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Crescimento, e respostas metabólicas de surubins híbridos (*Pseudoplatystoma* sp) alimentados com diferentes fontes e níveis de energia

Recife
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Tese apresentada para o cumprimento das exigências para obtenção do título de Doutor em Ciências Biológicas pela Universidade Federal de Pernambuco

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

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“*Se você quer ir rápido vá sozinho, se quer ir longe vá junto***”**

Ditado africano

RESUMO

Este trabalho teve o objetivo de avaliar a influência entre macronutrientes energéticos, com o desempenho, metabolismo e fisiologia digestiva de diferentes surubim híbridos (*Pseudoplatystoma corruscans* x *Pseudoplatystoma fasciatum* e *Pseudoplatysma fasciatum* x *Leiarius marmoratus*). Esses surubim híbridos são amplamente cultivados no Brasil, para abate sendo a carne destinada ao mercado interno e externo. No entanto, são poucos os dados na literatura em relação às exigências nutricionais para esses peixes, bem como o efeito da relação entre os macronutrientes com a fisiologia digestiva e metabolismo. Deste modo, o **capítulo um** avaliou o efeito da manga de uma fonte de carboidrato não amiláceo. Para tal foram fornecidas dietas contendo farinha de manga em substituição ao milho em quatro níveis diferentes (0; 33%; 66% e 100%) para alevinos de *Pseudoplatystoma* sp. Após o período de 60 dias para a análise de desempenho, foram realizadas as atividades das enzimas digestivas: protease total alcalina, tripsina e amilase. Como indicativos de mudança no metabolismo foram avaliados no plasma: triglicérides, glicose, proteínas totais, colesterol, amino ácidos livres; no fígado: glicogênio e alanina aminotransferase. Foi observado que não houve diferença significativa nos parâmetros de desempenho analisados. Entretanto foram observadas alterações nas atividades das enzimas digestivas e intermediários metabólicos, provavelmente devido à quantidade de fatores antinutricionais encontrados na casca da manga. Estes resultados demonstram que em longo prazo a inclusão de farinha de manga acarretará decréscimo no desempenho dos animais. No **capítulo dois** foi avaliado o efeito de diferentes valores da relação proteína(P):lipídio(L). Quatro dietas (45P 5L; 42P 9L; 39P 11L; 34P 19L%) foram fornecidas por 60 dias para observar o efeito sobre o desempenho, enzimas digestivas, intermediários metabólicos e parâmetros hematimétricos de alevinos do surubim híbrido *Pseudoplatysma fasciatum* x *Leiarius marmoratus*. O excesso de lipídio na dieta diminuiu o desempenho o que consequentemente afetou os demais parâmetros avaliados. Valores de proteínas totais e aminoácidos livres no plasma demostraram danos causados pela deficiência de proteína na dieta. Os valores de colesterol, triglicérides e glicose mostraram resposta consequente da baixa ingestão de nutrientes. A lipase foi a enzima digestiva mais influenciada pelos nutrientes dietéticos. Os parâmetros hematimétricos foram afetados pelas dietas e demonstraram que animais que ingeriram maior quantidade de lipídio tiveram adaptação metabólica e fisiológica para suprir a deficiência nutricional. No **capítulo três** foi avaliado o efeito de diferentes valores da relação proteína(P):carboidrato(C). Quatro dietas (28P 53C; 36P 44C; 40P 39C; 45P 37C%) foram fornecidas por 60 dias para observar o efeito sobre o desempenho, composição corporal, enzimas digestivas e intermediários metabólicos de alevinos do surubim híbrido *Pseudoplatysma fasciatum* x *Leiarius marmoratus*. O excesso de carboidrato na dieta diminuiu o desempenho, sem afetar demasiadamente os parâmetros fisiológicos e metabólicos avaliados, mostrando grande adaptação do híbrido avaliado. A eficiência energética das dietas avaliadas foi maior no tratamento com menor inclusão de carboidrato e maior inclusão proteica. Valores de atividades enzimáticas específicas foram maiores no tratamento com maior inclusão de proteína. A maior reserva de glicogênio hepático também foi observado no mesmo tratamento citado acima. Como conclusão geral pode-se afirmar que diferentes relações proteína:energia, independente de ser fonte lipídica ou de carboidrato, afeta o desempenho, fisiologia digestiva e metabolismo do surubim híbrido.

Palavra-chave: Siluriformes, desempenho, proteína, lipídio, carboidrato, fisiologia digestiva

ABSTRACT

This study aimed to evaluate the influence of macronutrients energetic, growth, metabolism and digestive physiology of different surubim hybrids (*Pseuduplatystoma corruscans* x *Pseudoplatystoma fasciatum* and *Pseudoplatysma fasciatum* x *Leiarius marmoratus*). These hybrids are highly raised in Brazil with its main products being sold to domestic and international market. However there is few information on literature published about data on nutrient requirements to this fish, as well as the effects among macronutrients and digestive physiology and metabolism. Thus, **chapter one** evaluated the effect of mango meal as a non-starch carbohydrate source. In order to accomplish it, diets containing mango meal replacing corn meal in four different levels (0; 33%; 66%; 100%) were given to *Pseudoplatystoma* sp. juvenile. After 60 days growth analysis were done as well as the digestive enzymes activities: total alkaline protease, trypsin and amylase. As indicative of metabolism changes were evaluated in the blood plasma: triglycerides, glucose, total proteins, cholesterol, free amino acids; in the liver: glycogen and alanine aminotransferase. It was observed no significant difference among growth parameters evaluated. However digestive enzymes activities and intermediary metabolic changes were observed, probably due to anti-nutritional factors found in mango skin. These results shows that, in long term feeding mango meal inclusion can lead to animal growth decrease. In **chapter two** it was evaluated the effect of different protein(P):lipid(L) ratios. Four diets (45P 5L; 42P 9L; 39P 11L; 34P 19L) were offered for 60 days to observe the effect on growth performance, digestive enzymes activities, intermediary metabolism and hematimetric parameters of hybrid surubim *Pseudoplatysma fasciatum* x *Leiarius marmoratus* juvenile. Dietary lipid excess decreased growth which consequently affected the others parameters evaluated. Plasma total protein and free amino acids values demonstrated injuries caused by protein deficiency in the diet. Cholesterol, triglycerides and glucose values showed consequent response to low nutrient ingestion. Lipase was the enzyme more influenced by dietary nutrients. Hematimetric parameters were affected by diets and showed that animals that ingested higher lipid amount had metabolic and physiologic adaptation to make up nutritional deficiency. In **chapter three** it was evaluated the effect of different protein(P):carbohydrate (C)values. Four diets (28C 53C; 36P 44C; 40P 39C; 45P 37C) were offered for 60 days in order to observe the effect on growth, whole body composition, digestive enzymes and intermediary metabolism of hybrid surubim *Pseudoplatysma fasciatum* x *Leiarius marmoratus* juvenile. Dietary carbohydrate in excess decreased growth without affecting all of others physiological and metabolic parameters evaluated, which shows high adaptation. Feed energetic efficiency evaluated was higher in the treatment with lower carbohydrate inclusion and higher protein inclusion. Specific digestive enzymes activities were higher in the treatment with higher protein inclusion. Higher hepatic glycogen reserve was also observed in the same treatment cited above. As a general conclusion it can be affirmed that different protein:energy ratio, no matter if it is lipidic or carbohydrate source, affects growth, digestive physiology and metabolism of hybrid surubim.

Keywords: Siluriformes, growth, protein, lipids, carbohydrates, digestive physiology

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Lista de abreviaturas e siglas

MPA	Ministério da Pesca e Aquicultura
FAO	Food and Agriculture Organization of the United Nations
MM	Mango meal
AOAC	Association of Official Analytical Chemists
CODEVASF	Companhia do Desenvolvimento dos Vales do São Francisco e Parnaíba
FBW	Final body weight
WG	Weight gain
SR	Survival rate
FCR	Feed convertion rate
FC	Feed consumption
ALT	Alanin amino transferase
TC	Total cholesterol
TP	Total protein
TG	Total triglycerides
Glc	Glycose
FAA	Free amino acids
GC	Glycogen
PE	Protein efficiency
EE	Energy efficiency
CF	Condition Factor
SGR	Specific growth rate
CY	Carcass yield
VSI	Viscero somatic index
MCV	Mean corpuscular volume

MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
FL	Final length
AST	Aspartate amino transferase
AFC	Apparent feed conversion
CP	Crude protein
LP	lipid
CHO	carbohydrate

Sumário

1. INTRODUÇÃO

A determinação da exigência de um único nutriente, seja macro ou micro, em dietas para peixes é avaliada pelo desempenho e metabolismo do animal. Sabe-se também que a interação entre os nutrientes influencia o desempenho dos peixes, independente do hábito alimentar ou do habitat natural da espécie (FRACALOSSI & CYRINO, 2013).

O equilíbrio das concentrações de energia e proteína é essencial em dietas para peixes, sejam de ambientes dulcícolas ou marinhos, de águas de clima temperado ou tropical (KARALAZOS et al., 2011; BICUDO et al., 2009). Essa relação expressa o ponto ideal de inclusão de macro nutrientes na dieta de tal modo a suprir todas as necessidades de aminoácidos para a síntese proteica, e de energia para o metabolismo. O excesso de proteína, que é um ingrediente oneroso, será usado como fonte de energia metabólica corpórea, além de excretado para o ambiente, causando impacto ambiental negativo (MELO et al., 2006). Da mesma forma, o excesso de energia, na forma de lipídio e/ou carboidrato, geralmente acarreta baixo crescimento e aumenta a percentagem de lipídio no corpo, gerando prejuízo ao produtor (PORTZ & FURUYA, 2013). Sendo assim, o excesso de energia também não é desejado.

Há relatos recentes na literatura indicando que quando diferentes níveis de proteína e lipídio em dietas para peixes tropicais foram avaliados em esquema fatorial, somente o nível de proteína na dieta influenciou o crescimento (ARSLAN et al., 2013; LIU et al. 2013; BICUDO et al., 2010). No entanto em peixes de águas de clima temperado esse efeito não foi observado (KARALAZOS et al., 2011). A influencia da relação energia:proteína sobre as enzimas digestivas tem sido pouco explorada, embora haja relato na literatura comprovando o efeito (ARSLAN et al., 2013; LI et al., 2012; MELO et al., 2012). Trabalhos avaliando o efeito desta relação têm focado no desempenho dos peixes e pouco nos efeitos desta relação sobre o metabolismo. Os estudos realizados, no entanto, comprovam também o efeito da relação entre os macro nutrientes sobre o metabolismo dos peixes (ALMEIDA et al., 2011; MORO et al., 2010; BICUDO et al., 2009; TAN et al., 2009). Macro nutrientes também exercem influência sobre os parâmetros hematológicos em peixes, indicando estado de saúde. (WANG ET AL., 2014; VEIVERBERG et al., 2010; BICUDO et al., 2009; NELSON & COX, 2007). O estresse na síntese proteica pode causar danos ao baço e consequentemente, anemia em peixes, os tornando mais susceptíveis a doenças (KRASNOV et al., 2013).

Devido à importância que a proteína tem na dieta de peixes carnívoros cultivados, muitos estudos têm sido feitos avaliando os efeito da relação energia:proteína sobre diversos parâmetros. No surubim híbrido (*Pseudoplatystoma fasciatum* x *Leiarius mamoratus*), um siluriforme, foi avaliado o efeito da relação proteína:carboidrato (1,24; 0,84; 0,56; 0,33) sobre alguns parâmetros de desempenho, no entanto não foi avaliado o efeito sobre as enzimas digestivas nem tampouco sobre parâmetros hematopoiéticos (SOUZA et al., 2014). No jundiá (*Rhamdia quelen*), também um siluriforme, foram avaliados o efeito da relação carboidrato:lipídio (0,3:1; 1,0:1; 2,0:1; 3,4:1; 4,6:1; 5,3:1; 5,6:1; 6,5:1) como fonte energética não proteica, sobre o crescimento, enzimas digestivas e a utilização destes nutrientes (MORO et al., 2010) e da proteína (20, 27, 34 e 41%) sobre o metabolismo nitrogenado (MELO et al., 2006) e enzimas digestivas (MELO et al., 2012). O efeito da relação proteína:lipídio em duas espécies de siluriformes carnívoros asiáticos (*Silurus meridionalis* e S. *asotus*) foi avaliado sobre o crescimento e utilização dos nutrientes (CONG et al., 2013). Na truta também foi avaliado o efeito da relação carboidrato(C):proteína(P) (20C50P; 35C35P%) sobre o fígado e músculo (SKIBA-CASSY et al., 2013). Os trabalhos supracitados afirmam que relação entre os níveis de nutrientes ingeridos nas dietas influenciam o crescimento e metabolismo.

Surubins híbridos têm sido cultivados em larga escala no Brasil, no entanto poucos ainda são os estudos realizados sobre as exigências nutricionais desses peixes. Devido ao custo da ração e visando a mitigação dos impactos ambientais negativos gerados por dietas contendo altos teores de proteína, torna-se imperativo fornecer ao surubim cultivado, uma dieta que maximize o aproveitamento corpóreo dos nutrientes fornecidos.

2 REVISÃO DE LITERATURA

2.1 A Piscicultura no Brasil

Discorrer sobre a piscicultura no Brasil é desafiador, uma vez que as estatísticas podem não ser muito confiáveis. Não pelo órgão que a realizou, mas pela dificuldade existente no fornecimento de dados confiáveis por parte dos próprios produtores. As estatísticas mais atuais foram divulgadas pelo Ministério da Pesca e Aquicultura (MPA) em 2011 no Boletim Estatístico da Pesca e Aquicultura. Semelhantemente, a Food and Agricultural Organization (FAO), lança bianualmente o boletim "The State of World´s Fisheries and Aquaculture". De acordo com o boletim da FAO de 2014, a população

mundial nunca consumiu tanto pescado e dependeu desse setor produtivo, para o seu próprio bem estar como nos dias de hoje. A média atual de consumo mundial de pescado *per capita* é de 19kg/ano, no Brasil ainda é de 12 kg/ano (MPA, 2011). O crescimento do consumo acarreta o aumento da oferta de empregos em toda a cadeia produtiva do setor, desde a produção de grãos para as rações, até o aumento da oferta de emprego para mulheres no setor de beneficiamento do pescado. De acordo com os dados da FAO, em 2013, a produção continental mundial de pescado foi de 41 292,167 milhões de toneladas. O Brasil se encontra no 9° lugar entre os maiores produtores de pescado, respondendo por 0,9% da produção mundial com 388 700 toneladas produzidas. O Boletim do Ministério da Pesca registra uma produção de 544 490 toneladas de pescado de água doce para o ano de 2011 no Brasil, respondendo por 38% da produção total nacional. No Nordeste o crescimento da aquicultura continental foi notável, passando de 78 578 toneladas em 2010 para 134 292 toneladas em 2011. Com esse valor a produção nordestina responde por 27% da produção nacional de pescado em águas continentais.

Os dados acima comprovam o crescimento contínuo da produção nas últimas décadas, concomitante com a estabilização da produção pesqueira. Essa é a nova realidade da produção de pescado para suprir a demanda de mercado cada vez mais crescente em decorrência não só do aumento da população, mas também de um público mais exigente com a própria alimentação.

