31 days *ID* values were higher in both systems and decreased at 63 and 96 days of cultivation. In the Figure 7C can be seen the intensity of pixels of the bands along the lanes of zymogram of farmed fish in the intensive (subfigure 1, 2 and 3) and semi-intensive (subfigure 4, 5 and 6) system. The images also confirm the five bands observed in Figure 7A. Integrated density values (*ID*) in their respective subfigures showed a statistical difference between the systems. Fish from intensive and semi-intensive systems showed the highest values of *ID* with 31 days of cultivation (95.95±8.63 and 88.24±12.65 respectively). The lowest values of *ID* were observed with fish from semi-intensive system with 63 and 94 days of cultivation (60.71±6.57 and 58.49±5.99 respectively).

Table 6 shows *ID* values related to the bands of amylase zymogram from intestine of Nile tilapia cultured in the intensive and semi-intensive systems. Amylase 1 (A1) showed the highest values for intensive and semi-intensive system with 31 days of culture (20.15±1.40 and 18.92±2.76,

respectively). The highest value for amylase 2 (A2) was observed for the intensive system with 31 days (22.05 ± 0.62), but did not differ statistically from the value of 94 days of cultivation for this system and neither from semi-intensive with 31 days. Amylase 3 (A3) showed the highest values for intensive system with 31 days (20.40±1.86). However, no statistical difference was observed with 63 and 94 days of cultivation and also for the semi-intensive system with 31 days. The highest integrated density for Amylase 4 (A4) was also observed for fish in intensive system (6.12±0.42) with 31 days, being statistically equal to the values obtained with 63 and 94 days of culture. In the evaluation of Amylase 5 (A5), the intensive system showed the highest values for 31 and 63 days of cultivation (3.70±0.52 and 3.71±0.45, respectively).

The two-dimensional electrophoresis (Figures 8 and 9) showed well-defined spots without trawling (horizontal and vertical) that could prevent detection. The proteins are distributed throughout the pH range of 3-10 and the molecular weights between 10 and 100 kDa.

The comparison of the proteomic profile of the intestine of animals cultured in intensive (Figure 8) and semi-intensive system (Figure 9) showed a total of 1,233 spots, 731 being different.

## 4. Discussion

Liti et al. (2005) showed that an inefficient management can promote reduction in weight gain in tilapia. However, animals can demonstrate some physiological compensation, thus the quality of food and adopted management may contribute for this kind of adjustment. Despite of the similarity observed between the final weight of fish cultured in both systems, the food and protein efficiency were better for animals cultured on semi-intensive system. Although it was observed a decrease in condition of fish from intensive system, experimental specimens reached similar final weight, as a way of adapting to the culture system employed (Santos, 2013). The digestive physiology of Nile tilapia may have contributed to this fact. Muhlia-Almazan et al. (2003) describe that changes in diet

of aquatic organisms can cause alterations in the expression and activity of digestive enzymes. Consequently, changes in management may induce animals physiological adaptations to maintain or achieve satisfactory levels of specimens nutritional status.

The Nile tilapia is considered an opportunistic omnivore species, able to assimilate nutrients from different sources such as benthic algae, phytoplankton, macrophytes, zooplankton, small invertebrates, detritus and cyanobacteria. The choice of feeding is primarily related to food availability in the environment, indicating that this species has high trophic plasticity (Bowen, 1982; Philippart and Ruwet, 1982; Fitzsimmons, 1997; Beveridge and Baird, 2000; Lowe-Mcconnell, 2000; Sampaio and Goulart, 2011). Therefore, the enzymatic arsenal of tilapia should be sufficiently diversified to digest the wide range of food sources.

This fact was taken into account to evaluate the digestive enzymes of Nile tilapia under different cultures. This study demonstrated a great diversity in the gut enzymes of Nile tilapia with the presence of trypsin, chymotrypsin, aminopeptidase, amylase and lipase. All substrates generated typical products of their respective reactions, however differences were detected in enzyme activity in the cultures analyzed. Total proteolytic activity remained constant as seen in Figure 1A. The different managements adopted were insufficient to cause detectable changes using azocasein as substrate. Buarque et al. (2009a), comparing total proteolytic activity between two stages of subadult shrimp (*Farfantepenaeus subtilis*), also found no statistical difference using the same substrate. Nevertheless, changes could be seen with the use of specific substrates.

Trypsin and chymotrypsin showed higher values in fish from semi-intensive system when compared to intensive system at the initial time (31 and 63 days of culture) even with a decrease in concentration of crude protein in the feed of semi-intensive system. In fact, this system is characterized by high natural food, especially at the beginning of cultivation (El Sayed, 2006). According to Liti et al. (2006) when tilapia feeding is supplemented with low protein concentrations in semi-intensive farming, the deficit is supplied by natural food. Luz and Zaniboni-Filho (2001)

447 emphasize that natural food has high nutritional value, and according to Kuz'mina and Golovanova
448 (2004) one of the major advantages of this diet is the presence of a high concentration of digestive
449 enzymes. França et al. (2010) also cite the importance of enzymes in the digestive processes of fish,
450 suggesting its presence and functional performance in the live food. Lauff and Hofer (1984) found
451 higher trypsin activity in *Coregonus sp*. larvae fed with *Monia sp*. Abolfathi et al. (2012) also
452 mention that the large proteolytic activity in omnivorous fish may be related to the need to digest
453 plant foods.

454 At 94 days of culture there was no statistical difference between the systems (activity of
455 trypsin and chymotrypsin), it is worth highlighting that the fish were in the intensive system with a
456 higher percentage of CP in the diet compared to semi-intensive and this fact may have contributed
457 to the increased enzyme activity in this system concomitantly with a possible decrease in the
458 concentrations of natural food in the semi-intensive system. Protein levels above 25% in the diet
459 induced proteolytic activity in *Labeo rohita* fingerlings (Debnath et al., 2007). Perez-Jimenez et al.
460 (2009) indicate that, independent of eating habits, the response of the digestive system of the fish
461 closely correlates with diet.

462 However, it should be noted that the activity of chymotrypsin showed an increase at 94 days of
463 cultivation for both systems, despite lower concentrations of CP in the diet and natural food
464 decrease. Some authors indicate trypsin, trypsin/chymotrypsin or chymotrypsin as nutritional
465 indicators in fish, commenting that its increase may occur to compensate for dietary deficiencies
466 (Cara et al. 2007). Papoutsoglou and Lyndon (2006) also observed an increase in the activities of
467 trypsin and chymotrypsin when there was a decrease in the concentration of protein in the diet for
468 *Anarhichas minor*. Corroborating this fact, Kumar et al. (2007) suggest that herbivorous fish can
469 compensate a low protein diet increasing its enzymatic activity. The authors mentioned that some
470 herbivorous fish have similar or higher trypsin activities when compared to carnivorous species,
471 possibly to maximize the efficiency of protein digestion. This can be extended for omnivorous fish

like tilapia, mainly because this species is characterized as opportunistic omnivorous (Bowen, 1982; Ruwet and Philippart, 1982; Fitzsimmons, 1997; Beveridge and Baird, 2000; Lowe-Mcconnell, 2000; Goulart and Sampaio, 2011).

According to Wilson (2002) the fish have no nutritional requirement of protein *per se*, but need minimum quantities and proportions of essential and non-essential amino acids in the diet for the deposition of muscle protein and other body proteins. After hydrolysis of proteins by proteases into long chain peptides, aminopeptidases further degrade into smaller peptides and free amino acids, increasing the absorption of these nutrients (Buarque et al. 2009b). According to Zambonino-Infante and Cahu (2007), activity of peptidases facilitates assimilation of amino acids in marine fish larvae. Thus, understanding the regulation of aminopeptidases becomes quite important from the nutritional and physiological point of view. Intestinal aminopeptidases of Nile Tilapia were capable of hydrolyzing substrates leucine, arginine, tyrosine, glycine, serine, alanine, histidine, isoleucine and proline β-naphthylamide. However, the activities of these enzymes differed between farming systems, not showing a logical relationship, except for His-, Iso-, Leu-, Arg- aminopetidase that showed a significant correlation with the requirement of amino acids, based on the ideal protein concept for Nile tilapia (Fagbenro, 2000; Celik, 2012), suggesting that the activity of these enzymes has a relationship with essential amino acid requirement for this species. From physiological and productive perspectives this fact is very important for aquaculture, as it may provide subsidies for works regarding digestibility and nutritional requirements for other species. Buarque et al. (2009a; 2009b) also found a correlation between the aminopeptidases of *Farfantepenaeus subtilis* and *F. paulensis* and their respective amino acid requirements.