2.2 O cultivo do surubim

Os peixes da Ordem Siluriformes, Família Pimelodidae, popularmente conhecidos como bagres, têm grande procura nos mercados do Brasil, principalmente onde fazem parte da cultura alimentar local. Podem ser citadas como exemplo, as regiões que são banhadas pelas bacias do Rio São Francisco, Rio Amazonas e do Rio Paraná. No entanto, devido à falta de estatísticas no Brasil e literatura sobre o comportamento de mercado de surubins, informações precisas sobre dados de produção são muito escassas. O Boletim do Ministério da Pesca e Aquicultura em 2011 registrou 8.824,3 toneladas produzidas no Brasil. No relato não há distinção da produção por estado e/ou espécie. Embora seja de conhecimento entre os atores da cadeia produtiva que os surubins cultivados atualmente são híbridos: pintachara (*Pseudoplatystoma corruscans* x *Pseudoplatystoma fasciatum*) ou cachandia (*Pseudoplatystoma fasciatum* x *Leiarius marmoratus*). Cruzamentos esses desenvolvidos pelos próprios produtores, com a intenção no ganho gerado pela heterose, mas sem algum tipo de controle genético e/ou biológico (DE RESENDE et al., 2010). A produção de surubins híbridos minimizou dificuldades encontradas na produção da espécie pura selvagem. Como exemplo, a larvicultura e

produção inicial em cativeiro que eram muito limitadas, onde o alto índice de canibalismo entre os alevinos e a baixa taxa de sobrevivência na larvicultura eram os principais gargalos.

Os espécimes da Família Pimelodidae, são encontrados em bacias hidrográficas da América Central e do Sul. Têm como características biológicas principais: a ausência de escamas, nadadeira adiposa presente, três pares de barbelas junto às narinas e os espinhos das nadadeiras peitoral e dorsal podem estar presentes ou não. Os Pimelodidae do gênero *Pseudoplatystoma* e do gênero *Leiarius* têm hábito alimentar noturno, se alimentando exclusivamente de peixes em seu habitat natural (LAYMAN et al., 2005; LUNDEBERG, 2003). Há também registrado na literatura o hábito alimentar onívoro para a espécie *Leiarius marmoratus* (RAMIREZ; AJIACA, 1997). Esse hábito, aliado a outros fatores, torna-os desejáveis no mercado, devido ao sabor da carne. A textura da carne é firme, pouco gordurosa e sem espinhos intramusculares. O rendimento de carcaça sem cabeça pode chegar a 70% e o rendimento do filé entre 30 e 40% (FANTINI et al., 2013). No entanto, por se tratar de animais originados de espécies de hábito alimentar carnívoro, o custo de produção é alto, não só devido ao alto teor de proteína exigido, mas também por terem como característica de desempenho, uma alta taxa de conversão alimentar quando cultivado comercialmente. Os valores da relação de conversão alimentar registrados variaram de 3,09-4,15 (LIRANÇO et al., 2011), 3,7-5,24 (COELHO & CYRINO, 2006), 1,49-4,6 (CREPALDI et al, 2006). Cultivos de peixes híbridos têm desempenho melhor, no entanto os lotes são mais heterogêneos causando aumento do coeficiente de variação na análise de dados e prejuízo para o produtor devido à falta de uniformidade dos lotes (OLIVEIRA et al., 2013; BARBOSA et al., 2011).

Em virtude da demanda desta espécie pelo mercado consumidor e visando o aumento da produtividade do mesmo, há um esforço mútuo e contínuo da comunidade científica brasileira no desenvolvimento de pesquisas que sanem os principais gargalos da cadeia produtiva do surubim. Atualmente os principais trabalhos estão sendo realizados nas áreas de sistema de produção (FARIA et al., 2011; FAGUNDES & URBINATI, 2008), manejo alimentar (LOURENÇO et al., 2013; BARBOSA et al., 2011) e sanidade (JERONIMO et al., 2013; VENTURA et al., 2013). A nutrição dos siluriformes será tratada no item 2.3.

2.3 Nutrição de peixes cultivados

Em um sistema de produção de peixes, incluindo os surubins, o item de maior custo variável é a ração, respondendo por aproximadamente 60-70% do custo total da produção (LIRANÇO et al., 2011; KUBITZA, 2000). Em decorrência deste que é um dos maiores gargalos na produção de peixes no Brasil, há um maior esforço de pesquisa e desenvolvimento de produtos na nutrição de peixes (FRACALOSSI & CYRINO, 2013). Segundo o Boletim Informativo do Sindicato dos Fabricantes de Rações Animal (SINDIRAÇÕES) de maio de 2014, o crescimento na produção de rações para peixes entre os anos de 2012 e 2013 foi de 13%, enquanto o de aves e suínos teve queda média de 2%. Estes percentuais crescentes fortalecem a justificativa para pesquisas nesse setor.

Por serem carnívoros, os surubins exigem elevadas concentrações de proteína em suas dietas em todas as fases de cultivo (CONG et al., 2013; COLLINS et al., 2012; CAMPOS, 2010), sendo este um nutriente limitante. No entanto, um dos maiores desafios é diminuir a concentração de proteína das dietas, por ser o nutriente de maior valor para o custo da ração. Aliado ao fato de ser o principal responsável pelo aumento da concentração de nitrogênio no ambiente (BALDISSEROTTO, 2013; MELO et al., 2006; TANTIKITTI et al., 2005; WOOD, 2001), causando impacto negativo e deteriorando a qualidade da própria água de cultivo (PORTZ & FURUYA, 2013). A proteína é, após os processos de digestão e oxidação metabólica, utilizada como energia para o metabolismo basal e crescimento corporal (BALDISSEROTTO, 2013; MORAES & ALMEIDA, 2014). Portanto, quando em excesso nas rações, a proteína gera prejuízo financeiro aos fabricantes de rações e consequentemente aos produtores.

As exigências em proteína dietética para peixes variam com a fase de crescimento dos animais e com o hábito alimentar (FRACALOSSI & CYRINO, 2013; NRC, 2011). Os principais fabricantes nacionais de rações têm produtos diferenciados respeitando os fatores supracitados, à medida do que lhes é permitido devido aos custos de produção. Em recente revisão sobre os avanços em nutrição de peixes carnívoros de água doce, Cyrino et al. (2013) relatam a escassez e falta de conectividade dos trabalhos publicados sobre a nutrição do surubim. Os trabalhos mais recentes relatam resultados sobre níveis de proteína, lipídio e carboidrato. Souza et al. (2014) avaliaram quatro níveis (1,24; 0,84; 0,56; 0,33) da relação proteína:carboidrato sobre o desempenho e metabolismo do híbrido cachandia e concluíram que a relação de 0,84 é a mais indicada. Arslan et al. (2013) avaliaram o efeito de nove dietas contendo diferentes níveis de proteína (40, 45, 50%) e de lipídio (12, 16, 20%) sobre o desempenho e

composição corporal de alevinos de *Pseudoplatystoma* sp. com 1g de peso inicial. Como resultado sugerem 45% de proteína e 16% de lipídio como níveis de inclusão. Níveis de exigência em energia (18; 18,8; 19,6; 20,5; 21.3 $MJ.kg^{-1}$) para *Pseudoplatystoma* sp. de 90g foram testados por Teixeira et al. (2013). No mesmo trabalho foram testados níveis de proteína (36; 40; 44; 48; 52%) para juvenis com peso inicial de 170g. O nível recomendado de energia foi de 20,3 $MJ.kg^{-1}$ e entre 36 e 40% de proteína. Lundsted et al (2004) avaliaram o efeito de dietas contendo níveis crescente de proteína bruta (20, 30, 40 e 50%) sobre as enzimas digestivas e metabólitos em *Pseudoplatystoma corruscans*. Algumas atividades de enzimas digestivas sofreram efeito dos tratamentos, assim como os metabólitos intermediários mostrando que houve adaptação fisiológica em resposta aos diferentes níveis de nutrientes ingeridos.

Alimentos energéticos são compostos pelos grupos dos lipídios e dos carboidratos. Embora sejam estruturalmente distintos, na nutrição de peixes desempenham papel semelhante que é poupar a proteína da rota do metabolismo basal, liberando-a somente para o crescimento muscular (MORAES & ALMEIDA, 2014). Esse efeito é altamente desejável, devido ao valor do custo dos ingredientes proteicos. Atualmente há grande esforço para aumentar o nível de carboidratos nas rações para peixes carnívoros, devido ao baixo valor do milho e trigo, que são as principais fontes utilizadas (BOSCOLO et al., 2011; ENES et al., 2010; MARTINO et al., 2005; KROGDAHAL et al, 2004). Peixes não apresentam exigência nutricional para carboidratos (NRC, 2011), no entanto apresentam adaptações digestivas para aproveitar metabolicamente este nutriente (KAMAL et al., 2012; POLAKOF et al., 2012; LUNDSTEDT et al., 2004). O excesso de carboidratos na dieta acarreta longos picos de glicemia pelos animais, limita o crescimento dos mesmos (BOOTH et al., 2013) e aumenta a quantidade de glicogênio hepático (ENES et al., 2012).

De modo oposto, os lipídios estão disponíveis no ambiente natural dos peixes, que já estão adaptados a extrair energia desta fonte e o fazem de forma mais eficiente, principalmente os carnívoros (ARSLAN et al., 2013). Consequentemente a esse fato e também devido ao alto valor energético dos lipídios, os mesmos são rapidamente metabolizados pelos peixes e disponibilizados para metabolismo, manutenção da estrutura celular, reprodução, absorção de vitaminas lipossolúveis e esteróides (GARCIA et al., 2013; NRC, 2011). Devido a essas vantagens sobre os carboidratos, a resposta dos peixes em utilizar lipídios dietéticos como energia é melhor, independente do hábito alimentar (MORAES & ALMEIDA, 2014). Assim sendo, a concentração da proteína dietética pode ser menor, diminuindo o custo das

mesmas e também a excreção de compostos nitrogenados no ambiente de cultivo, otimizando o crescimento (ARSLAN et al., 2013; CHATIZIFOTIS et al., 2010; MELO et al, 2006).

Rações para peixes são compostas por todos os macro nutrientes (proteínas, carboidratos e lipídios), bem como por micronutrientes (vitaminas e minerais). No entanto, atualmente, a resposta que fabricantes de ração e produtores necessitam é: "Qual a quantidade ideal de cada um dos macronutrientes a ser inserida na ração para que a espécie de peixe cultivada tenha crescimento muscular maximizado, excretando a menor quantidade possível de amônia no ambiente aliado ao menor custo desse insumo?" Para obter esta resposta estudos avaliam a relação energia:proteína nas dietas para peixes, onde os níveis dos nutrientes são variados, ou pode-se fixar algum deles. O efeito da proteína (46, 43, 40, 37%) e lipídio (13, 10, 7%) dietéticos foi avaliado em dois bagres asiáticos *Silirus meridionalis* e S. *asotus*. Os melhores resultados para as espécies foram respectivamente: 43:10 e 43:7 (LIU et al., 2013). Li et al.(2012) similarmente avaliaram o efeito de níveis de proteína (27, 31, 35 %) e lipídio (4, 7, 10 %) sobre o desempenho, atividade de enzimas digestivas e metabolismo em alevinos de *Megalobrama amblycephala*. Os resultados indicam que o aumento lipídico de 4 para 7% favorece o crescimento, reduzindo o catabolismo proteico. Também o decréscimo proteico de 35 para 31% diminui o conteúdo lipídico do fígado.

O principal objetivo em estudos de relação energia:proteína, é avaliar o chamado efeito poupador de proteína, onde há maximização da energia dietética direcionada para o metabolismo basal do peixe, e a proteína dietética é aproveitada ao máximo para o crescimento muscular (AMIM et al., 2014; SOUZA et al., 2014; KARALAZOS et al., 2011). A falta de balanceamento da relação energia:proteína pode ainda acarretar dois fatores severos: se a quantidade de energia for alta, o peixe sacia precocemente e não ingere a quantidade de nutrientes necessárias; se a quantidade de energia for baixa, a proteína dietética será utilizada para manutenção basal (PORTZ & FURUYA, 2013).

2.4 Fisiologia digestiva de peixes cultivados

A disponibilidade dos nutrientes ingeridos nos alimentos pelos peixes para o crescimento e manutenção do metabolismo depende do processo de digestão desses alimentos. A digestão consiste em uma série de processos físicos, químicos e enzimáticos para a quebra do alimento até que os nutrientes estejam disponíveis para entrar na corrente sanguínea, e serem distribuídos para o corpo do animal. Como exemplo, a quebra dos carboidratos em monossacarídeos. Semelhantemente, a quebra dos lipídios em

ácidos graxos. Também a quebra da proteína bruta ingerida em aminoácidos (MORAES & ALMEIDA, 2014).

Semelhantemente ao que ocorre em mamíferos, em peixes o alimento entra pela boca onde começa a digestão mecânica e passa pelo esôfago. Em seguida, inicia-se a digestão química do alimento no estômago. Neste órgão, inicialmente os alimentos são mecanicamente triturados (BAKKE et al., 2011). Existe variação na morfologia dos estômagos a depender do hábito alimentar do peixe. Os carnívoros tem estômago com a parede mais grossa. Nos espécimes da família Pimelodidae o estômago é comumente dividido nas regiões cárdica, fúndica e pilórica (SANTOS et al., 2007). O estômago também possui glândulas gástricas que produzem HCl e enzimas, iniciando a digestão química propriamente dita, com pH variando entre 2 e 3. Em peixes carnívoros como as trutas, o pH é em torno de 0,6 (BALDISSEROTTO, 2013). Algumas espécies já apresentam secreção enzimática no estômago, principalmente pepsina, além de amilase e lipase (MORAES & ALMEIDA, 2014). As diferenças morfofisiológicas no trato digestório dos peixes influenciam na adaptação da espécie a determinado alimento. O que, consequentemente, influencia o desempenho e o custo de produção da espécie em cultivo comercial.

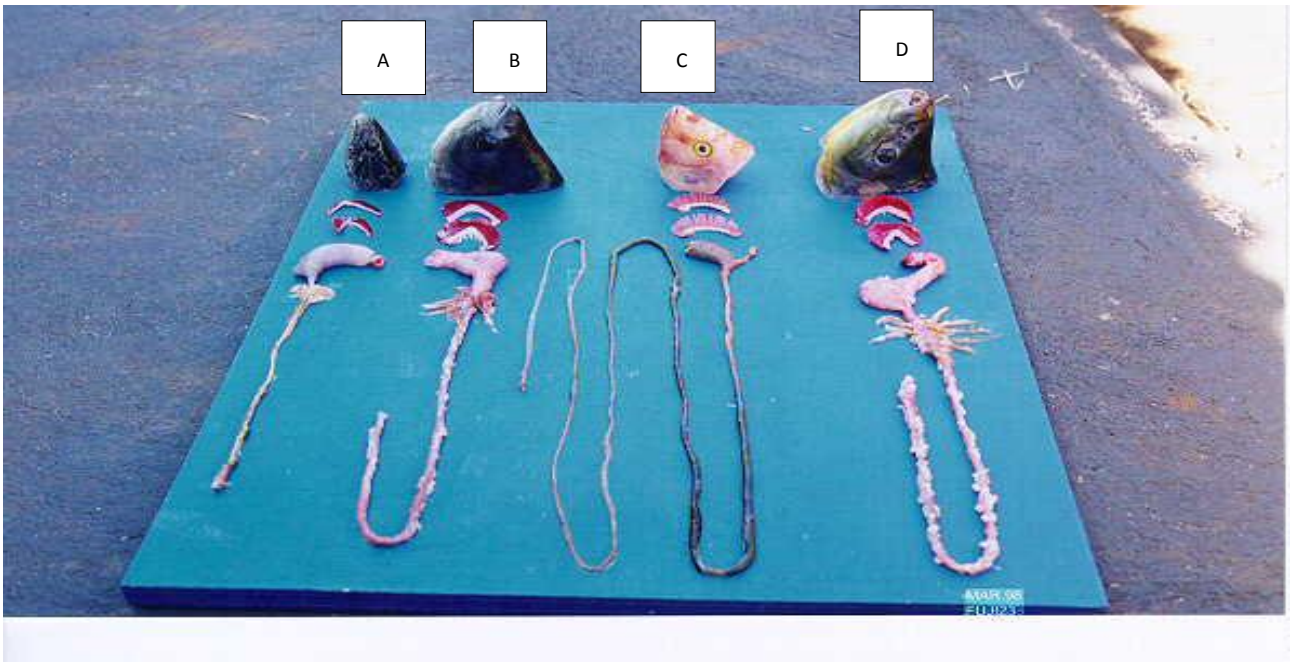
O intestino localiza-se após o estômago e é responsável pela finalização da digestão química e absorção dos nutrientes. É caracterizado por ser um tubo longo, com pregas, mas que a depender do hábito alimentar, pode exibir particularidades (DABROWSKI & PORTELLA, 2006). O alimento ingerido entra no intestino com o nome de quimo, que recebe as secreções enzimáticas do intestino, pâncreas e vesícula biliar. No intestino de algumas espécies carnívoras e onívoras apresentam-se os cecos pilóricos (Figura 1). Estes são projeções do intestino que aumentam a área para absorção de nutrientes e podem produzir enzimas digestivas e recebem secreções pancreáticas e biliares (BALDISSEROTTO, 2013). Em peixes o pâncreas aparece como um órgão difuso no mesentério e mantém as características de secreção endócrina e exócrina, principalmente nos espécimes que não têm cecos pilóricos. Os ductos pancreáticos são diretamente ligados ao intestino para a secreção das enzimas digestivas em forma de zimogênio, digerindo lipídios, polissacarídeos e proteínas. Assim como o ducto da vesícula biliar tem a mesma ligação com o intestino, secretando os sais biliares que auxiliam na digestão dos lipídios (BAKKE et al., 2011).

De modo geral, os intestinos são divididos em duas partes, a proximal e a distal. A proximal está mais ligada à digestão enzimática e a segunda à absorção dos nutrientes. Há também diferença na morfologia

e comprimento intestinal dos peixes, a depender do hábito alimentar de cada um. Geralmente os carnívoros e herbívoros tem o intestino mais comprido (DABROWSKI & PORTELLA, 2006). Essa característica permite maior absorção dos nutrientes. No entanto os carnívoros possuem a parede intestinal mais espessa e com mais dobras, e os herbívoros tem parede intestinal mais fina (BALDISSEROTTO, 2013).

A eficiência de processo ocorrido no trato digestório está ligada à soma dos fatores citados acima, ou seja, o hábito alimentar, a morfologia digestória e alimento ingerido pela espécie. Somente com a eficiência máxima desse conjunto, os nutrientes ingeridos nas rações serão hidrolisados e solubilizados em moléculas que possam ser transportadas pelas paredes intestinais (BAKKE et al., 2011) e assim utilizados para a manutenção corpórea.

Figura 1. Tratos digestivos de peixes de diferentes hábitos alimentares. A–Traíra (*Hoplias marabalicus*); B- Tambaqui (*Colossoma macropomum*); C- Tilápia (*Oreochromis niloticus*); D –Piauçu (*Leporinus macrocephalus*). Foto – Paulo Carneiro.



2.5 Enzimas digestivas

Após os processos mecânicos e químicos ocorridos na boca e estômago, a ração ingerida pelo peixe, i.e., o alimento na forma de quimo, recebe a ação das enzimas digestivas principalmente no intestino (BALDISSEROTTO, 2013). Estas transformarão os nutrientes em moléculas, que estarão disponíveis para serem absorvidas pelas paredes em forma de pregas no intestino, ou seja, em pequenos peptídeos, aminoácidos, monossacarídeos e ácidos graxos (BAKKE et al., 2011).

As proteases são enzimas que digerem proteínas, quebram as cadeias polipeptídicas que formam as proteínas e são basicamente divididas em endopeptidases e exopeptidases (NELSON & COX, 2007). Como resultado desse processo tem-se os aminoácidos que são então absorvidos no intestino e liberados na corrente sanguínea. Diferentes fatores afetam a atividade das proteases. Santos *et al*. (2013) avaliaram o efeito de diferentes níveis de inclusão de hidrolisado proteico de camarão (0; 1.5; 3; 6%) em dietas para alevinos de tilápia (*Oreochromis niloticus*). Os autores observaram que o perfil de atividade de enzimas proteolíticas variou em decorrência do nível de inclusão. A fonte de proteína também alterou as atividades proteolíticas de juvenis de salmão alimentados com dieta contendo farelo de soja em substituição à farinha de peixe, após cinco dias de alimentação (CHIKWATI et al., 2013). A atividade da tripsina e da quimotripsina foram afetadas quando juvenis de salmão (*Salmo salar* L.) foram submetidos a diferentes condições de ambiente interno (estágio de vida e fenótipos de tripsina) e externo (jejum, alimentação e temperatura). Neste trabalho, Rungruangsak-Torrisen et al. (2006) afirmaram que as atividades das proteases estudadas são determinantes no processo digestivo de forma a afetar o crescimento de salmão. Inibidores da tripsina podem causar em peixes uma diminuição do crescimento e de retenção proteica na carcaça (HART et al., 2010). Sunde et al. (2004) avaliando fonte proteica de alta e baixa qualidade em salmão de 150g e 2kg, afirmaram que após 60 dias a alta eficiência da conversão alimentar nos grupos que receberam dietas contendo proteína de alta qualidade, foi consequência da alta atividade de tripsina e quimotripsina. A tripsina em particular tem sido muito estudada por ser enzima chave no processo de digestão de proteínas, ativando o próprio tripsinogênio, além da quimotripsina e demais proteases específicas (MORAES & ALMEIDA, 2014).

A principal enzima a digerir os carboidratos em peixes é a amilase, que quebra os polissacarídeos de glicose em oligossacarídeos (BAKKE et al 2011). A digestão do amido pelos peixes tem relação com o hábito alimentar e com o clima no qual a espécie vive, sendo que os onívoros tropicais são os que mais facilmente digerem o carboidrato da dieta (MORAES & ALMEIDA, 2014; JI et al., 2012). Há grande

interesse em avaliar a amilase em conjunto com a digestibilidade de carboidratos e crescimento em peixes (SERRANO, 2013; PÉREZ-JIMÉNEZ et al., 2009), pelo fato de este ser um alimento barato para compor as rações comerciais, potencialmente diminuindo a porcentagem de inclusão de proteína animal.

A lipase, secretada na forma ativa no intestino dos peixes, é a enzima responsável pela digestão dos lipídeos até estarem degradados em ácidos graxos para serem agrupados em micelas. Estas são desintegradas ao chegarem à mucosa intestinal e absorvidas (BAKKE et al., 2011). Em sua maioria as lipases são não específicas e atuam no lipídio juntamente com sais biliares (MORAES & ALMEIDA, 2014). Em peixes, além das lipases diferirem entre os peixes de diferentes hábitos alimentares, também difere entre o habitat dulcícola e marinho (JI et al., 2012; BAKKE et al., 2011; DABROWSKI & PORTELLA, 2006). O tipo e concentração de lipídios na dieta também influenciam a atividade da lipase (LI et al., 2011; ASKARIAN et al., 2011).

2.6 Intermediários metabólicos

Intermediários metabólicos são os produtos encontrados em um organismo vivo, provenientes dos processos anabólicos e catabólicos ocorridos nas células (NELSON & COX, 2007). Muitos destes produtos sofrem influências, por exemplo, dos alimentos ingeridos por um animal, podendo servir como indicadores da condição nutricional do mesmo ou demanda energética (FRACALOSSI et al., 2013). Na nutrição, esses intermediários metabólicos são oriundos dos processos de digestão e metabolismo dos macronutrientes ingeridos em determinada dieta, i.e., proteínas, lipídios e carboidratos.

A concentração de aminoácidos livres e proteínas totais no plasma podem ser resultantes do processo de metabolismo da proteína ingerida na dieta. Há uma relação direta entre a síntese de proteínas no fígado e a concentração de proteínas totais no sangue, sendo que o valor de albumina e globulina no sangue tem maior influência nesse valor (BANAEE et al 2011). O perfil de aminoácidos também pode alterar os valores metabólicos. Alevinos de "red sea bream" *Pagrus major*, alimentados com dietas isoproteicas e isoenergéticas, mas elevando os níveis de valina (0.27; 0.79; 1.22; 1.9; 2.04; 2.38%) mostraram aumento de proteína plasmática até o valor de 2.04% (RAHIMNEJAD et al., 2013). Os valores de aminoácidos livres no plasma podem estar relacionados com a digestão e absorção da proteína ingerida na dieta, ou do perfil de aminoácidos disponível na mesma. Esta afirmação foi constatada por Larsen et al. (2012) que avaliaram o perfil de aminoácidos na dieta e no plasma, concomitantemente com a digestibilidade em alevinos de truta arco-íris alimentados com dietas contendo proteína animal e vegetal. Do mesmo modo, Mach et al. (2011), alimentaram alevinos de beijupirá (*Rachycentron canadum*) com dietas com silagem de peixe substituindo a farinha de peixe em 0; 13; 26; 39%) e que continham diferentes perfis de aminoácidos. Este fato também alterou o perfil de aminoácidos no plasma, fígado e músculo. O processo de deaminação muscular em decorrência da deficiência de proteína na dieta para a gluconeogênese também pode afetar os valores de aminoácidos livres no plasma. Níveis maiores de aminoácidos livres no plasma foram observados em *Solea senegalensis* em jejum por 21 dias, do que nos animais que estavam em jejum por 24 horas (COSTAS et al., 2011).

Dentre os intermediários metabólicos que são resultantes do processo de digestão e metabolismo dos lipídios pode ser citado como principal, o colesterol. Este é um esteroide, encontrado nas membranas

celulares e precursor de hormônios da reprodução. Desta forma é essencial para a função estrutural da membrana celular e de reprodução animal, sendo o principal esteroide em peixes (GARCIA et al., 2013). Independente do hábito alimentar natural em peixes, a origem do lipídio da ração pode ou não influenciar na taxa de colesterol plasmático. Juvenis (55g) de tilápia do Nilo não apresentaram mudança no perfil de colesterol plasmático quando alimentados, por 55 dias, com dietas contendo óleo de soja ou de dendê com diferentes níveis de inclusão (1,3; 2,1 e 2,5%) (AZEVEDO et al., 2013). Diferentemente, juvenis de tilápia do Nilo (72g) alimentados, por 160 dias, obtiveram nível mais elevado de colesterol plasmático quando alimentado com dieta semipurificada contendo 5% de inclusão de óleo de peixe, em comparação a óleo de soja, milho ou linhaça. O menor nível de colesterol foi no grupo alimentado com óleo de oliva (FERREIRA et al., 2011). Morais et al. (2011) afirmam que a fonte de óleo, vegetal ou animal, pode modificar a rota metabólica do colesterol em salmão do Atlântico. No mesmo trabalho, o grupo que se alimentou, por 55 dias, com dieta contendo óleo de peixe apresentou maiores valores de colesterol plasmático. A fonte de proteína dietética pode influenciar a taxa de colesterol no plasma de peixes carnívoros. Salmão do Atlântico alimentado com dietas contendo níveis crescentes de substituição de farinha de peixe por concentrado de proteína de grão de bico e farinha de krill (0; 15,8; 32,7; 48,7 e 75%) tiveram diminuição no nível de colesterol plasmático (15,7; 16,4; 13,7; 12,2 e 11,7 mM). Este fato foi provavelmente devido à fonte proteica vegetal que tem baixa concentração de colesterol (HANSEN et al., 2011). Semelhantemente foi observado em peixes da mesma espécie alimentados com dietas contendo farinha de peixe como fonte de proteína animal e glúten de trigo e farinha de tremoço como fonte vegetal (GU et al., 2014).

O aumento da inclusão do nível de carboidratos em dietas para peixes tem sido estudado nas mais diferentes formas. Alevinos de *Seriola lalandi* alimentados com dietas contendo níveis de inclusão de 10 a 40% de carboidratos, tendo como fontes principais o amido de trigo pré-gelatinizado ou o trigo extrusado, tiveram desempenho mais influenciado pela percentagem de carboidrato no tratamento do que pela fonte (BOOTH et al., 2013). Doses crescentes de insulina (0,35 e 0,7 $IU.kg^{-1}.d^{-1}$) foram injetadas em truta arco-irís alimentadas com dietas contendo elevada concentração de carboidrato (POLAKOF et al., 2013). Neste trabalho os autores reforçaram a hipótese de que a insulina é que estimula a lipogênese hepática em peixes carnívoros. Entre os principais fatores externos que afetam o metabolismo de carboidratos e, consequentemente, afeta o nível de inclusão, é a temperatura. Qiang et al. (2014) avaliaram o efeito de diferentes temperaturas (22, 28 e 34 °C) sobre o metabolismo

enzimático de carboidratos e o desempenho de alevinos de tilápia. Constataram que a 28 °C houve melhor desempenho e maior capacidade glicolítica e a 22 °C a maior gliconeogênese e lipogênese no fígado. Intermediários metabólicos em peixes alimentados com dietas contendo diferentes níveis de carboidratos também foram afetados (SOUZA et al., 2014; MORO et al., 2010). Dentre os principais produtos avaliados são: as triglicérides, a glicose e o glicogênio hepático.