Most aminopeptidases analyzed showed no difference between systems in beginning of the cultivation, except Pro- and Iso- aminopeptidase. However, with 63 and 94 days of culture the majority of these enzymes had higher activity in fish intensive system. In intensive system was not present abundant natural food (less exogenous enzymes), this fact may have contributed to different

497 regulation of these enzymes in this system and the nutrients ingested by the fish need to be better
498 utilized since the contribution of natural food is minimal. In order to assess the function of digestive
499 enzymes in *Salmo salar*, Refstie et al. (2006) fed the animals with different concentrations of
500 protein made from yellow or narrow leafed lupins and verified no statistical difference in Leu-
501 aminopeptidase activity. Moreover, Ezquerra et al. (1999) observed an increase in Met-, Val-, Pro-,
502 Lys- and Leu- aminopeptidase activity in white shrimp fed with menhaden fish meal although this
503 was not in accordance with the nutritional standards required for this species. The authors also
504 mention that when the shrimps were fed with soybean meal, the activities of these enzymes
505 decreased mainly Gly- and Met- aminopeptidase. Methionine is usually the first limiting amino acid
506 in diets based on soybean protein to fish (Furuya et al. 2001).

507 Several animal and vegetable foods can be used in feed for aquatic organisms, partially or
508 totally replacing fishmeal traditionally used in aquaculture feed. Thus, several studies have been
509 conducted to evaluate new protein sources that could be used in the manufacture of feed (Jackson et
510 al., 1982; Gaber, 1996; Olvera-Novoa et al., 1997; Olvera-Novoa et al., 2002 ; Jatomea-Plascencia
511 et al., 2002, El-Saidy and Gaber, 2003; Gaber, 2006, Leal et al., 2009). Thus, in diets for Nile
512 tilapia, several sources can be used in the manufacture of feed, due to its ability to digest proteins
513 from plant and animal origin, therefore contributing to changes in enzymatic profile.

514 In order to observe the gut proteases of Nile tilapia under different culture conditions, more
515 sensitive tools were used to detect possible changes in the activities. According to Santos et al. (*in*
516 *press*) and Lemos et al. (2000) zymogram can be considered a more sensitive technique than
517 conventional quantitative analyzes. In fact, this technique allowed a good view of the behavior of
518 proteases from Nile tilapia, especially when the bands were analyzed using the intensity of pixel
519 values (integrated density - *ID*). Corroborating the data observed in quantitative analysis, the values
520 of *ID* observed for Nile tilapia were higher at all times in semi-intensive system. Making a
521 comparison of these results with the condition factor, both remained constant for these specimens.

522 The fish of intensive system showed the lowest values of *ID* and also had a decrease in the
523 condition with 31 days of cultivation. This suggests that the adopted management can interfere with
524 the condition/enzymatic activity of the experimental specimens. However, Nile tilapia through his
525 great capacity of adaptation, overcome the changes in management as evidenced by results of
526 growth. But it is noteworthy the importance of natural food in semi-intensive cultures, since
527 zooplankton can assist in the digestion process through their digestive enzymes, either by autolysis
528 or as zymogens that activate endogenous digestive enzymes (Kolkovsk, 2001). Furthermore, in
529 terms of cost/benefit, the use of natural foods in farm ponds is one of the most efficient ways to
530 reduce costs and environmental impacts of aquaculture (Beyruth et al., 2004). Its continuous
531 availability allows the fish to adjust the intake and nutritional supply to their metabolic needs. The
532 increase in feed frequency implies in more regular nutrient supply, increasing the efficiency of
533 digestion and metabolism of nutrients (Beyruth et al., 2004).

534 Santos et al. (*in press*) identified eight out of twelve proteases found in the intestine of Nile
535 tilapia. Based on this information, was assessed the integrated density of bands in intensive and
536 semi-intensive farming systems. Several bands showed similarity between the systems,
537 demonstrating the sensitivity of the method. Most enzymes from fish of semi-intensive system
538 presented higher *ID*, including enzymes with aminopeptidase activity (not shown in the quantitative
539 analyzes, excepting Pro- aminopeptidase activity with 31 days of cultivation). However, some
540 identifications cited in Santos et al. (*in press*) were not conclusive, therefore the authors suggest that
541 the Nile tilapia enzymes may have low compatibility with mammalian protease inhibitors.

542 In quantitative terms, amylase and lipase showed no statistical difference between the systems
543 (Figures 3 and 4, respectively). Bowyer et al. (2012) report that the lipase activity of *Seriola lalandi*
544 was statistically lower in fish fed with canola oil.

545 Through the amylase zymograms some differences in enzyme activity could be detected.
546 Figure 7B shows a similar trend of activity for the two culture systems, where the values of *ID*

decreased with the course of time. The intensive system showed higher values of integrated density, though these values showed no statistical difference when compared to the mean of semi-intensive system with 31 days (Figure 7C). Evaluating the bands separately, most showed higher values of *ID* in intensive system (Table 6), excepting amylases 1, 2 and 3 of the semi-intensive system with 31 days of cultivation.

According to Le Moullac and VanWormhoudt (1994) amylase activity increases with the increase of carbohydrates in the diet. Kuz'mina et al. (1996) also found a correlation between amylase activity and concentration of carbohydrates in the diet for freshwater fish. This leads to a very important point: the carbohydrates can be digested as they may have a positive relationship with amylases. This may reflect the satiation of animals harming their growth. In fact the condition of the animals in intensive system was minor than that of the semi-intensive with 31 days of culture, suggesting a better energy: protein ratio for the semi-intensive system at this time. However, according to Ceccaldi (1997) when optimal levels of dietary carbohydrates are overcome, the enzyme activity tends to decrease. Nakamura (1970) and Reed et al. (2010) also reported the inhibition of amylases by their own substrates. Sands and Lukes (1974) also mention that in some microorganisms, the carbohydrate may even prevent expression of the enzyme. Thus, increases in the carbohydrate diet may have caused a regulation/inhibition of enzyme activity. In addition, the fact of amylolytic activities were slightly higher in the intensive system can be related to results obtained by Ribeiro et al. (2002). These authors evaluated the enzymatic activities of *Solea senegalensis* fed with live food and diet observed that amylase secretion was significantly higher in the treatment with diet. In fact, the use of natural food for fish farmed in cages is more difficult than in cultured fish in ponds due to density, fish movement, etc. Corroborating the results, Fountoulaki et al. (2005) showed that differences in enzyme profiles are related to the nutrients present in feed.

These differences could also be confirmed by the analysis of proteomic profile of culture systems, demonstrating how the environment/diet can promote changes in protein profile.

Moreover, in semi-intensive system there is a greater occurrence of microorganisms in the water. Such microorganisms besides serving as food for fish, can also form symbiotic relationships with them. Therefore, it is possible the existence of proteins belonging to these microorganisms in the digestive tract, which could not be present in fish intensive system. Lucitt (2008) compared proteomic profiles of two different embryonic developmental stages of zebrafish using two-dimensional electrophoresis and found more than 660 proteins, being 477 different. The level of changes that may occur in the proteomic profile of a species is very variable. Gómez-Requeni (2011) subjected the zebrafish feeding unbalanced in only one amino acid (lysine) and the proteomic profile of the body showed 207 proteins in total, being 45 significantly different.

Following the advances of technology in the areas of genomics and proteomics, the study of the fish physiology has increased, making possible to analyze the effects of stress on these animals (Parrington et al., 2002). Thus, not only the food, but also the environment in which animals are exposed may cause quantitative and qualitative changes in global protein expression of a species.

## 5. Conclusion

The complexity of digestive functions allows the animal to modify the expression/activity of the enzymes to achieve more efficiently the reaction products necessary for their metabolism. This fact could be observed in both production systems through adaptations made for Nile tilapia. Thus, the expression of these proteins appears to have a performance according to environmental and feeding conditions, so that the digestive process can be as efficient as possible in accordance with the conditions of cultured animals.

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## 7. Tables

Table 1: Proximate analysis of the commercial diets used in the experiment.

| Proximate analysis (as-fed basis, g . $kg^{-1}$)* | Crude Protein | | |
|---|---|---|---|
| | 35% | 32% | 28% |
| Pellet diameter | 3 - 4 | 4 - 6 | 6 - 8 |
| Moisture | 130 | 130 | 130 |
| Crude protein | 350 | 320 | 280 |
| Ether extract | 65 | 50 | 50 |
| Crude fiber | 50 | 50 | 50 |
| Ash | 120 | 120 | 120 |
| Calcium | 15 | 15 | 15 |
| Phosphorus | 6 | 6 | 6 |
| Vitamin C | 0.5 | 0.5 | 0.5 |

*Data from manufacturer

Table 2: Nutritional plan used as feed for Nile tilapia (*O. niloticus*) subjected to different culture systems.

| Time (days) | Nutrition plan | |
|---|---|---|
| | Intensive culture | Semi-intensive culture |
| 0 – 31 | 35% CP | 35% CP |
| 32 – 63 | 35% CP | 32% CP |
| 64 – 94 | 32% CP | 28% CP |

879 Table 3: Growth performance and nutrient utilization of Nile tilapia (*O. niloticus*) cultivated in
880 intensive and semi-intensive system.

881

| Parameters | Intensive culture | Semi-intensive culture |
|---|---|---|
| Initial weight (g) | 88.33 ± 1.54[a] | 86.90 ± 1.50[a] |
| Final weight (g) | 543.58 ± 48.11[a] | 501.74 ± 4.37[a] |
| WG (g)[1] | 455.24 ± 41.68[a] | 414.84 ± 3.48[a] |
| Survival (%) | 75.0[b] | 85.0[a] |
| FCR[2] | 1.59 ± 0.12[a] | 1.57 ± 0.11[a] |
| PER[3] | 4.23 ± 0.37[b] | 6.51 ± 0.30[a] |
| FE[4] | 1.6±0.02[b] | 2.30±0.11[a] |

882 Values are mean ± SE. Within a row, means with different superscript letters differ significantly (P
883 < 0.05) by Wilcoxon-Mann-Whitney test and Student's *t* test

884 [1] Weight gain, [2] Feed conversion ratio, [3] Protein efficiency ratio, [4] Feed efficiency.
885 Data described in Santos (2013).

886

887 Table 4: Fulton's condition factor of Nile tilapia cultivated in intensive and semi-intensive systems
888 at times "0" (zero), 31, 63 and 94 days.

889

| Time (days) | Intensive culture | Semi-intensive culture |
|---|---|---|
| 0 | 1.97±0.06[a] | 2.00±0.01[a] |
| 31 | 1.95±0.02[b] | 2.17±0.07[a] |
| 63 | 2.03±0.06[a] | 2.11±0.04[a] |
| 94 | 2.10±0.08[a] | 2.08±0.01[a] |

890 Values are mean ± SE. Within a row, means with different superscript letters differ significantly (P
891 < 0.05) by Student's *t* test.

892 Data described in Santos (2013).

893

894

Table 5: Integrated density (*ID*) of the bands of protease zymograms from the intestine of Nile tilapia (*O. niloticus*) cultured in intensive and semi-intensive systems.

| Bands | Intensive culture | | | Semi-intensive culture | | | Enzyme* |
|---|---|---|---|---|---|---|---|
| | 31 days | 63 days | 94 days | 31 days | 63 days | 94 days | |
| P1 | 2.98±0.24$^{b}$ | 3.48±0.56$^{c}$ | 4.29±0.48$^{b}$ | 4.60±0.37$^{a}$ | 4.39±0.25$^{b}$ | 4.67±0.39$^{b}$ | Not identified$_{1}$ $^{**}$ |
| P2 | 15.71±0.39$^{ab}$ | 15.88±0.71$^{ab}$ | 15.15±0.19$^{b}$ | 15.70±0.36$^{ab}$ | 16.24±0.35$^{ab}$ | 16.34±0.38$^{a}$ | Not identified$_{2}$ $^{**}$ |
| P3 | 18.57±0.15$^{ab}$ | 17.21±0.36$^{b}$ | 18.71±1.24$^{ab}$ | 18.89±0.73$^{ab}$ | 19.84±0.57$^{a}$ | 19.37±0.43$^{a}$ | Trypsin/Aminopeptidase |
| P4 | 13.77±0.58$^{bc}$ | 12.27±0.60$^{c}$ | 14.87±0.32$^{b}$ | 13.26±0.76$^{c}$ | 16.51±0.51$^{a}$ | 15.29±0.60$^{ab}$ | Trypsin/Aminopeptidase |
| P5 | 23.43±0.63$^{c}$ | 22.68±0.44$^{c}$ | 24.19±0.89$^{c}$ | 24.91±1.06$^{bc}$ | 26.91±1.12$^{ab}$ | 28.36±1.40$^{a}$ | Aminopeptidase |
| P6 | 7.90±0.09$^{b}$ | 6.87±0.20$^{c}$ | 8.34±0.19$^{b}$ | 9.26±0.50$^{a}$ | 9.29±0.11$^{a}$ | 9.81±0.51$^{a}$ | Trypsin/Aminopeptidase |
| P7 | 18.81±0.35$^{c}$ | 17.96±0.44$^{d}$ | 21.04±1.21$^{b}$ | 21.70±0.73$^{a}$ | 21.72±0.74$^{a}$ | 21.77±0.51$^{a}$ | Trypsin/Aminopeptidase |
| P8 | 13.11±0.08$^{ab}$ | 11.04±0.22$^{c}$ | 12.17±0.42$^{bc}$ | 12.95±0.81$^{ab}$ | 13.01±0.96$^{ab}$ | 13.80±0.20$^{a}$ | Trypsin/Aminopeptidase |
| P9 | 9.82±0.40$^{bc}$ | 9.42±0.22$^{c}$ | 11.23±0.21$^{a}$ | 10.88±0.67$^{ab}$ | 10.79±0.59$^{ab}$ | 10.68±0.31$^{ab}$ | Aminopeptidase |
| P10 | 15.93±0.31$^{c}$ | 9.66±0.74$^{e}$ | 14.42±0.56$^{d}$ | 18.32±0.78$^{a}$ | 17.34±1.03$^{b}$ | 17.10±0.84$^{b}$ | Not identified$_{10}$ $^{**}$ |
| P11 | 16.12±0.37$^{b}$ | 11.49±0.24$^{f}$ | 14.62±0.48$^{e}$ | 17.03±0.89$^{a}$ | 15.58±0.63$^{d}$ | 15.92±0.33$^{c}$ | Trypsin/Chymotrypsin |
| P12 | 9.51±1.20$^{bc}$ | 8.53±0.22$^{c}$ | 10.40±0.50$^{abc}$ | 11.19±0.52$^{ab}$ | 12.80±0.74$^{a}$ | 11.48±1.73$^{ab}$ | Not identified$_{12}$ $^{**}$ |

Values are mean ± SE of Integrated density mean. Within a row, means with different superscript letters differ significantly ($P < 0.05$) by Kruskal-Wallis test or One-way analysis of variance (ANOVA) followed Tukey test.

*Santos et al. (*in press*).

$^{**}$Numbering defined by the authors.

Table 6: Integrated density (*ID*) of the bands of amylase zymograms from the intestine of Nile tilapia (*O. niloticus*) cultured in intensive and semi-intensive systems.

| Bands | Intensive system | | | Semi-intensive system | | | Enzyme* |
|---|---|---|---|---|---|---|---|
| | 31 days | 63 days | 94 days | 31 days | 63 days | 94 days | |
| A1 | 20.15±1.40$^{a}$ | 7.88±2.02$^{b}$ | 12.98±1.7$^{b}$ | 18.92±2.76$^{a}$ | 11.59±1.73$^{b}$ | 9.61±2.14$^{b}$ | Amylase 1 |
| A2 | 22.05±0.62$^{a}$ | 16.65±1.67$^{bc}$ | 17.77±1.64$^{abc}$ | 21.19±2.21$^{ab}$ | 15.35±1.90$^{c}$ | 15.29±1.55$^{c}$ | Amylase 2 |
| A3 | 20.40±1.86$^{a}$ | 18.45±2.13$^{ab}$ | 16.58±1.02$^{abc}$ | 18.37±3.20$^{ab}$ | 13.01±0.46$^{c}$ | 14.52±1.66$^{bc}$ | Amylase 3 |
| A4 | 6.12±0.42$^{a}$ | 5.55±1.02$^{ab}$ | 4.86±0.62$^{ab}$ | 4.17±0.79$^{bc}$ | 2.40±0,27$^{d}$ | 3.08±0.32$^{cd}$ | Amylase 4 |
| A5 | 3.70±0.52$^{a}$ | 3.71±0.45$^{a}$ | 2.47±0.46$^{b}$ | 2.66±0.35$^{b}$ | 2.59±0.36$^{b}$ | 1.93±0.09$^{b}$ | Amylase 5 |

Values are mean ± SE of Integrated density mean. Within a row, means with different superscript letters differ significantly ($P < 0.05$) by One-way analysis of variance (ANOVA) followed Tukey test.