Um dos gargalos na utilização de carboidratos em dietas para peixes é a hiperglicemia observada nos mesmos, quando alimentados com dietas contendo altos níveis do nutriente (SOUZA et al., 2014; KROGDAHL et al., 2004). Este fato demonstra a resistência pelos peixes na utilização de glicose como fonte de energia (SKIBA-CASSY et al., 2013; ENES et al., 2012). Após ser absorvida no intestino, a glicose, pode ter dois destinos: circulação sanguínea e ser metabolizada nos tecidos, e/ou quando em excesso, é sintetizada em glicogênio no fígado (CAMPBELL, 2010 a). A formação do glicogênio é de cadeias ramificadas de moléculas de glicose e é a fonte de reserva energética em um animal. O metabolismo carboidrato-glicogênio é critico em um animal em se tratando de suplemento energético emergencial para o sistema nervoso central (CRUZ et al., 2010). O glicogênio pode ser encontrado no fígado, e também nos tecidos musculares, servindo também para a manutenção de glicose na ausência da entrada da mesma (CAMPBELL, 2010 b). Os nutrientes contidos na dieta, principalmente proteína e carboidrato, têm relação direta com a concentração de glicogênio, pelo excesso ou deficiência (SANTOS et al., 2014; CHEN et al., 2013; FELIP et al., 2012). No entanto quando há substituição de ingredientes, mantendo-se as dietas isoproteicas e isoenergéticas, o nível de glicogênio hepático não é alterado (ADAMINOU et al., 2009).

Triglicerídeos também são um composto resultante da digestão e metabolismo de carboidratos, formado por três ácidos graxos ligados a um glicerol (CAMPBELL, 2010b). Deste modo, o carboidrato dietético pode influenciar nas taxas de triglicérides no sangue (CHEN et al., 2013; CORREA, 2007). Assim como também a relação entre carboidrato e lipídio na dieta pode influenciar a taxa de triglicérides, uma vez que afeta o transporte de lipídio no organismo (GAO et al., 2010). As respostas encontradas na literatura sobre a influência dos nutrientes e hábito alimentar natural de determinada espécie de peixe sobre a taxa de triglicérides ainda não é conclusiva e podem ser vistas como respostas metabólicas adaptativas (TIAN et al., 2012; ALMEIDA et al., 2011).

3. OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar o efeito dos macronutrientes dietéticos sobre o desempenho e intermediários de alevinos de surubins híbridos – *Pseudoplatystoma* sp.

3.2 OBJETIVOS ESPECÍFICOS

- Avaliar o efeito de uma fonte de carboidrato não amiláceo sobre o desempenho zootécnico, a atividade das enzimas digestivas e intermediários metabólicos de um peixe carnívoro tropical
- Avaliar o efeito da relação dietética proteína:lipídio e proteína: carboidrato dietético sobre o desempenho zootécnico e o metabolismo de um peixe carnívoro tropical

- **CAPÍTULO I**

Corn meal replacement by mango meal in diets for juvenile surubim, *Pseudoplatystoma* sp – effect over growth performance, digestive enzymes and metabolism

A ser submetido à Aquaculture

Corn meal replacement by mango meal in diets for juvenile surubim, *Pseudoplatystoma* sp – effect over growth performance, digestive enzymes and metabolism

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HIGHLIGHTS:

- Fish species showed physiological adaptation when different carbohydrate sources were ingested
- The change in qualitative carbohydrate pattern caused metabolic adaptations such as decrease in cholesterol and triglycerides, although growth was maintained during the 60 days trial
- Growth performance cannot be the only biological tool used to prove that an alternative ingredient can be introduced in a fish species diet.

Abstract - This study aimed to analyze the effect of mango meal, a non-starch polysaccharide, as a carbohydrate source on growth performance parameters, digestive enzymes and intermediary metabolism for hybrid surubim (*Pseudoplatystoma* sp) a tropical water carnivorous fish. Juvenile (±14.5g initial weight) were fed 3% of total biomass for 60 days in four experimental diets replacing 0, 33, 66 and 100% of corn meal. By growth final weight was evaluated, but statistical difference was not observed among treatments. The activity of total alkaline protease and trypsin decreased when 100% of corn meal was replaced by mango meal, but without statistical significance. Zymogram showed bands of protease and amylase activities for all treatments. Cholesterol (0%- 141.83±46.10; 100%- 71.36±14.40 $mg.dL^{-1}$) and total protein (0%- 3.98±0,94; 100%- 1.70±1.03 $mg.dL^{-1}$) in the plasma, decreased as mango meal levels increased in the fed. In the opposite glucose (0%- 105.08±31.24; 100%- 128.11±24.51 $mg.dL^{-1}$) and free amino acids (0%- 24.51±2.62; 100%- 38.13±8.94 $nmoles.ml^{-1}$) in the plasma increased. The result suggests that up to 33% of mango meal can replace corn meal in diets for *Pseudoplatystoma* sp.

1. Introduction

Corn meal is the most commonly used carbohydrate source in fish feeds. Although it is not the most expensive ingredient in the diet, corn carbohydrate is a starch polysaccharide. There are metabolic and performance signs of carbohydrate overdose in several tropical fish (Dabrowski and Portella, 2006). Diets rich in non-starch polysaccharide showed to improve nitrogen retention in fish as well as to improve protein, fat and starch digestibility for rainbow trout (Meriac et al., 2014), higher growth and lower hepathossomatic index for sea bass (Gatesoupe et al., 2014).

The level and type of carbohydrate in fish affect several metabolic and enzymatic parameters (Kamalam et al., 2012; Saravanan et al., 2012). Some might have anti-nutritional factors, which affects proteolytic enzyme activity during digestion and consequently affect fish growth performance (Francis et al., 2001). It has already been reported that plasma glucose and liver glycogen are affected by carbohydrate type, no matter what the natural food habit of the specie (Enes et al., 2009; Krogdahl et al., 2004). However there is little information about carbohydrate metabolism in tropical water carnivorous fish (Booth et al., 2013).

The amount of carbohydrate used in the diet might affect amylase activity in carnivorous intestine (Ren et al., 2011; Perez-Jimenez et al., 2009; Krogdahl et al., 2004) and decrease fish growth. In order to improve fish wellfare and growth, alternative sources of carbohydrate have been tested to offer to fish feed industry, ingredients of lower cost and/or better digested by the animal (Adamidou et al., 2009; Melo, et al., 2012).

It is well known that carnivorous fish do not require carbohydrate in their diet as a nutrient, due to their inadequate regulation of hepatic glucose utilization and production (Enes et al., 2009; Krogdahl et al., 2004; Moon, 2001). In general, as more carnivorous is the species, the longer time needed to clear a glucose load (Moon, 2001). One hypothesis is that fish has a persistent high level of endogenous glucose production (Enes et al., 2009) and it is specie food habit dependent (Dabrowski and Portela, 2006).

Mango worldwide production is over 25 million tons per year and its production has increased (FAOSTAT, 2013). This situation might be an opportunity for its use as an ingredient in animal feed, since mango and its by-products are a source of fiber and carbohydrate (Pereira et al., 2013; Kassahun et al., 2012; Rêgo et al., 2010). Mango has 17.5 % of carbohydrate and it is one of the fruit with highest percentage of it (Kumar et al., 2012). However care must be taken when using mango or its by-product as an ingredient in animal feed due to its anti-nutritional factors found in the skin such as polyphenols (Garcia-Magaña et al., 2013).

Hybrid surubim (*Pseudoplatystoma fasciatum* x *Pseudoplatystoma corruscans* ; *Pseudoplatystoma fasciatum* x *Leiarius marmoratus*) is an important commercial and carnivorous specie in South America, for its tender and bone less meat. Total production in Brazil is over 2,000 tons per year (MPA, 2011). Recent researches were with the aim to improve its performance on production, related to nutrient requirement or alternative ingredients and their impact on the specie physiology and metabolism (Bicudo et al., 2012; Teixeira et al., 2010; Arslan et al., 2009).

This study aimed to analyze the effect of a non-starch polysaccharide as a carbohydrate source on performance parameter, digestive enzymes and metabolic answers for a hybrid surubim in order to improve its growth.

2. Material and Methods

2.1. Experimental diets

Four diets containing 0, 10, 20 and 30 % of mango meal (MM), respectively, as replacement of 0, 33, 66 and 100 % of corn meal (designated as MM0, MM33, MM66 and MM100) were formulated. The formulation was made in order to have isonitrogen (35 %) and isoenergetic (18.67 $kJ.g^{-1}$) basis. Dietary formulation, proximate composition and mango meal anti-nutritional factors are presented in Table 1.

Table 1. Mango meal chemical compositon and characterization

Chemical Composition (%)	
Crude Protein	4.31
Ether Extract	1.46
Ash	3.92
Fiber	7.08

Antinutritional factors

	Poliphen	Total tanin
	$g.kg^{-1}$. dry matter	$g.kg^{-1}$ dry matter
Mango with skin	3.67	2.81

Mango meal was made by cutting whole mangos without the seed with a sharp knife, in sequence the mangos were put in an air forced recirculated stove at 60 ˚C for 24 h. After removal it was ground into powder in a mill. All ingredients were ground to pass through a 0,5mm mesh screen and thoroughly

mixed before adding hot water (60 ˚C). Pellets of about 4.0-mm-diameter were made with a meat grinder and taken to an air forced recirculated stove at 60 ˚C for 24 h. After leaving the stove and cooling to room temperature, the diets were frozen (-18 ˚C) until the beginning of the trial. Proximate composition of ingredients was determined according to AOAC (1990) for moisture, ash, crude protein and ether extract analyses (Table 2).

Table 2. Formulation and proximate analysis of experimental diets (% dry matter).

Content %	Experimental Diets			
	MM0	MM33	MM66	MM100
Ingredients				
Fish meal	43.31	43.31	43.31	43.31
Soybean meal	14.01	14.01	14.01	14.01
Corn meal	30.00	20.00	10.00	0.00
Mango meal	0.00	10.00	20.00	30.00
Soybean oil	5.97	5.97	5.97	5.97
Vitamin and Mineral Premix[a]	2.00	2.00	2.00	2.00
Vitamin C	0.01	0.01	0.01	0.01
BHT	0.02	0.02	0.02	0.02
NaCl	0.50	0.50	0.50	0.50
Lysine	2.57	2.57	2.57	2.57
Metionine	1.60	1.60	1.60	1.60
Proximate composition				

Moisture	8.97	7.67	8.19	8.07
Crude Protein	35.54	36.03	35.72	36.07
Crude lipid	12.01	11.74	12.03	12.69
Ash	9.13	10.72	13.25	11.96
Carbohydrate[b]	34.35	33.84	30.81	31.21
Crude Energy[c] ($kJ.g^{-1}$)	19.07	18.99	18.50	18.92

[a] Vitamin and Mineral Premix: Vitamin A = 1.200.000 UI; vitamin D3 = 200.000 UI; vitamin E = 12.000 mg; vitamin K3 = 2400 mg; vitamin B1 = 4800 mg; vitamin B2 = 4800 mg; vitamin B6 = 4000 mg; vitamin B12 = 4800 mg; folic acid = 1200 mg; calcium pantothenate = 12.000 mg; vitamin C = 48.000 mg; biotin = 48 mg; choline = 65.000 mg; nicotinic acid = 24.000 mg; Fe = 10.000 g; Cu = 600 mg; Mn = 4000 mg; Zn = 6000 mg; I = 20 mg; Co = 2 mg; Se = 20 mg.

[b]Carbohydrate% = 100-(crude protein% + crude lipid% + moisture% + ash%)

[c]Energy ($kJ.g^{-1}$ diet) = (% crude protein x 23,6) + (% crude lipid x 39,5) + (% carbohydrates x 17,3)

2.2. Fish and Feeding

Fish were obtained from CODEVASF (Companhia de Desenvolvimento do Vale do São Francisco e Parnaíba), a Federal Brazilian development company. Juvenile surubim arrived in the laboratory, were acclimated for 20 days and fed a commercial diet (45% crude protein) before the trial. To initiate the trial all fish were individually weighed (average 14.53g). After this procedure they were sorted in 800L PVC tanks, with 10 fish per tank. Three replicate groups of fish were used for each diet. Tanks were built in a total open water system. Water parameters were monitored weekly (average temperature was 27.5˚C; pH 7.3 and dissolved oxygen 5.7 $mg.l^{-1}$). Fish were fed twice a day, at 3% of total biomass, (0830 am and 0400 pm) for 60 days. Everyday each tank was cleaned daily by scrubbing and siphoning of accumulated wastes, algae or any dirt that possibly came from the water.

2.3. Sample collection

At the end of the trial fish were starved for 24h. All fish in each tank were individually weighed for performance parameters: final body weight (FBW), weight gain (WG), survival rate (SR) and feed conversion ratio (FCR). Feed consumption (FC) was also measured. Fish were anesthetized (8ml/100L of water) and blood samples from 4 fish, randomly selected, from each tank were draw from the causal vein, subsequently euthanized with eugenol. Glucose was immediately measured with an automatic monitor. In sequence those blood samples were centrifuged at 1000 rpm for 5 minutes and the plasma stored in a freezer (-20˚C) for a short period until the beginning of the total cholesterol, total protein, triglycerides and free amino acids.

Subsequently blood draw, liver and total intestine samples were collected from the same fish and immediately stored in a freezer (-20˚C) for short period until the beginning of glycogen and alanin amino transferase analysis (liver) and the enzymes activities and zymogram analyzes (intestine).

2.4. Performance

Performance was measured by the following equations:

WG (g) = [final body weight (g) – initial body weight (g)]

SR (%) = 100 x (final fish number/ initial fish number)

FCR = [feed consumed (g)/ weight gain (g)]

FC = feed consumed (g)/time

2.5 . Enzymes Activities

After collected intestine were immediately stored in -20°C. At the laboratory it was homogenized in 0.01M Tris-HCl pH 8.0 buffer using a tissue homogenizer. The resulted preparation was centrifuged at 10,000xg for 10 min at 4°C. The supernatants (crude enzyme extract) were used for analysis assays (Santos et al., 2013).

2.5.1. Total alkaline protease activity

The total enzymatic activity of proteases present in crude extracts was performed using 1% azocasein as substrate, prepared in 10 mM Tris-HCl, pH 8.0. Aliquots containing 30 µL of the crude extract were incubated with 50 µL of substrate solution for 1 hour at 25 °C. Then, 240µL of 10% trichloroacetic acid was added to stop the reaction. After 15 minutes the mixture was centrifuged at 8.000 xg for 5 minutes. The supernatant was collected and 70 µL of it was mixed in 130 µL 1M sodium hydroxide solution (revealing solution) in microplates. The absorbance was measured on a microplate reader (x-Mark Bio-Rad) at a wavelength of 450 nm. A negative control (blank) was performed, replacing the enzyme extract by a solution of 10 mM Tris-HCl, pH 8.0 with added 0.15 M NaCl. The activities were carried out in triplicate and one unit (U) of enzyme activity was defined as the amount of enzyme required to hydrolyze azocasein and produce a change of 0.001 units of absorbance per minute.

2.5.2. Trypsin Activity (E.C. 3.4.21.4)

The activity of trypsin was determined using 8.0 mM BApNA (Nα-benzoyl-DL-arginine-p-nitroanilide) in DMSO (Dimethyl sulfoxide). Intestine crude enzyme extract (30 µL) was incubated with 0.1M Tris-HCl buffer pH 8.0 (10 µL) and respective substrates (30 µL) in a microtiter plate reader (Bio-Rad 680, Japan). The absorbance was measured at 405 nm *versus* a similarly prepared blank in which 0.1M Tris-HCl pH 8.0 replaced the crude extract sample. Enzyme activity was determined in triplicate. Trypsin units of activity were expressed as a change in absorbance per minute per milligram of protein (Bezerra et al., 2005).

2.5.3. α-Amylase Activity (E.C. 3.2.1.1)

α-Amylase activity from the intestine of *Pseudoplatystoma* sp. was based on the method of Bernfeld (1955) using 2% (w/v) starch solution as substrate. The enzymatic preparations were in 0.2 M citrate-phosphate buffer, pH at 7.0 at 37 °C at 30 minutes. Then the assay was by addition of 3,5-dinitrosalicylic acid (DNSA) at 100 °C for 10 minutes. The absorbance was measured at 570nm using a microplate reader (Bio-Rad 680). Both a substrate free control and an enzyme free control were run. The amount of maltose released from this assay was determined from the standard curve using commercial maltose. One milliunit of specific activity was defined as the amount of enzyme needed to release 1µg maltose per minute per milligram of soluble protein in enzyme solution at 37°C ($mU.min^{-1}.mg^{-1}$ Protein).

2.6. Zymograms

2.6.1. Protease zymogram

Protease zymogram was performed to characterize the protease present in the crude enzyme, in a procedure described by Garcia-Carreño et al. (1993). Zymogram was initiated by electrophoresis (SDS-PAGE) under immersion in an ice bath. Crude enzyme extracts were mixed with sample buffer containing 0.5M Tris–HCl at a pH of 6.8, 20 % (w/v) glycerol, 10% (w/v) SDS, and 0.5 % (v/v) bromophenol blue. A quantity of 20 µL of the sample buffer mixture was loaded into SDS–PAGE gel with a thickness of 1.0 mm. The gel consisted of 4 % (w/v) stacking gel and a 12.5 % (w/v) separating gel. Electrophoresis was conducted at constant current of 12 mA with an electrophoresis buffer comprising Tris–glycine–sodium dodecyl sulfate. After electrophoresis, the gel was immersed in 100 mL of 2.5 % (v/v) Triton X-100, diluted in Tris-HCl 0.1 M, pH 8.0, for a period of thirty minutes at 4 °C to remove the SDS. Then Triton X-100 was removed by washing the gels with Tris-HCl 0.1 M, pH 8.0. The gel was incubated in 100 mL of casein 3 % (w/v) diluted in Tris-HCl 0.1 M, pH 8.0, for 30 minutes at 4 °C to determine the proteolytic activity. Soon after the gel was kept in the same casein solution at 25 °C for 90 minutes to permit the digestion of casein by active fractions. Finally the gel was stained with a solution composed of Coomassie Brilliant Blue 0.01 % (v/v), methanol 25 % (v/v) and acetic acid 10 % (v/v) and after 24 hours was bleached in a solution with the same composition but devoid of the dye.

2.6.2. Amylase zymogram

Amylase zymogram was carried out according to the modified methodology described by Fernández et al. (2001). Enzyme preparations were applied to a 12.5% (w/v) separating gel. After electrophoresis performed at 4 °C and a constant current of 12 mA, the gel was immersed in 2.5 $mL.L^{-1}$ (100 mL) Triton X-100 in 0.1M Tris-HCl (pH 8.0, for 30 min at 4 °C) to remove the SDS. Triton X-100 was removed by washing the gel three times with 100 mL of 0.1 M Tris-HCl buffer, pH 8.0. Next, the SDS-free, Triton X-100-free gel was incubated with a starch solution 2 % (w/v) containing 10 mM phosphate buffer, pH 8.0, and 1 mM $CaCl_2$ for 60 min at 37 °C to allow the digestion of starch by the active fractions. Finally, the gel was washed with distilled water, stained with an iodine/KI solution 10 % (w/v) for 5 minutes and added acetic acid solution a 13 % acetic acid solution (v/v) was added to stop the reaction.

2.7. Plasma and liver metabolites

Metabolite concentration was carried out in plasma and liver tissue extracts. Total plasmatic cholesterol was determined by colorimetric enzimatic method, using a Labtest®, Brazil commercial kit with the reading done at 500 nm wave. Ten (10) microliters of plasma sample was incubated for 10 minutes at 37 °C and the measure unit was expressed in $mg.dL^{-1}$. Total protein was determined by Biureto method (Hiller, 1948). Twenty microliters of plasma was incubated for 10 minutes at 37 °C. The reading was at 545 nm and the measure unit was expressed in $g.dL^{-1}$. Plasma triglycerides were analyzed by enzymatic colorimetric method using a commercial kit by Labtest®, Brazil. It was used 10 microliters of plasma samples containing reagent in 1ml of buffer pH 7.0, incubated for 10 minutes at 37 °C. The samples were read at 500nm and the units of measurement were in $mg.dL^{-1}$.

Glucose was measured at the end of the trial, in the fish blood using an automatic glucose reader Accuchek®, Brazil. Free amino acids were determined in neutral extracts at 570 nm (Copley, 1941). Glycogen was assayed in alcoholic precipitates from tissues alkaline homogenates (Bidinotto et al., 1997).

Alanin amino transferase (ALT) activities were determined by a commercial kit by Labtest®, Brazil. The reaction principle of ALT is based on the conversion of alanine to pyruvate by transamination, followed by reduction to lactate by LDH auxiliary enzyme which is monitored optically by extinction of NADH. The reaction cocktail for ALT contained: tris-HCl buffer, pH 7.5 - 100 mM, 13.7 mM 2-oxoglutarate, 0.18 mM NADH, 0.5 mM L-alanine, 21 mKat LDH/L, and sufficient volume enzyme. The enzymatic activity was expressed in $U.L^{-1}$.

2.8. Statistic

All data were analyzed by one-way analysis of variance (ANOVA). Linear, polynomial and exponential models were tested for all regressions, and the best model was selected based on R^2. For enzymes activities, significant differences between means were determined by the Tukey´s multiple range test. The probability level of 0.05 was used for rejection of the null.

3. Results

In this study it could be observed the influence of a non-starch polysaccharide carbohydrate source on a tropical carnivorous siluriforme. Statistical difference among treatments in relation to performance was not observed (Table 3). No death was notified during the trial.

Among intestinal enzyme activities analyzed, it was observed that amylase was the lowest one independent of group treatment. Total protease (MM0 0.139 ± 0.03^{A}; MM33 0.154 ± 0.05^{A}; MM66 0.154 ± 0.06^{A}; MM100 0.035 ± 0.00^{B} $U.mg^{-1}$), trypsin (MM0 0.812±0.49; MM33 0.559±0.57; MM66 0.781±0.32; MM100 0.325±0.22 $U.mg^{-1}$) and amylase activity (MM0 0.008±0.00; MM33 0.009±0.00; MM66 0.011±0.00; MM100 0.007±0.00 $um.min^{-1}.mg^{-1}$) were lower ($p<0,05$) at the group were 100% of corn meal was replaced by mango meal (Figure 1). Statistical difference was only observed in total protease activity analysis.

Table 3. Growth performance and metabolic intermediates of hybrid surubim *Pseudoplystoma* sp. fed diets containing various levels of mango meal in replacement for corn meal.

	MM0	MM33	MM66	MM100	
Performance					
FBW (g.fish^{-1})	32.50 ± 1.37	31.35 ± 1.79	30.09 ± 2.81	30.09 ± 2.18	
WG (g)	17.62 ± 1.11	17.08 ± 1.60	15.84 ± 2.81	15.41 ± 1.92	
SR (%)	100	100	100	100	
FCR	0.76 ± 0.04	0.75 ± 0.06	0.82 ± 0.17	0.86 ± 0.09	
FC (g.day^{-1})	4.4 ± 0.12	4.2 ± 0.06	4.2 ± 0.15	4.4 ± 0.12	
Metabolism					
Blood Plasma					
TC (mg.dL^{-1})	141.83 ± 46.10	104.87 ± 37.77	81.32 ± 26.49	71.36 ± 14.40	y=-0,7044+134,89 / R^2= 0,93
TP (mg.dL^{-1})	3.98 ± 0.94	4.13 ± 0.68	3.28 ± 0.46	1.70 ± 1.03	$y = -0,0004x^2 + 0,0152x + 3,9968$ $R^2 = 0,9985$
TG (mg.dL-1)	163.87 ± 10.98	139.95 ± 17.58	121.77 ± 22.62	110.69 ± 25.23	
G (mg.dL^{-1})	105.08 ± 31.24	100.11 ± 14.84	141.11 ± 36.09	128.11 ± 24.51	$y = -0,0005x^3 + 0,066x^2 - 1,835x + 105,08$ $R^2 = 1$
FAA (nmoles.ml^{-1})	24.51 ± 2.62	23.51 ± 4.03	41.10 ± 8.44	38.13 ± 8.94	$y = -0,0002x^3 + 0,0262x^2 - 0,6995x + 24,51$ $R^2 = 1$
Liver					
GC (umols glicose.g^{-1})	39.91 ± 8.28	32.49 ± 4.10	15.70 ± 4.45	20.30 ± 4.24	$y = 0,0001x^3 - 0,0192x^2 + 0,2469x + 39,91$ $R^2 = 1$
ALT (U.L^{-1})	5.01 ± 1.80	3.92 ± 1.13	4.58 ± 1.49	4.58 ± 1.49	

Values are means ± SD. FBW = Final body weight, WG = weight gain, SR = survival rate, FCR = feed conversion rate, FC = feed consumption, TC = total cholesterol, TP = total protein, TG = triglyceris, G = glucose, FAA = free amino acids, GC = liver glycogen, ALT = liver alanin amino transferase

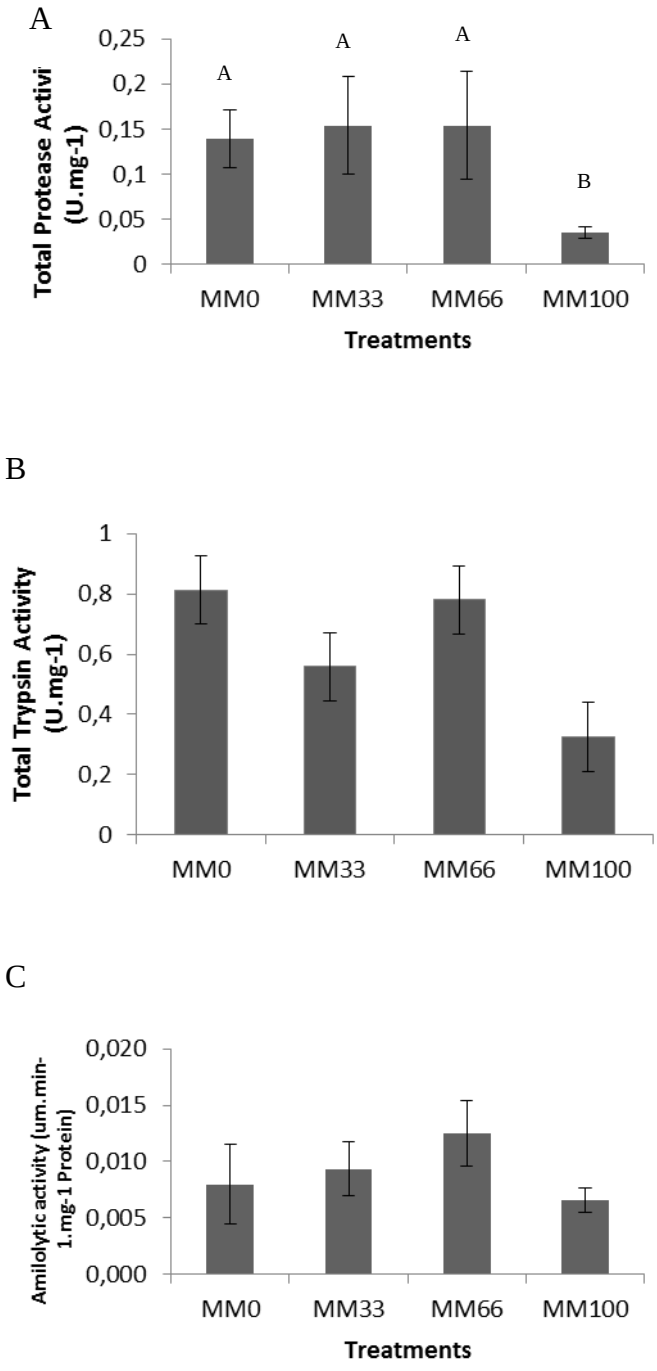

Figure 1. Intestine digestive acitivity (means±S.D) of surubim juvenile fed diets containg mango meal replacing corn meal. **A**. Total alkaline protease activity. **B**. Trypsin activity. **C**. Amylase activity. Different letters in the same line indicates statistical difference ($P<0,05$) among treatments (Tukey).

A caseinolytic zymogram was prepared to compare the proteolytic activities of the treatments (Figure 2A). Similar patterns were observed for MM0 and MM33. Five caseinolytic bands were observed for MM0 and MM33; six for MM66, five of them with higher intensity (A) and two for MM100.
The amylase zymogram (Figure 2B) revealed that the surubim *Pseudoplatystoma* sp. has two types of amylase. The intensity of one of the amylases was always higher than the other. It can also be observed in the fourth line, which is the treatment MM100, the activity was a little lower than the others.

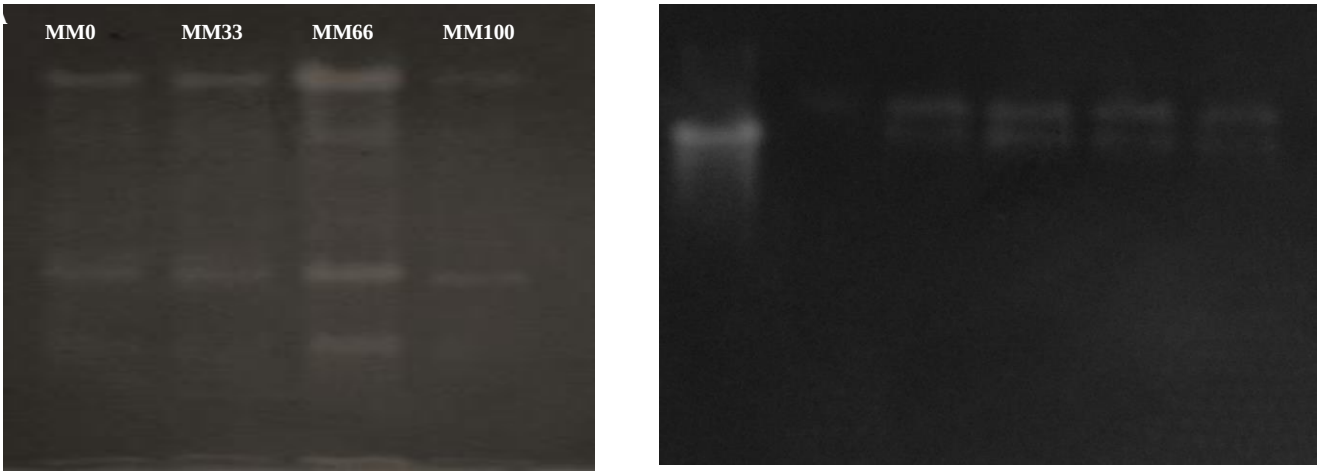

Figure 2. A. Zymogram of digestive protease (3% casein as substrate) of intestine enzyme extract from surubim *Pseudoplatystoma corruscans* fed on diets containing different inclusion levels of mango meal. Lanes correspond to dietary mango meal inclusion (%): MM0% ; MM33%; MM66%; MM100%. **B.** Zymogram of digestive amylase (2% starch as substrate) of intestine enzyme extract from surubim *Pseudoplatystoma corruscans* fed on diets containing different inclusion levels of mango meal. Lanes correspond to dietary mango meal inclusion (%): MM0% ; MM33%; MM66%; MM100%.