*Nomenclature defined by the authors.

## 8. Figures

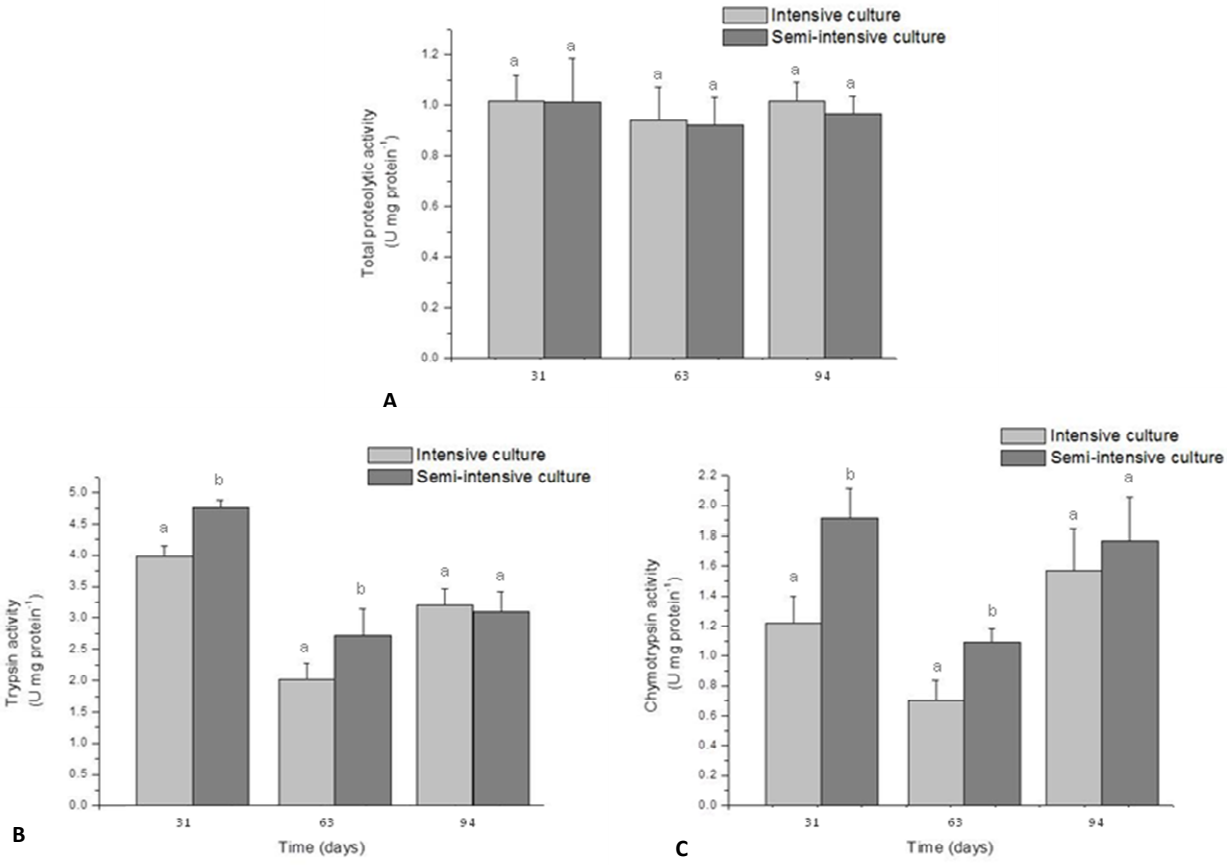
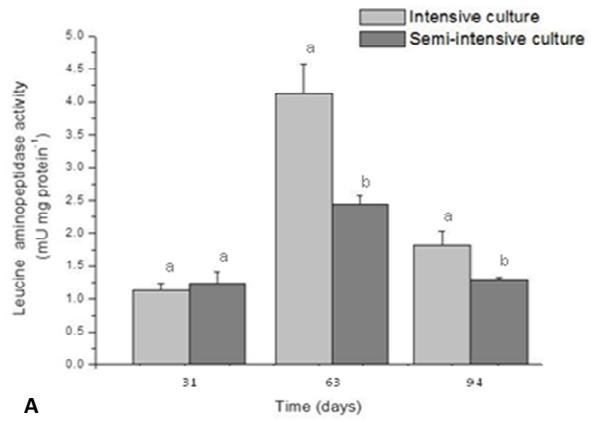
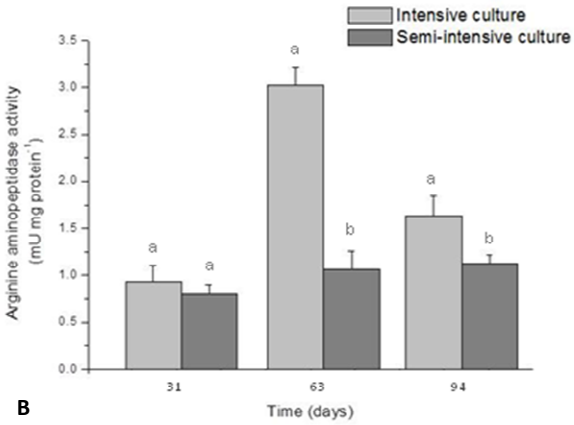


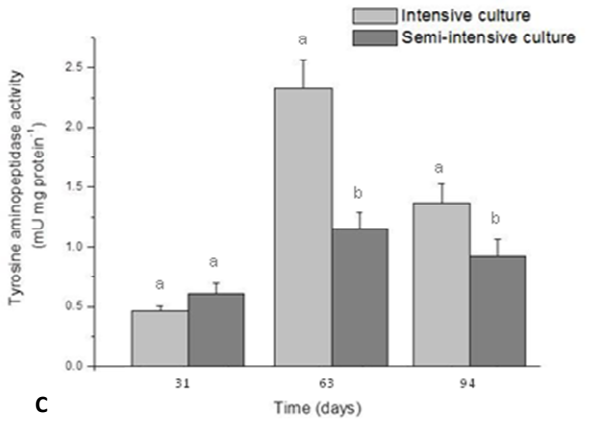

Figure 1 : Alkaline proteolytic activity of the crude extract from the intestine of Nile tilapia (*Oreochromis niloticus*) cultured in intensive and semi-intensive system. Values are mean ± SD, using azocasein (A), BApNA (*Nα*-benzoyl-$_{DL}$-arginine-*p*-nitroanilide) (B), and SApNA (Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide) (C) as substrate, expressed as U mgProtein$^{-1}$. For the same times, means with different superscript letters differ significantly ($P < 0.05$) by Student's t test ($n = 3$).


Intensive culture
Semi-intensive culture
Leucine aminopeptidase activity (mU mg protein$^{-1}$)
Time (days)
31
63
94
a
a
a
b
a
b

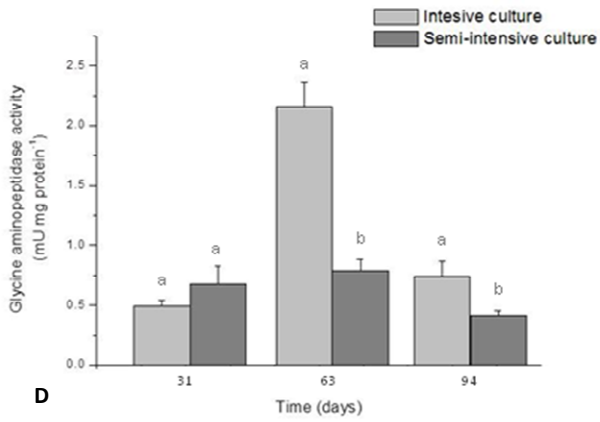
A


Intensive culture
Semi-intensive culture
Arginine aminopeptidase activity (mU mg protein$^{-1}$)
Time (days)
31
63
94
a
a
a
b
a
b