All metabolic parameters showed significant difference among treatments (Table 3), with exception of triglycerides. Total cholesterol showed a linear regression. At the treatment with 100% mango meal inclusion, total cholesterol was lower. Plasma total protein showed a second order polynomial regression model, although lower level was also observed at the treatment with 100% mango meal

inclusion. Glucose and free amino acids in the plasma showed a third order polynomial regression model. At both parameters analyzed lowers levels were observed at treatments with 0 and 33% inclusion of mango meal. Liver glycogen also showed a third order polynomial regression model with higher levels observed at treatments with 0 and 33% inclusion of mango meal.

Discussion

Despite the small nutritional importance that carbohydrate seems to have in carnivorous fish, in this present study the change in the qualitative pattern of carbohydrate source modified the metabolism of a carnivorous tropical fish. There was physiological adaptation to the diet, although growth has not changed.

Carbohydrate inclusion may improve the protein efficiency of fish feed (Kalaman et al., 2012). It is known that the amount of carbohydrate influences the performance, digestive enzymes, digestibility and metabolism in carnivorous fish (Ren et al., 2011). Alternative carbohydrate source also has impact over the performance and metabolism of carnivorous fish species (Adaminou et al., 2009). Searching for an alternative carbohydrate source for commercial fish feed may not only have economic importance, but mainly physiological importance for growth and metabolic maintenance (Polakof et al., 2012; Adamidou et al., 2011), which will also reflect in an economic answers for the producer.

In this present study there was not any statistic difference among the performance parameters evaluated, during the 60 days trial. When corn meal was replaced by mango meal with skin, juvenile tilapia performance was negatively influenced by this source of carbohydrate used in the feed when fed 66 and 100% inclusion (Souza et al., 2013). Although, that is not the same for all fish species and the natural feeding behavior must be considered. Fruit skin has a high amount of tannins which is an anti-nutritional factor, but usually some fish species can tolerate certain amount of tannins (Francis et al., 2001; Siddhuraju and Becker 2001). Those substances are enzymes inhibitor that bind to proteases in its substrate *loci* and negatively affects fish growth, but some species can compensate it by increasing the trypsin activity in the intestine (Zheng et al., 2012; Francis et al., 2001).

Although this study showed that the carbohydrate source evaluated did not influence the performance, *Pseudoplatystoma corruscans* growth is affected by the source of feed energy ingested (Arslan et al., 2009). A diet for a carnivorous tropical fish might attend the energy requirement for the species using either carbohydrate or lipid, but the correct fatty acid and amino acid profile is essential for optimal

performance and survival. Since no deformities were observed in the fish from this study, probably the fish oil from the fish meal used and soybean oil added fatty acid profile, providing the requirement for the specie.

High protease activity, especially trypsin is an indicator of growth in fish (Torrisen et al., 2006). In this study, protease activity was lower in the group that had 100% of corn meal replaced by mango meal and growth parameters were not different. There was no difference among digestive enzyme levels in the treatments from 0 through 66% of corn replacement by mango meal. Differences observed might be probably due to the random selection of fish sampled for the assays.

In carnivorous fish species, protease activity will always be higher when compared to the other digestive enzymes activities no matter what the macronutrients sources are, due to their natural feeding behavior (Lundsted et al., 2004). Digestion potential ratio analysis shows that a carnivorous fish exhibits higher amylase activity than an herbivore (1:1.28) and an herbivore shows higher activity of acid protease than a carnivore (1:1.25) (Chacrabarti et al., 1995). It seems that fish in general have an evolutionary adaptation to digest any kind of food, which is, clearly, an advantage over any kind of food deprivation.

Juvenile tilapia fed diets with 50% of rapeseed meal inclusion, which is rich in anti-nutritional factors such as tannins, also had their protease activity decreased when compared to other plant protein sources without high amounts of anti-nutritional factors (Lin et al., 2010). Total alkaline protease activity is the sum of specific proteases such as trypsin, chymotrypsin and others, which cleave the peptides from protein diet into amino acids that are then bioavailable for fish growth or fish metabolism. Trypsin activity has been set as a key factor to supply amino acids and peptides for fish growth (Sunde et al., 2001). Also trypsin activity and growth rate are linked through trypsin´s effect on the capacity of fish to convert food into body component (Lemieux et al., 1999). As it can be seen from the results found in this present work, the highest trypsin activity was found in the group where 66% of the corn meal was replaced by mango meal. In these same group free amino acids in the plasma was also higher than the other groups, which might also indicate that amino acids from feed protein were available for fish growth.

Different amounts of the dietary macronutrients used in carnivorous fish feed influence the activity of its digestive enzymes, including amylase and lipase. Indeed digestive tract pH may also affect it (Pérez-

Jimenéz et al., 2009). But there are also studies showing that the amount of starch in the feed does not influence protease or amylase activity in carnivorous fish (Couto et al., 2012). In this present study, amylase activity was not statistically different among the tested groups. It may be due to the amount of carbohydrate found in the diet (Pérez-Jimenéz et al., 2009). However tilapia fed different sources of plant did not have amylase activity affected by it (Lin et al., 2010).

Zymogram has proved to be an effective tool in detecting changes in digestive enzyme activity in fish submitted to different diets (Santos et al., 2013). Both protease and amylase zymograms showed the same enzyme activity found in the crude extract assays. It also confirmed the inhibitory effect of the anti-nutritional factors found in the diets with 100% of mango meal. In short, there was a clear physiological adaptation of surubim digestive enzymes as an answer to the different carbohydrate patterns found in the diet. This adaptation promoted alteration in feed nutrients bioavailability which changed metabolic profile of the fish.

The metabolic responses of the surubim were highly influenced by the level of mango meal. Fish total cholesterol decreased with the increase of mango meal, as it has also been observed in mammals (Salgado et al., 2008). Fruits in general have high amounts of soluble fibers which link to cholesterol irreversibly and to bile acids carrying them to the feces, thus cholesterol is not absorbed in the liver (Zhang et al., 2011).

Total glucose in the plasma was lower in fish groups that had the lowest amounts of mango meal in their diets. In those same groups, liver glycogen was higher than the others groups. On the groups where mango meal inclusion was higher, it was observed higher glucose levels and lower glycogen levels, probably due glycolysis. Liver glycogen reduction for glucose maintenance has already been observed in tambaqui (*Colossoma macropomum*) (Almeida et al., 2011). Unlike the results observed in this study, rainbow trout fed low and high amount of starch did not have glucose and total free amino acids values changed, but triglycerides changed (Kalamam et al., 2012). However like the result found in this study, triglycerides level in sea bass fed non-starch polysaccharide diet was lower than fish fed starch polysaccharide diet (Gatesoupe et al., 2014). Triglycerides, which indicates that excess carbohydrate from the diet was not used as energy source and was converted in fat acids and glycerol, also had a slightly change when intra-peritoneal glucose was injected in rainbow trout. Only 24h after the injection the level of this parameter started to increase and was about the same as 2h after the

injection (Enes et al., 2012). This proves that glucose influences the level of triglycerides, but nothing has been specified about the influence of the carbohydrate source. However carbohydrate concentration may affect triglycerides levels (Correa, 2007). Levels of glucose in fish has been related to their tolerance in time rather than temperature, fish species and it´s feeding habit than to its diet (Enes et al., 2012).

Kumar et al. (2009) found that carps fed diets with non-gelatinized corn had lower glucose levels than the groups fed gelatinized corn. Which is a similar result found in this study, since the treatments with lower mango meal had higher amounts of starch. Mango meal is compound by short chains, non-starch polysaccharides (sacharose and fructose) which improve its digestibility and energy availability to the animal (Couto et al., 2012). When liver glycogen concentration is low it might mean that glucose available in the body is being used for metabolic energy (Enes et al., 2009). It has already been proved for carnivorous fish that the amount of carbohydrate in the diet influences liver glycogen and plasma glucose, but not the source of carbohydrate (Kalanam et al., 2012; Ren at al., 2011). Recent studies have shown that there is no difference between ingested carbohydrate and gene expression of enzymes from the gluconeogenesis pathway (Enes et al., 2009). The fact that the values of ALT have not changed among the different treatments tested means that no damage was caused in the liver (Fuentealba et al., 2011). Also carbohydrate source and the amount evaluated did not influence transamination by ALT. The activities of transaminases and deaminases are useful to evaluate the feeding status in some fish (Moyano et al., 1991; Melo et al., 2006).

Total protein in the plasma was lower than in the groups with 0 and 33% inclusion level of mango meal in the diet. This fact might also indicate a change in protein and free amino acids metabolism and their synthesis in the liver (Youself et al., 2006). Total plasma protein is related to globulins levels, so the lower levels found in the MM66 and MM100 groups might indicate a fish immunity decrease.

Conclusion

Although growth performance was not affected during the 60 days trial, the decrease in plasma protein and glycogen, as well as the increase in glucose in the treatments MM66 and MM100, showed negative metabolic responses to these diets. Therefore diet nutrients caused metabolic changes, even at 60 days feeding trials. These changes are a sign that performance can be compromise at long term feeding periods with diets that contains more than 33% inclusion of mango meal. It was proved through the analyses performed that only growth performance cannot answer about the inclusion of an alternative ingredient in fish diet.

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CAPÍTULO II

Protein:lipid ratio changes growth, digestive enzymes activities and metabolic profile in hybrid surubim (*Pseudoplatystoma fasciatum* x *Leiarius marmoratus*)

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CAPÍTULO II

Protein:lipid ratio changes growth, digestive enzymes activities and metabolic profile in hybrid surubim (*Pseudoplatystoma fasciatum* x *Leiarius marmoratus*)

Abstract- The effect of protein:lipid ratio on growth, nutrient utilization, digestive enzyme activities, intermediary metabolism and erythogram of the hybrid surubim *Pseudoplatystoma corruscans* x *Leiarius marmoratus* was evaluated. Juveniles (±8.90g initial weight) were fed 3% of total biomass for 60 days in four isoenergetic experimental diets with different protein: lipid levels 9.00; 4.60; 3.54 and 1.78. Final weight was higher in groups with high protein:lipid ratio. The activity of intestine total alkaline protease and trypsin ($U.mg^{-1}$ protein) were not statistically different. Chymotrypsin ($U.mg^{-1}$ protein) was higher for high protein:lipid ratio groups. Lipase ($U.mg^{-1}$ protein) was higher for low protein:lipid ratio groups. Amylase was higher for intermediary groups which ingested higher carbohydrate content. Blood glucose, plasma total protein, triglycerides and cholesterol were significant lower for low protein:lipid ratio. There was no difference in plasma free amino acids among treatments. Liver glycogen was lower at 9.00 and 1.78 and higher at 4.60 and 3.54. Hematocrit was higher at high protein:lipid ratio and the only hematopoietic parameter with significant difference. Hybrid surubim showed metabolic adaptation to the different protein:lipid ratio ingested. The result suggests that 4.6 protein:lipid ratio showed better protein spare effect of lipid. It was noticed that nutrient requirements for cold water carnivorous fish species cannot be used for tropical carnivorous.

Key Words- surubim; alkaline protease, amylase, lipase, intermediary metabolism

INTRODUCTION

Evaluation of protein:energy ratio for fish has been widely study to provide macronutrients values for best protein and energy efficiency of diets in order to improve growth as well as decrease nitrogen compounds release in the environment (Almeida et al., 2011; Chatzifotis et al., 2010; Melo et al. 2006). Studies that evaluate protein:lipid ratio commonly analyze growth and nutrient utilization (Arslan et al., 2013; Liu et al., 2013; Teixeira et al., 2013; Karalazos et al., 2011; Bicudo et al., 2010). Digestive enzyme activities and metabolic responses assays has also been performed for tropical species such as pacu *Piaractus mesopotamicus*, tambaqui *Colossoma macropomum*, blunt snout bream *Megalobrama amblycephala* and for the cold water fishes like rainbow trout (Li et al., 2012; Saravanan et al., 2012; Almeida et al., 2011; Bicudo et al., 2009). Studies that show combined results of growth performance, nutrient utilization, digestibility, enzyme activities and metabolic responses, provide a more complete information of how fish body is responding to different protein:energy ratios and its whole physiological adaptation. Therefore producers, researchers and fish feed industry can have the perspective of the effect on the fish of a long term feeding period of different diet.

Studies about carnivorous fish nutrient utilization are more common in cold water fishes, such as salmon and rainbow trout. Tropical carnivorous fish are less studied and there is a lack of information about it. Performance, carcass composition, nutrient utilization, metabolic profile and digestive enzyme activities fed different protein, lipid and carbohydrate levels have been studied for the South American siluriforme *Pseudoplatystoma corruscans* (Martino et al., 2005; Lundsted et al., 2004; Martino et al., 2002) and *Pseudoplatystoma reticulatum* (Cornelio et al., 2014), Mediterranean meagre (*Argyrosomos regius*) and Asian siluriforme *Silurus meridionalis* (Liu et al., 2013). When lipid feed percentage is over metabolic and growth demand for fish, it is stored mainly as visceral fat and also affects lipase activity, cholesterol and triglycerides levels and decrease growth. It is desirable that protein from the diet is mainly used for body growth and not as metabolic energy. Protein in excess is discharged from the body as nitrogen compound, mainly ammonia, which causes negative environment impact to the water body where fish is raised. Also in this situation the most expensive feed ingredients is being discharged.

Studies with hybrids are still published (Oliveira, 2014; Essa et al., 2011). The hybrid surubim *Pseudoplatystoma corruscans* x *Leiarius marmoratus* is largely raised in Brazil due to its higher

growth index, boneless, tender meat and it accepts easy the feed since its larval phase. Its production is over 800 tons per year which is consumed in Brazil and also exported (MAPA, 2011). Carnivorous fish feed has high percentage of protein inclusion during all phases compared to omnivorous fish species. *Pseudoplatystoma corruscans* and *Leiarius marmoratus* are piscivorous species, but L. *marmoratus* can have herbivorous feeding behavior as well (Fishbase, 2014). Rarely are the information published about this promising hybrid species (Maciel et al., 2014; Oliveira et al., 2013; Souza et al., 2014; Ventura et al., 2013) and also in the recent literature there is little information about the whole effect on growth of dietary macronutrient and its utilization by carnivorous tropical fish.

In response of the facts listed above, the aim of this study was to evaluate the effect of protein:lipid ration on growth, nutrient utilization, digestive enzyme activities, intermediary metabolism and erythogram of the surubim hybrid *Pseudoplatystoma corruscans* x *Leiarius marmoratus*.

MATERIAL AND METHODS

1. Experimental diets

Four isoenergetic (20kJ.g^{-1}) on crude energy basis diets containing different levels of protein and lipid were made as experimental treatments. It was formulated in order to have different lipid:protein values: 9.00 (45.44% crude protein/5% lipid); 4.60 (42.15% crude protein/9.87% lipid); 3.54 (39.39% crude protein/11.07% lipid); 1.78 (34.64% crude protein/19.19% lipid). The dietary formulation and proximate composition are presented in Table 1. Feed were made by a commercial fish feed industry, Poytara®- São Paulo – Brazil. The chemical composition of the ingredients was determined according to the AOAC (1990) for the analyses of moisture, ash, crude protein, crude fiber, energy and ether extract (Table 1).

Table 1. Formulation and proximate analysis of experimental diets (% dry matter).

	9.00	4.60	3.54	1.78
Ingredients				
Fish meal 66%	31.50	27.00	21.00	13.00
Corn meal	34.00	34.00	34.00	34.00
Poultry by product 67%	15.00	11.00	9.00	7.00
Soybean meal 34%	17.00	21.00	21.00	25.00
Vitamin and Mineral*	2.00	2.00	2.00	2.00
NaCl	0.25	0.25	0.25	0.25
Antioxidant	0.25	0.25	0.25	0.25
Soybean oil	-	5.00	10.00	15.00
Cellulose	-	-	4.00	-
Proximate composition (%)				
Moisture	1.50	2.19	2.10	1.23
Crude protein	45.44	42.15	39.39	34.65
Ether extract	5.02	9.87	11.07	19.19
Nitrogen free-extract	25.10	24.45	30.00	21.00
Ash	11.74	10.49	9.53	8.55
Crude Fiber	7.89	12.01	7.39	13.72
Energy ($kJ.g^{-1}$)	20.10	19.94	20.48	20.43

***** Rovimix fish:vit. A: 5.000.000 UI; vit. D3: 200.000 UI; vit. E: 5.000 UI; vit. K3: 1.000 mg; vit. B1: 1.500 mg; vit. B2: 1.500 mg; vit. B6: 1500 mg; vit. B12: 4.000 mg; vit. C: 15.000 mg; folic acid: 500 mg; pantotenic acid: 4.000 mg; B.H.T.: 12,25 g; biotin: 50 mg; inositol: 1.000 mg; nicotinamide: 7.000 mg; coline: 40 g; cobalt: 10 mg; cupper: 500 mg; iron: 5.000 mg; iode: 50 mg; manganese: 1.500 mg; selenium: 10 mg; zinc: 5.000 mg; q.s.q.: 1.000 g.

2. Fish and Feeding

Fish were obtained from CODEVASF (Companhia de Desenvolvimento do Vale do São Francisco e Parnaíba), a Brazilian Federal development company. Juvenile hybrid surubim were acclimated in the laboratory for 15 days and fed a commercial diet (45% crude protein) before trial began. To initiate the trial all the fish were individually weighed (average 8.9g). After this procedure they were sorted in 1000L PVC tanks, with 10 fish per tank. Three replicate groups of fish were used for each diet. Tanks were built in a closed recirculated water system with a biofilter. Water parameters were monitored weekly (average temperature was 28.3˚C; pH 7.0 and dissolved oxygen 4.5 $mg.l^{-1}$). Fish were fed twice a day, at 3% (800h and 1700h) for 60 days. Each tank was cleaned daily by scrubbing and siphoning the accumulated wastes, algae or any dirt that possibly came from the water.

3. Sample collection

At the end of the trial fish were starved for 24h. All fish in each tank were individually weighed for performance parameters: final body weight (FBW), weight gain (WG), apparent feed conversion rate (AFC), protein efficiency (PE), energy efficiency (EE), condition factor (CF), specific growth rate (SGR), carcass yield (CY), viscera somatic index (VSI). Fish were anesthetized and blood samples from 4 fish, randomly selected, from each tank were draw from the causal vein. Subsequently fish were euthanized with eugenol (0.08 mL.L) for tissue removal. Glucose (Glc) was immediately measured with an automatic monitor (Accu-Ckech®). In sequence those blood samples were centrifuged at 1000 rpm for 5 minutes and the plasma stored in a freezer (-20˚C) for a short period until the beginning of the total protein (PT), triglycerides (TG), cholesterol (TC), free amino acids (FAA).

Subsequently blood draw, liver and total intestine samples were collected from the same fish and immediately stored in a freezer (-20˚C) for short period until the beginning of the glycogen (GC) and enzymes activities analyzes.

4. Performance

Performance was measured by the following equations:

WG (g) = [final body weight (g) – initial body weight (g)]

FC = feed consumed (g)/time

AFC = [feed consumed (g)/ weight gain (g)] x 100

PE = [weight gain (g)/ total protein intake ($g.kg^{-1}$, dry weight)] x 100

EE = [weight gain (g)/ total energy intake ($KJ.kg^{-1}$, dry weight)] x 100

CF = {final weight (g) x [final total length (cm)]^3} x 100

SGR = {ln [final weight (g)] – ln [initial weight (g)]/time x 100

CY = final weight (g) – viscera weight (g)

VSI = [viscera weight (g)/ final body weight (g)] x 100

SR (%) = 100 x (final fish number/ initial fish number)

5. Intestine Digestive Enzyme Activities

After collected intestine were immediately stored in -20°C. At the laboratory it was homogenized in 0.01M Tris-HCl pH 8.0 buffer using a tissue homogenizer. The resulted preparation was centrifuged at 10,000xg for 10 min at 4°C. The supernatants (crude enzyme extract) were used for analysis assays (Santos et al., 2013).

5.1 Total alkaline protease activity

The total enzymatic activity of proteases present in crude extracts was performed using 1% azocasein as substrate, prepared in 10 mM Tris-HCl, pH 8.0. Aliquots containing 30 μL of the crude extract were incubated with 50 μL of substrate solution for 1 hour at 25 °C. Then, 240μL of 10% trichloroacetic acid was added to stop the reaction. After 15 minutes the mixture was centrifuged at 8,000 xg for 5 minutes. The supernatant was collected and 70 μL of it was mixed in 130 μL 1M sodium hydroxide solution (revealing solution) in microplates. The absorbance was measured on a microplate reader (xMark Bio-rad) at a wavelength of 450 nm. A negative control (blank) was performed, replacing the enzyme extract by a solution of 10 mM Tris-HCl, pH 8.0 with added 0.15 M NaCl. The activities were carried out in triplicate and one unit (U) of enzyme activity was defined as the amount of enzyme required to hydrolyze azocasein and produce a change of 0.001 units of absorbance per minute.

5.2 Trypsin Activity (E.C. 3.4.21.4) and Chymotrypsin Activity (E.C.3.4.21.1)

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

5.3 Lipase Acitivity (E.C. 3.1.1)

No specific lipase activity from intestine was based on Albro et al. (1985). The reaction was incubated in 0,4 mM p-nitrophenil meristate with a 24 mM ammonium bicarbonate buffer solution and 0,5% Triton X-100. After 30 min, the reaction was stopped with NaOH 25 mM. The absorbance was measured at 405 nm with a microplate reader (Bio Rad xMark™). The specific activity was expressed as the amount of enzyme needed to form 1 μmol of hydrolyzed per minute (U) per protein mg (U/min/ protein mg).

5.4 α-Amylase Activity (E.C. 3.2.1.1)

α-amylase activity was based on the method of Bernfeld (1995) using 2% (w/v) starch solution as substrate. The enzymatic preparations were in 0.2 M citrate-phosphate buffer, pH at 7.0 at 37 °C at 30 minutes. Then the assay was by addition of 3,5-dinitrosalicylic acid (DNSA) at 100°C for 10 minutes. The absorbance was measured at 570nm using a microplate reader (Bio-Rad 680). Both a substrate free control and an enzyme free control were run. The amount of maltose released from this assay was determined from the standard curve using commercial maltose. One milliunit of specific activity was defined as the amount of enzyme needed to release 1μg maltose per minute per milligram of soluble protein in enzyme solution at 37°C ($mU.min^{-1}.mg^{-1}$ Protein).

6. Metabolic intermediates

Metabolite concentration was carried out in plasma and liver tissue extracts free of protein.

Glucose was measured at the end of the trial, in the fish blood using an automatic glucose reader Accuchek®, Brazil. Total protein was determined by Biureto method (Hiller, 1948). Twenty (20) microliters of plasma was incubated for 10 minutes at 37 degrees Celsius. The reading was at 545 nm and the measure unit was expressed in $g.dL^{-1}$. Plasma triglycerides were analyzed by enzymatic colorimetric method using a commercial kit by Labtest®, Brazil. It was used 10 microliters of plasma samples containing reagent in 1ml of buffer pH 7.0, incubated for 10 minutes at 37 degrees. The samples were read at 500nm and the units of measurement were in $mg.dL^{-1}$. Total plasmatic cholesterol was determined by colorimetric enzimatic method, using a Labtest®, Brazil commercial kit with the reading done at 500 nm wave. Ten (10) microliters of plasma sample was incubated for 10 minutes at 37 degrees Celsius and the measure unit was expressed in $mg.dL^{-1}$. Free amino acids were determined in neutral extracts at 570nm (Copley 1941). Glycogen was assayed in alcoholic precipitates from tissues alkaline homogenates (Bidinotto et al. 1997).

7. Hematological Parameters

Blood samples were collected from caudal vein using 1 ml syringe. Heparin was used as anti-coagulant. Part of blood collected was intended to hematocrit analyses.

7.1 Hematocrit

It was realized through capillary centrifugation according to Goldenfarb et al. (1971). Capillary tubes were previously rinsed with heparin. After filled with blood sample the tubes were closed with an appropriate material and centrifuged at 10.000xG for 5 minutes. Reading was done in a standardized scale.

7.2 Hemoglobin Index

Hemoglobin concentration was determined according to Collier (1944). Ten microliters of blood were added to 2ml Drabkin solution, homogenized in Vortex and reading at the spectrophotometer was done at 540 nm. Hemoglobin concentration was following the expression:

$$g\% = Abs_{sample} \times 0.143 \times dilution$$

In order to determine erythrocytes number, blood smear was done (Tavares-Dias et al., 1999). Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin

concentration (MCHC) were determined from erythrocytes number, hemoglobin index and hematocrit (Wintrobe, 1934). Identification and cells nomenclature followed Tavares-Dias et al. (2002).

8. Statistic

All data were analyzed by one-way analysis of variance (ANOVA). Significant differences between means were determined by the Tukey´s multiple range test. Linear, polynomial and exponential models were tested for all regressions, and the best model was selected based on R^2. The probability level of 0.05 was used for rejection of the null.

RESULTS

Analysis of the results shows that protein:lipid ratio affected directly hybrid surubim growth and metabolism. Weight gain and apparent feed conversion ratio at 20 days after fish started to be fed with the different diets until the last day of the feeding trial were affected by different ratio evaluated (Table 2). Data show that during the feeding trial fish groups had different growth adaptation to treatments diets. High protein:lipid ratio (9.00 and 4.60) showed higher growth during the whole trial. Weight gain decreased as protein:lipid ratio decreased, lower value was at 1.78 ratio. In agreement with it, apparent feed conversion ratio increased as protein:lipid ratio decreased, highest value was at 1.78 ratio. During this partial evaluation it could be noted an adaptation to the feed by hybrid surubim.

At the end of the growth trial final weight was higher at 9.00 and 4.60 protein:lipid ratio (Table 3). At these same ratios, hybrid surubim feed consumption, protein efficiency, energy efficiency, condition factor, specific growth rate and carcass yield were also higher (Table 3). Protein efficiency was high up to 39% of crude protein inclusion. Energy efficiency was high up to 42% of crude protein inclusion level and 9% of lipid inclusion level. Viscero somatic index was higher at treatments with 3.54 and 1.78 protein:lipid ratio. Regression analysis showed second order polynomial equation for all parameters evaluated. High lipid and low protein content negatively affected growth of the carnivorous tropical siluriforme juvenile.

Table 2. Comparative weight gain and apparent feed conversion of hybrid surubim fed different protein:lipid ratio feed during trial period. (mean ± S.D.)

Weight Gain (g)						
	Protein:lipid ratios				Regression	
Days	9.00	4.60	3.54	1.78	Equation	R^2
20	18.37±1.0	14.59±0.7	13.90±0.6	8.35±0.4	$y = -0,3803x^3 + 5,1227x^2 - 15,732x + 22,267$	1.0
40	38.32±2.2	34.79±0.9	26.86±7.1	15.86±3.6	$y = -0,9691x^3 + 13,305x^2 - 43,229x + 56,147$	1.0
60	115.07±9.9	107.20±5.6	80.73±13.48	35.09±1.8	$y = -3,8484x^3 + 52,389x^2 - 168,11x + 190,04$	1.0
Apparent feed conversion rate						
	Protein:lipid ratios				Regression	
Days	9.00	4.60	3.54	1.78	Equation	R^2
20	0.57±0.0	0.72±0.0	0.76±0.0	1.26±0.0	$y = 0.0341x^3 – 0.4544x^2 + 1.3822x + 0.0469$	1.0
40	1.25±0.0	1.28±0.1	0.98±0.2	0.72±0.1	$y = -0.0182x^2 + 0.1634x + 0.5804$	0.84
60	0.93±0.0	0.91±0.0	1.12±0.1	1.64±0.2	$y = 0.0413x^3 – 0.5555x^2 + 1.7508x + 0.0506$	1.0

Table 3. Growth parameters of hybrid surubim fed with diferent protein:lipid ratio feed. (mean±S.D)

	Protein:lipid ratios				Regression	
	9.00	4.60	3.54	1.78	Equation	R^2
FW (g)	123.30±9.3	116.29±4.7	89.12±13.9	45.07±1.3	$y = -3.0827x^2 + 44.251x – 25.029$	0.99
FC (g)	107.0±1.5	96.91±1.4	89.41±5.4	57.19±4.0	$y = -1.7642x^2 + 25.793x + 17.608$	0.99
PE	2.36±0.1	2.63±0.17	1.64±0.2	0.71±0.0	$y = -0.0649x^2 + 0.9404x – 0.7845$	0.97
EE	2.42±0.2	2.24±0.1	1.64±0.2	0.71±0.0	$y = -0.0649x^2 + 0.9404x – 0.7845$	0.99
CF	0.87±0.0	0.84±0.0	0.78±0.0	0.67±0.0	$y = -0.0071x^2 + 0.1046x + 0.5046$	0.99
SGR (%)	4.51	4.25	3.93	2.51	$y = -0.0829x^2 + 1.1658x + 0.7297$	0.99
CY (g)	111.35±12.5	103.34±8.5	85.35±15.3	44.21±5.5	$y = -2.6308x^2 + 37.679x – 14.643$	0.99
VSI (%)	11.38±1.4	13.84±1.6	14.90±0.8	16.21±2.2	$y = 0.0317x^2 – 1.018x + 17.962$	0.99

FW=final weight; FC= feed consumption; PE=protein efficiency; EE=energy efficiency; CF=condition factor; SGR=specific growth rate; CY=carcass yield; VSI=viscera somatic index

Five intestine digestive enzyme activities were analyzed (Table 4). Total alkaline protease and trypsin were the only enzymes that did not show significant difference among treatments. Although for both enzymes a slight decreased was observed in the last treatment. Chymotrypsin ($U.mg^{-1}$) activity was lower in the treatments were feed protein levels were also lower. Chymotrypsin showed the highest activity values among proteases analyzed. Lipase (U.min.protein mg^{-1}) activity was higher in the treatment were feed lipid levels was higher. Amylase activity was higher in 3.54 and 1.78 protein:lipid ratio.