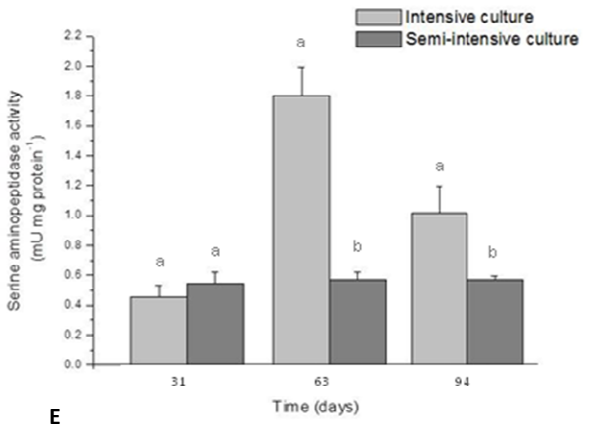
B


Intensive culture
Semi-intensive culture
Tyrosine aminopeptidase activity (mU mg protein$^{-1}$)
Time (days)
31
63
94
a
a
a
b
a
b

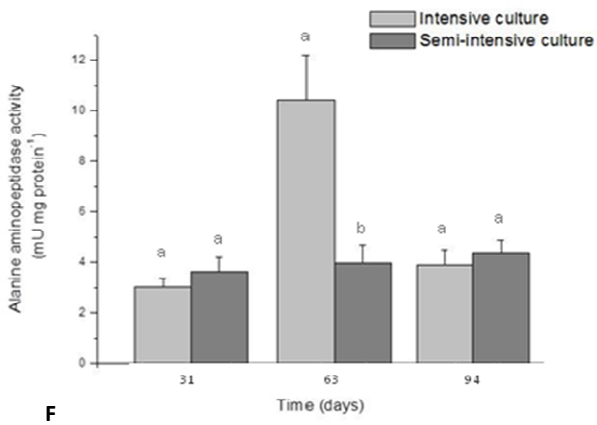
C


Intesive culture
Semi-intensive culture
Glycine aminopeptidase activity (mU mg protein$^{-1}$)
Time (days)
31
63
94
a
a
a
b
a
b

D


Intensive culture
Semi-intensive culture
Serine aminopeptidase activity (mU mg protein$^{-1}$)
Time (days)
31
63
94
a
a
a
b
a
b

E


Intensive culture
Semi-intensive culture
Alanine aminopeptidase activity (mU mg protein$^{-1}$)
Time (days)
31
63
94
a
a
a
b
a
a

F

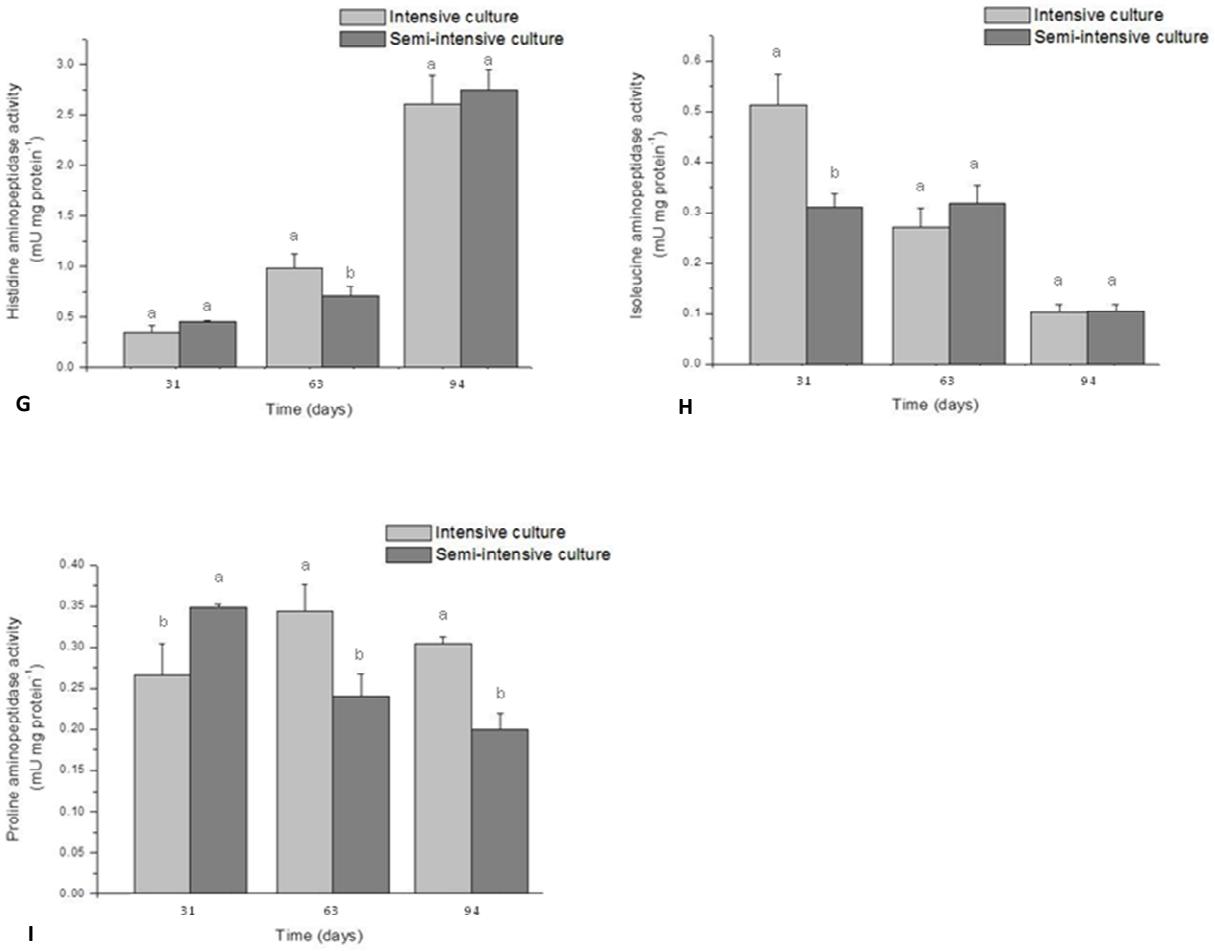



Figure 2: Aminopeptidase activity of the crude extract from the intestine of Nile tilapia (*Oreochromis niloticus*) cultured in intensive and semi-intensive system. Values are mean ± SD, using aminoacyl-β-naphthylamide as substrates with Leu (A), Arg (B), Tyr (C) Gly (D), Ser (E), Ala (F) His (G) Iso (H) and Pro (I) as specific substituents and expressed as mU mgProtein$^{-1}$. For the same times, means with different superscript letters differ significantly ($P < 0.05$) by Wilcoxon-Mann-Whitney test and Student's t test ($n = 3$).

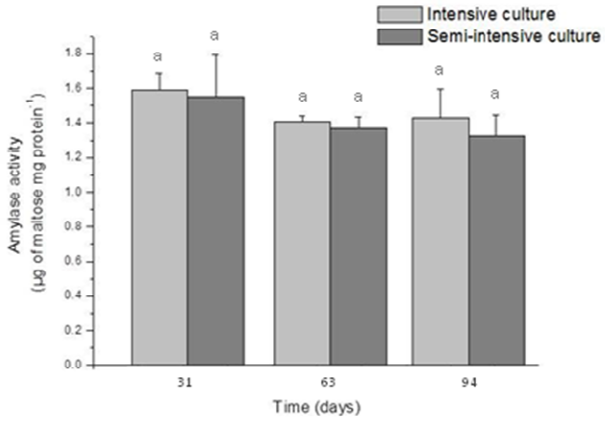



Figure 3: Amylase activity of the crude extract from the intestine of Nile tilapia (*Oreochromis niloticus*) cultured in intensive and semi-intensive system. Values are mean ± SD, using starch as substrate, expressed as µg of maltose.min$^{-1}$.mg Protein$^{-1}$. For the same times, means with different superscript letters differ significantly ($P < 0.05$) by Student's t test ($n = 3$).

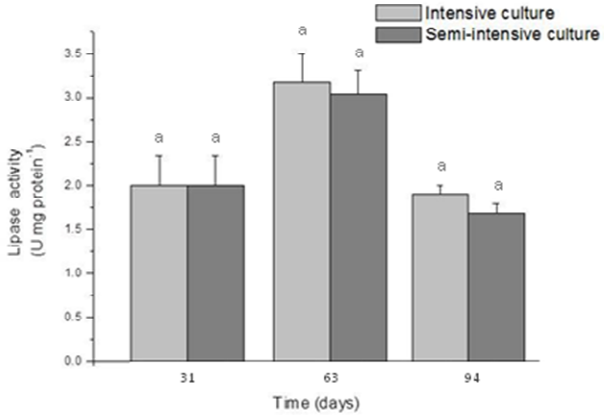



Figure 4: Lipase activity of the crude extract from the intestine of Nile tilapia (*Oreochromis niloticus*) cultured in intensive and semi-intensive system. Values are mean ± SD, using *p*-nitrophenyl palmitate (*p*-NPP), as substrate, expressed as U mgProtein$^{-1}$. For the same times, means with different superscript letters differ significantly ($P < 0.05$) by Wilcoxon-Mann-Whitney test ($n = 3$).

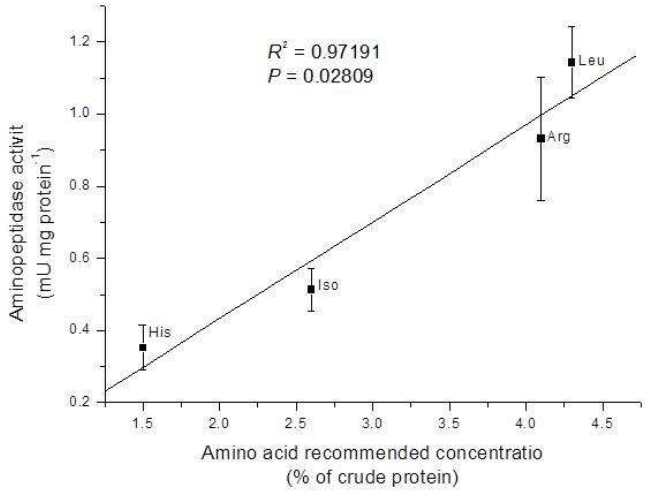



Figure 5: Correlation between aminopeptidase activity of Nile tilapia (*Oreochormis niloticus*) using aminoacyl-β-naphthylamide as substrates and recommended concentration of essential amino acids for Nile tilapia assessed by the Ideal Protein Concept. Values are shown as mean ± SD of three crude extracts obtained from intensive culture. Concentration of amino acids expressed in % of dietary protein: arginine (4.1), leucine (4.3), isoleucine (2.6) and histidine (1.5) *Celik (2012), based on Fagbenro (2000).

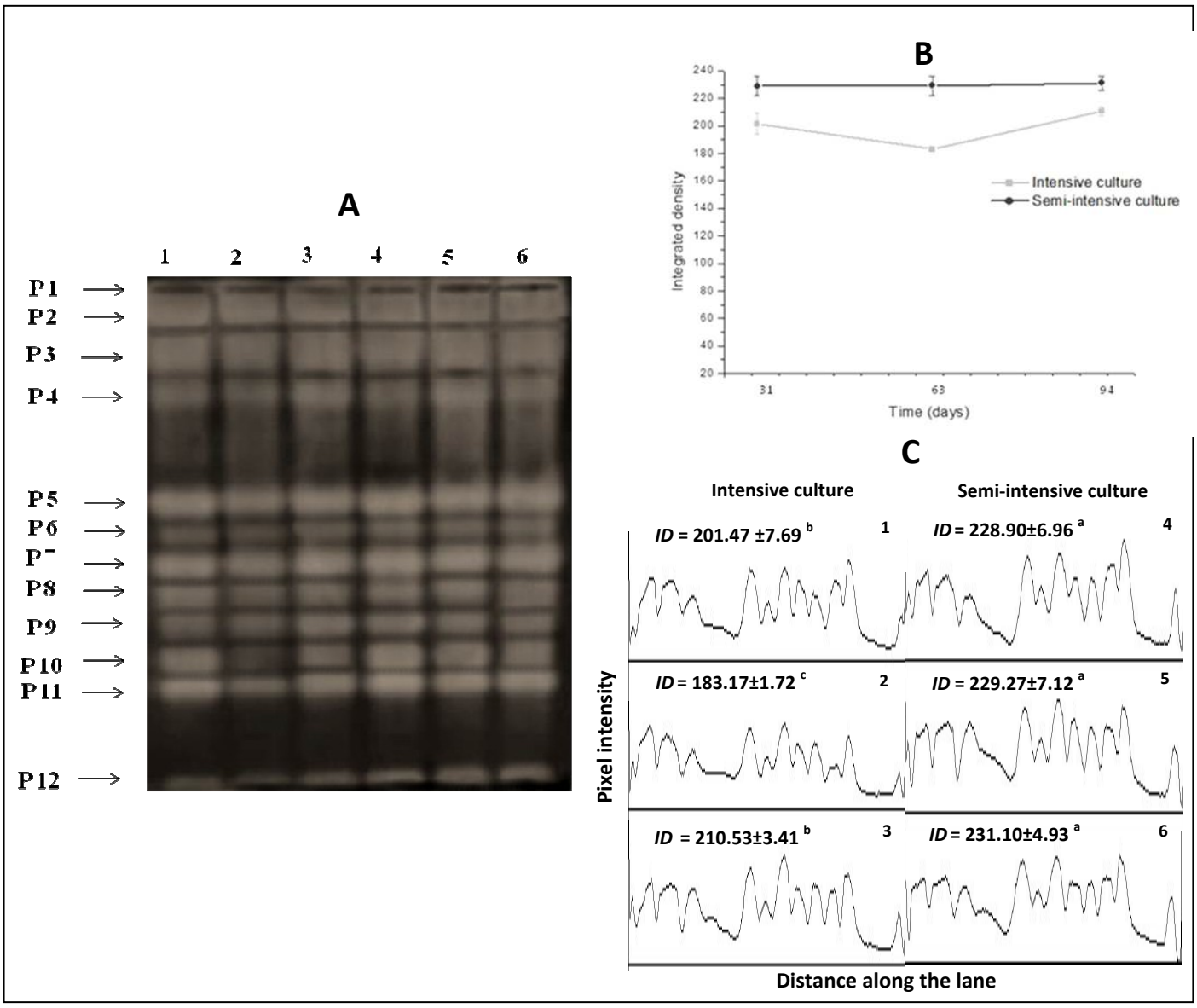



Figure 6: (A) Zymogram of digestive proteases of the crude extract from the intestine of Nile tilapia (*Oreochromis niloticus*) cultured in intensive and semi-intensive system, using casein as substrate. Lanes 1, 2 and 3 correspond to intensive culture in the times 31, 63 and 94 days, respectively; Lanes 4, 5 and 6 correspond to semi-intensive culture in the times 31, 63 and 94 days, respectively. (B) Evolution of the integrated density of the bands of proteases zymograms from the intestines of Nile tilapia cultivated in the intensive and semi-intensive system. (C) Pixel intensity and respective means values of integrated density (*ID*) for each lane of protease bands (lanes 1-6), subfigures 1, 2 and 3 correspond to the intensive system and 4, 5 and 6 semi-intensive system, means with different superscript letters differ significantly ($P < 0.05$) using One-way analysis of variance (ANOVA) followed by Tukey test ($n = 3$).

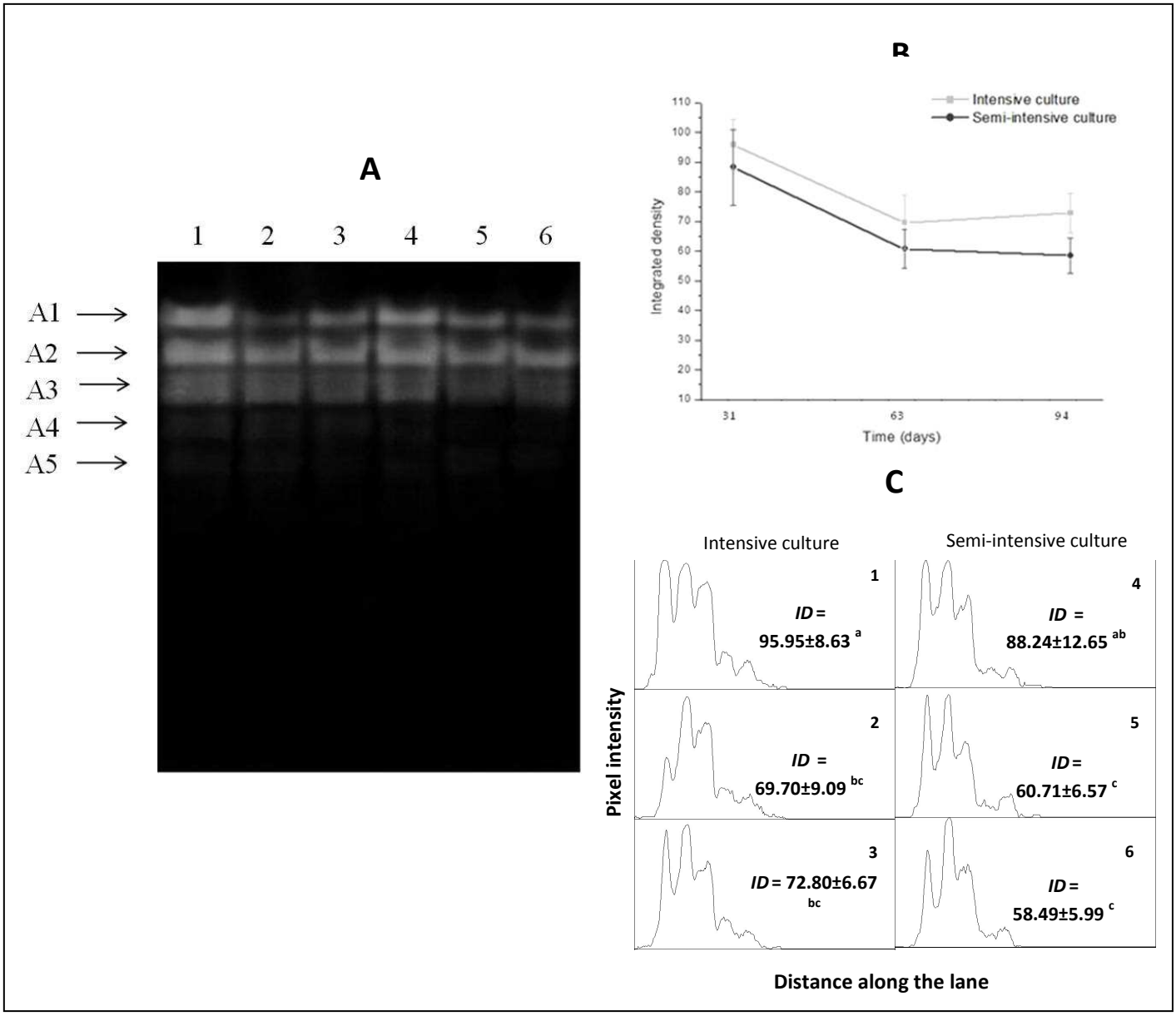



Figure 7: (A) Zymogram of amylase activity of the crude extract from the intestine of Nile tilapia (*Oreochromis niloticus*) cultured in intensive and semi-intensive system, using starch as substrate. Lanes 1, 2 and 3 correspond to intensive culture in the times 31, 63 and 94 days , respectively; Lanes 4, 5 and 6 correspond to semi-intensive culture in the times 31, 63 and 94 days, respectively. (B) Evolution of the integrated density of the bands of amylase zymograms from the intestines of Nile tilapia cultivated in the intensive and semi-intensive system. (C) Pixel intensity and respective means values of integrated density (*ID*) for each lane of amylase bands (lanes 1-6), subfigures 1, 2 and 3 correspond to the intensive system and 4, 5 and 6 semi-intensive system, means with different superscript letters differ significantly ($P < 0.05$) using One-way analysis of variance (ANOVA) followed by Tukey test (n=3).

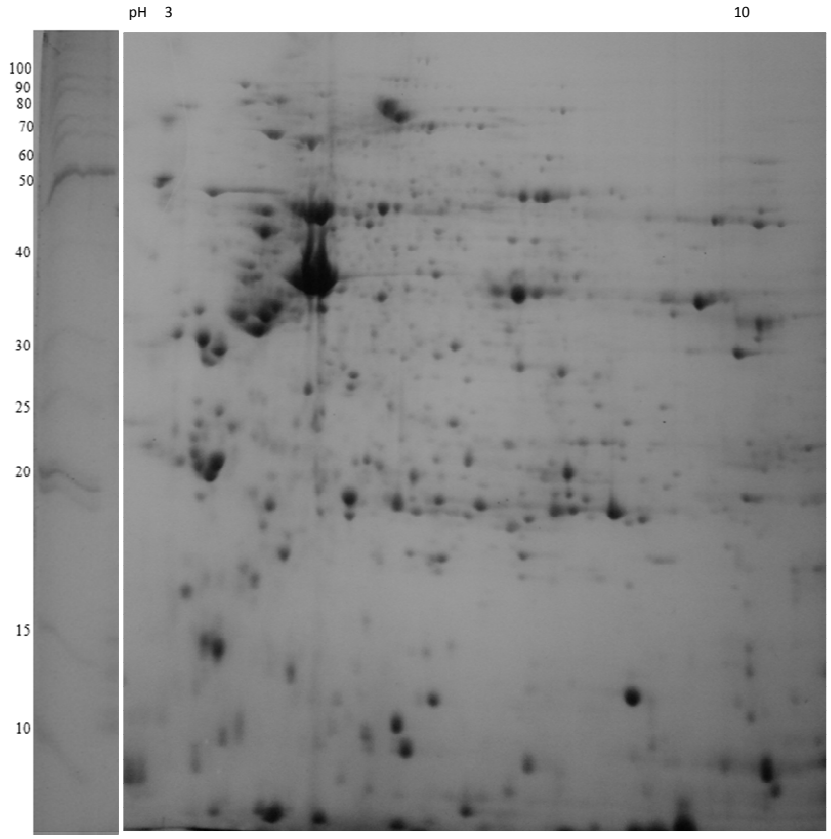



Figure 8: Two-dimensional gel electrophoresis profile of the crude extract from the intestine of Nile tilapia (*Oreochromis niloticus*) cultured in intensive system. First proteins were separated by charge in the first dimension (immobiline Drystrip pH 3–10) and then by molecular weight in the second dimension (12% SDS-PAGE).

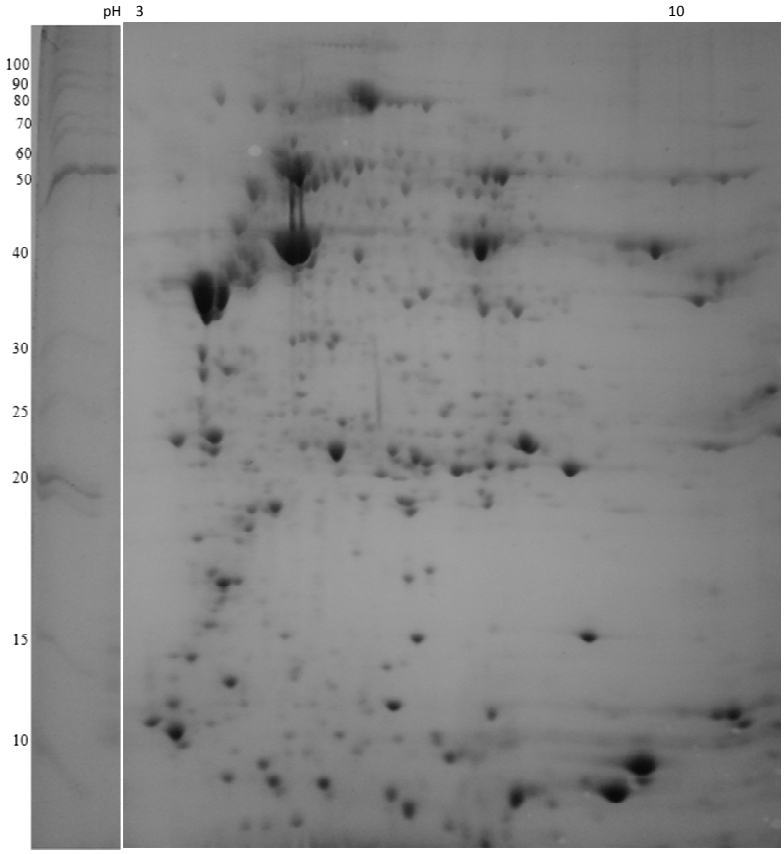



Figure 9: Two-dimensional gel electrophoresis profile of the crude extract from the intestine of Nile tilapia (*Oreochromis niloticus*) cultured in semi-intensive system. First proteins were separated by charge in the first dimension (immobiline Drystrip pH 3–10) and then by molecular weight in the second dimension (12% SDS-PAGE).

## 8. CONCLUSÃO

Avaliando o desempenho das tilápias do Nilo alimentadas com diferentes concentrações de hidrolisado proteico de camarão e cultivadas em sistemas semi-intensivo e intensivo, foram observadas as mesmas tendências de crescimento.

Contudo, avaliando os sistemas intensivo e semi-intensivo a partir de outros parâmetros (fator de condição e seus derivados) algumas mudanças puderam ser observadas. Os peixes do sistema semi-intensivo apresentaram melhores resultados no início do cultivo, contudo os espécimes experimentais demonstraram compensações que levaram a uma similaridade no crescimento na avaliação final dos sistemas. O monitoramento contínuo destes parâmetros aliados ao estudo do peso relativo nos leva a refletir sobre a qualidade dos espécimes de tilápia do Nilo cultivados atualmente, já que este peixe mostrou, em algumas situações, peso relativo inferior a espécimes do ambiente natural.

As funções digestivas permitiram que a tilápia pudesse modificar suas atividades enzimáticas de forma a obter mais eficientemente os produtos das reações necessárias para o seu metabolismo, demonstrando que este peixe pode se beneficiar de várias fontes alimentares, já que seu arsenal enzimático se apresentou diversificado/adaptado suficientemente para digerir a grande variedade de comida ingerida.

## 9. ANEXOS

9.1 Normas da Revista Aquaculture

**INTRODUCTION**

***Types of paper***

*Original Research Papers* should report the results of original research. The material should not have been previously published elsewhere. Articles are expected to contribute new information (e.g. novel methods of analysis with added new insights and impacts) to the knowledge base in the field, not just to confirm previously published work.

*Review Articles* can cover either narrow disciplinary subjects or broad issues requiring interdisciplinary discussion. They should provide objective critical evaluation of a defined subject. Reviews should not consist solely of a summary of published data. Evaluation of the quality of existing data, the status of knowledge, and the research required to advance knowledge of the subject are essential.

*Short Communications* are used to communicate results which represent a major breakthrough or startling new discovery and which should therefore be published quickly. They should not be used for preliminary results. Papers must contain sufficient data to establish that the research has achieved reliable and significant results.

*Technical Papers* should present new methods and procedures for either research methodology or culture-related techniques.

The *Letters to the Editor* section is intended to provide a forum for discussion of aquacultural science emanating from material published in the journal.

***Contact details for submission***

Papers for consideration should be submitted via the electronic submission system mentioned below to the appropriate Section Editor:

**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. 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. Improvement of production systems that results in greater efficiencies of resource usage in aquaculture. Effective applications of technologies and methods of aquaculture

production for improved stocking regimes, the use of new species and species assemblages, and research on the efficient and sustainable usage of system space with the objective of minimizing resource usage in aquaculture. Investigations to minimize aquaculture wastes and improve water quality, technologies for nutrient recycling in aquaculture ecosystems, and the synergy of aquaculture and other food production systems using methods such as polyculture and integrated aquaculture.

**Physiology and Endocrinology:**

E.M. Donaldson

The Physiology and Endocrinology Section welcomes high quality research papers presenting novel data as well as original reviews, on various aspect of the physiology and endocrinology of cultured aquatic animals and plants, providing that their content is relevant to solving aquaculture problems.

Manuscripts submitted to the journal 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. When a study involves the administration of a pharmaceutical or other commercial product, please use generic or scientific names rather than trade names especially in the Title. The trade name can be mentioned in the Materials and Methods together with an exact description of its composition.

Topics covered by this section include, but are not limited to, physiological and endocrine aspects of:

- Reproductive development including both endocrine and environmental control
- Induced ovulation and spermiation
- Gamete quality, storage and cryopreservation
- Control of sex differentiation
- Physiology and endocrinology of gynogenetic and triploid aquatic organisms
- Physiology and endocrinology of transgenic organisms
- Molecular genetic assessment of physiological processes
- Larval physiology and the ontogeny of endocrine systems
- Malpigmentation
- Metamorphosis, smolting (salmonids) and molting (crustacea)
- Nutritional physiology
- Osmoregulation
- Stress
- Anesthesia, transport, handling

- Physiology of harvest techniques, product quality, flesh quality and pigmentation
- Endocrine and environmental regulation of growth
- Rearing density
- Temperature tolerance
- Disease physiology
- Pollutant physiology and water quality (when directly relevant to aquaculture)
- Respiratory, muscle and exercise physiology of cultured organisms
- Immunology ( manuscripts on the physiological effects of probiotics must be of high scientific quality with statistically valid conclusions)

**Diseases:**

B. Austin

The Diseases Section welcomes high quality research papers presenting novel data as well as original reviews, on various aspect of the diseases of aquatic animals and plants, so long as their content is relevant to solving aquaculture problems.

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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

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**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.

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State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

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A Theory section should extend, not repeat, the background to the article already dealt with in the Introduction and lay the foundation for further work. In contrast, a Calculation section represents a practical development from a theoretical basis.

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Results should be clear and concise.

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• Number the illustrations according to their sequence in the text.

• Use a logical naming convention for your artwork files.

• Provide captions to illustrations separately.

• Produce images near to the desired size of the printed version.

• Submit each figure as a separate file.

A detailed guide on electronic artwork is available on our website:

http://www.elsevier.com/artworkinstructions

**You are urged to visit this site; some excerpts from the detailed information are given here.**

*Formats*

Regardless of the application used, when your electronic artwork is finalised, please 'save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):

EPS: Vector drawings. Embed the font or save the text as 'graphics'.

TIFF: Color or grayscale photographs (halftones): always use a minimum of 300 dpi.

TIFF: Bitmapped line drawings: use a minimum of 1000 dpi.

TIFF: Combinations bitmapped line/half-tone (color or grayscale): a minimum of 500 dpi is required.

If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is'.

**Please do not:**

• Supply files that are optimised for screen use (e.g., GIF, BMP, PICT, WPG); the resolution is too low;

• Supply files that are too low in resolution;

• Submit graphics that are disproportionately large for the content.

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Please make sure that artwork files are in an acceptable format (TIFF, EPS or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color on the Web (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version. **For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article.** Please indicate your preference for color: in print or on the Web only. For further information on the preparation of electronic artwork, please see http://www.elsevier.com/artworkinstructions.

Please note: Because of technical complications which can arise by converting color figures to 'gray scale' (for the printed version should you not opt for color in print) please submit in addition usable black and white versions of all the color illustrations.

*Figure captions*

Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

*Text graphics*

Text graphics may be embedded in the text at the appropriate position. Further, high-resolution graphics files must be provided separately whether or not the graphics are embedded. See further under Electronic artwork.

***Tables***

Number tables consecutively in accordance with their appearance in the text. Place footnotes to tables below the table body and indicate them with superscript lowercase letters. Avoid vertical rules. Be sparing in the use of tables and ensure that the data presented in tables do not duplicate results described elsewhere in the article.

***References***

*Citation in text*

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

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As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

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Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

*Reference management software*

This journal has standard templates available in key reference management packages EndNote (http://www.endnote.com/support/enstyles.asp) and Reference Manager (http://refman.com/support/rmstyles.asp). Using plug-ins to wordprocessing packages, authors only need to select the appropriate journal template when preparing their article and the list of references and citations to these will be formatted according to the journal style which is described below.

*Reference style*

*Text:* All citations in the text should refer to:

1. *Single author:* the author's name (without initials, unless there is ambiguity) and the year of publication;

2. *Two authors:* both authors' names and the year of publication;

3. *Three or more authors:* first author's name followed by 'et al.' and the year of publication. Citations may be made directly (or parenthetically). Groups of references should be listed first alphabetically, then chronologically. Examples: 'as demonstrated (Allan, 2000a, 2000b, 1999; Allan and Jones, 1999). Kramer et al. (2010) have recently shown ....' *List:* References should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.

*Examples:*

Reference to a journal publication:

Van der Geer, J., Hanraads, J.A.J., Lupton, R.A., 2010. The art of writing a scientific article. J. Sci. Commun. 163, 51–59.

Reference to a book:

Strunk Jr., W., White, E.B., 2000. The Elements of Style, fourth ed. Longman, New York. Reference to a chapter in an edited book: Mettam, G.R., Adams, L.B., 2009. How to prepare an electronic version of your article, in: Jones, B.S., Smith , R.Z. (Eds.), Introduction to the Electronic Age. E-Publishing Inc., New York, pp. 281–304.

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The following list will be useful during the final checking of an article prior to sending it to the journal for review. Please consult this Guide for Authors for further details of any item.

**Ensure that the following items are present:**

One author has been designated as the corresponding author with contact details:

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All necessary files have been uploaded, and contain:

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