Table 4. Digestive enzymes activities ($U.mg^{-1}$ protein) from hybrid surubim (*Pseusoplatystoma fasciatum* x *Leiarius marmoratus*) intestine, fed different protein:lipid levels. (means±S.D)

	9.0	4.60	3.54	1.78
Total Alkaline Protease	0.107±0.03	0.144±0.02	0.103±0.02	0.098±0.02
Trypsin	0.597±0.17	0.807±0.35	0.764±0.38	0.428±0.12
Chymotrypsin	$3.41±1.86^{AB}$	$5.79±1.45^{A}$	$3.23±1.19^{B}$	$1.85±1.35^{B}$
Lipase	$0.078±0.00^{AB}$	$0.059±0.01^{A}$	$0.059±0.01^{A}$	$0.098±0.03^{B}$
Amylase	$0.416±0.06^{AB}$	$0.521±0.09^{A}$	$0.513±0.12^{A}$	$0.348±0.1^{AB}$

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

All plasma and liver intermediary metabolites were affected by dietary treatments, but plasma free amino acids (Table 5). The differences observed showed metabolic adaptation to the dietary treatments in order to maintain growth. Blood glucose and plasma total protein decreased as protein level decreased and lipid levels increased in each experimental treatment. The same data pattern was observed for triglycerides. Cholesterol was higher only at fish fed 9.0 protein:lipid ratio. Triglycerides and cholesterol showed linear regression. Plasma free amino acids had lower values at lower protein:lipid ratio treatment (1.78). Glycogen (umols glicose.g^{-1}) in the liver showed quadratic effect,

with higher values at treatments 4.60 and 3.54. Regression analysis showed second order polynomial equations for all others parameters.

Table 5. Blood, plasma and liver metabolic responses of hybrid surubim juvenile fed with different protein:lipid ratio. (mean±S.D)

	Protein:lipid ratios				Regression	
	9.00	4.60	3.54	1.78	Equation	R^2
Blood						
Glc ($mg.dL^{-1}$)	191.50±43.95	170.67±27.72	173.00±31.24	139.50±11.08	$y = -1,246x^2 + 20,284x + 109,44$	0.93
Plasma						
TP ($mg.dL^{-1}$)	3.64±0.35	3.15±0.39	3.15±0.17	2.41±0.09	$y = -0,0281x^2 + 0,4661x + 1,7095$	0.95
TG ($mg.dL^{-1}$)	102.10±17.37	59.18±20.05	72.69±10.63	55.93±18.88	$y = 6,2315x + 43$	0.82
TC ($mg.dL^{-1}$)	46.31±17.80	27.17±2.38	30.58±5.77	23.56±1.72	$y = 3,1133x + 17,179$	0.91
FAA ($nmoles.ml^{-1}$)	32.33±4.4	34.08±4.9	33.95±3.7	28.57±3.8		
Liver						
Glycogen (umols glicose.g^{-1})	22.69±9.2	67.78±5.9	50.22±5.2	37.4±5.0	$y = -2,5612x^2 + 25,888x - 2,4432$	0.92

Hematological parameters (Table 6) were influenced by the dietary treatments. Hemoglobin and hematocrit were higher in the treatments with higher protein:lipid ratio. Hematocrit was significantly higher at 9.00 and 4.60 protein:lipid ratio. Erythrocytes percentage, mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration did not show difference among treatments.

Table 6. Hematopoietic parameters of hybrid surubim fed with diferent protein:lipid ratio feed. (mean±S.D)

	9.00	4.60	3.54	1.78
Hemoglobin ($g.dL^{-1}$)	5.18±1.90	5.33±1.60	4.44±1.57	4.06±0.99
Erythrocyte ($x10^6$ µL)	500.47±150.93	610.42±230.11	650.04±230.11	520.68±190.57
Hematocrit (%)	37^{A}	32^{AB}	25^{B}	23^{B}
MCV (fL)	$1.03x10^6$	$1.58x10^6$	$1.90x10^7$	$2.25x10^7$
MCH ($g.dL^{-1}$)	1.032	0.951	0.777	0.848
CMCV ($g.dL^{-1}$)	10.31	9.51	7.77	8.48

*Different letters in the same line indicates statistical difference (*P<0,05*) between treatments (Tukey). MVC (mean corpuscular volume); MCH (mean corpuscular hemoglobin); CMVC (concentration of mean corpuscular hemoglobin).

DISCUSSION

Growth performance was influenced by protein:lipid ratio during all trial period. This influence was observed at the first 20 days and lasted for the 60 days trial period, showing different nutrient utilization by the different treatments groups along the time. Early answer by dietary treatments was also observed in others trial with fish of different feeding behavior (Teixeira et al., 2013; Johansen et al., 2011; Karalazos et al., 2011). When feed consumption decreases all growth parameters are affected

due to low nutrient intake. This fact is evident when 9.00 and 1.78 protein:lipid ratio treatments growth parameters are compared in the study. Feed consumption affected not only growth, but also feed efficiency, protein efficiency, energy efficiency and condition factor. Apparent feed conversion ratio was higher in lower lipid inclusion treatments. This was also observed for channel catfish *Ictalurus puctatus* fed diets with 2.50- 1.50% of catfish offal oil inclusion (Li et al., 2010) and for surubim *Pseudoplatystoma corruscans* fed diets with 6-10-14-18% of lipid levels (Martino et al., 2002). It is noticed that high lipid levels leads the fish to early satiation affecting feed consumption (Martino et al., 2002; Liu et al., 2013) as it was also observed in this study. Although the same studies showed that the interaction between protein and lipid did not affect feed intake. Energy levels (18-21 $MJ.kg^{-1}$) also did not affect feed intake, feed conversion and protein efficiency of 90g surubim in a 45 days trial (Teixeira et al., 2013). Lipid level did not affect the growth performance of surubim (P. *corruscans*) when it was fed level from 12-20% inclusion (Arslan et al., 2013). In the present study lipid levels of 11 and 19% and low protein percentage decreased growth performance while 5 and 9% showed higher growth.

Protein and energy efficiency decrease as protein:lipid ratio decreased was observed in the present study. These can explain the decrease in the specific growth rate and increase in the viscerosomatic index also when these ratios decreased. Whereas 90g surubim growth performance decrease when fed increasing levels of protein (36-52%) and decreasing levels of soybean oil (9-6%) (Teixeira et al., 2013). A protein spare effect by lipid is highly desirable by producers, but growth parameters shows that there is a limit for lipid utilization. All feeds from the trial were formulated in order to have the same crude energy values, therefore lipid level was the limiting factor for feed intake by the fish. The same was found for the Asian catfish *Pangasius hipophythalmus* fed different protein and lipid levels diet (Liu et al., 2011).

Values of viscerosomatic index were significantly higher at the 1.78 protein:lipid ratio treatment where protein levels were low and lipid levels were high, which means that feed energy was stored as fat. At this same treatment carcass yield was lower compared to other treatments. All diets had the same carbohydrate level of inclusion, therefore these data contributes to prove that nutrients are differently used by fish and that there is a metabolism change due to feed nutrients (Melo et al., 2012; Almeida et al., 2011). Although feed intake and feed conversion ratio was also affected by protein and lipid levels in diets for a Asian siluriforme, there was no difference in values for viscerosomatic index, hepatosomatic index, protein and energy retention efficiency (Liu et al., 2011).

Nutrients from the feed are only bioavailable to growth and metabolism after the complete digestion process. Protein, carbohydrate and lipid concentration from the diet influence digestive enzymes activity (Santos et al., 2013; Li et al., 2012; Pérez-Jimenes et al., 2009). Among the proteases evaluated in this study, chymotrypsin was the only one that showed significant difference. It decreased as dietary protein decreased. Total protease activity also decreased as the percentage of dietary protein decreased in diets for *Labeo rohita* fingerlings (Debnath et al., 2007). Proteases break down polypeptides into amino acids which are bioavailable for fish growth and metabolism. Several factors, such as other nutrients, feed rate and water temperature influence the correlation of proteases and growth, therefore it is common to find in the literature different results about it. In the present study protein efficiency and feed conversion were higher in the same treatment that chymotrypsin activity was higher. Similar results were found for salmon *Salmo salar* and surubim *Pseudoplatystoma corruscans* (Rungruangsak-Torrissen et al., 2006; Lundsteadt et al., 2004; Lemieux, 1999), on the other hand contrary results were found for carp (Mohanta et al., 2008).

Treatment with 1.78 protein:lipid ratio had higher lipid content which stimulated lipase activity and probably increased fatty acids availability as in the same treatment, feed consumption was lower. As consequence viscerosomatic index was higher and energy efficiency and weight gain lower. Growth of blunt snout bream *Megalobrama amblycephala* fingerling, silver barb *Puntius gonionotus* fingerlings, Atlantic cod larvae, were positively affected by diets with high lipid levels which promoted higher lipase activities (Li et al., 2012; Mohanta et al., 2008; Mac Donald et al., 2006). Carbohydrate inclusion in the experimental diets was the same for all treatments and the significant difference among treatments might be due to the relationship between the other enzymes activities as it was observed for *Labeo rohita* fingerlings (Debnath et al., 2007). The results above, from the present study and others, confirms the fact that several factors affect the relation between digestive enzymes activity and growth. Lipid and carbohydrate are energy sources and both percentages in the diet influences digestive enzymes activities, promoting increase or decrease of fatty acid, monosaccharides, glucose and other saccharides availability to body growth and metabolism. Thus lipid and carbohydrate and its specific enzymes activities influence protein and energy efficiency.

Blood, plasma and liver metabolic parameters indicate how those nutrients are being used by animal metabolism. Glucose level was higher in the treatment with high protein:lipid ratio (9.00). However not true for *Labeo rohita* fingerlings (Debnat et al., 2007), raibow trout *Onchoryncus mykiss* (Skiba-Cassy

et al., 2013), surubim *Pseudoplatystoma curruscans* (Lundsted et al., 2004) fed different protein level but the same lipid level and pacu *Piaractus mesopotamicus* (Bicudo et al., 2009), fed different protein and oil level. It seems that there is a metabolic adaptation in response to different diets according to fish natural food habit and percentage of each nutrient inclusion. Due to the percentage of protein inclusion, high levels of glucose might also indicate gluconeogenesis as it was observed for *Rhamdia quelem* (Melo et al., 2006). Higher levels of feed protein also resulted in higher levels of plasma total protein, as has been noticed for carp *Cyprinus carpio*, *Rhamdia quelen* and pacu *Piaractus mesopotamicus* (Nasir & Al-Sraji 2013; Coldebella et al., 2011; Bicudo et al., 2009). Plasmatic protein levels are related to globulins levels. Therefore it is a possible indicator of fish immune system. Plasma free amino acids levels are also a response to fish nutrient metabolism (Mach & Nortvedt, 2011). In the present study free amino acids did not show significant difference at different protein:lipid levels. However, growth parameters and nutrient utilization at 1.78 protein:lipid ratio treatment were lower compared to the others. Therefore, value of plasmatic free amino acids in this treatment might indicate that fish mobilized body protein as an adaptation process to low nutrient intake (Costas et al., 2011).

Triglycerides levels were lower as lipid level increased and protein levels decreased. Pacu *Colossoma macropomum* also fed diets with different protein:lipid ratios (7.1; 3.71; 2.23; 1.41), showed triglycerides decreased values as lipid levels increased in the dietary treatments (Almeida et al., 2011). *Rhamdia quelem* fed different carbohydrate:lipid levels (0.3-6.5) did not show triglycerides difference or growth parameters (Moro et al., 2010). Cholesterol levels also decreased as lipid levels increased in the diets. Triglycerides and cholesterol levels decreased as carbohydrate:lipid (202.5; 17.1; 7.5; 4.4; 2.8; 1.7) levels increased in diets for herbivorous grass carp *Ctenopharyngodon idella* (Gao et al., 2010). According to the authors it was due to lipid transport in the tissues as lipid levels increased in the diet. This is the opposite found in this study, probably because in the present study feed intake decreased as lipid levels increased. Different results for these metabolic variables in different fish species and feeding habit also strengthen the fact of fish have a high metabolic adaptation ir order to improve growth.

In the present study at 9.0 protein:lipid ratio glycogen level was the lowest, as growth parameter and nutrient utilization were higher than 4.60 and 3.54 ratios. These might indicate that at 9.0 ratio, nutrients provided from energetic ingredients were used to maintain basal metabolism and protein was used for body growth. At 4.60 and 3.54 ratios it can be observe higher glycogen deposition in the liver,

probably due to higher carbohydrate intake from the diet. Almeida et al. (2011) observed similar results from present work (1.78 ratio) when tambaqui *Colossoma macropomum* was fed different protein:lipid ratio diets. The authors affirms that the nutrient balance evaluated seemed to lead to glycogenolysis and glycolysis in tambaqui liver, as it was also observed in this study. Grass carp fed diet with decreasing levels of carbohydrate:lipid showed lower growth parameters concomitant to higher liver lipid, viscerosomatic and hepatossomatic index (Gao et al., 2010). This results support the fact also found in this study that increasing lipid levels increases fat deposition in fish body and decreases growth.

Protein:lipid ratio also influenced blood parameters which prove that macronutrients can influence it and as a consequence the immune system. Hematocrit, hemoglobin, mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) were higher in the treatments with higher protein level in the diets (9.0 and 4.60 ratio). Data were in accord with results found for common carp fingerlings fed different levels of protein (23.68; 19.86; 17.26; 13.82%) that also had higher red blood cells and mean corpuscular volume in the groups fed 23.68% crude protein (Nasir & Al-Sraji, 2013). Erythrogram reference range values for adults *Pseudoplatystoma* sp. is 1.720-2.020 $x10^6$ µL for erythrocytes, 30-35% for hematocrit, 5.2-6.1 $g.dL^{-1}$ for hemoglobin, 159.2-180.3 fL for mean corpuscular volume (MCV), 16.8- 18.8 $g.dL^{-1}$ for MCHC (Tavares-Dias et al., 2009). Lanbarrere et al (2012) evaluated erythrogram of 150g and 400g *Pseudoplatystoma* sp. kept in different density stocks, and found values in agreement of reference range for the species. Values for erythrogram found in the present study were lower than reference range values for adults probably due to age difference. In 1.78 protein:lipid ratio treatment, which had lower growth parameters, erythrogram value did not show significant difference from others treatment as well as hemoglobin, MCV and MCHC. This might indicate that fish body had a physiological mechanism to adapt to lower nutrient intake and maintain growth. High hemoglobin and MCV value means that high blood capacity to transport oxygen in an attempt to supply higher energetic demand also as a response to a stress situation (Lanbarrere et al., 2012). This fact evidenced the hypothesis of adaptation to poor nutrient intake in 1.78 ratio treatment.

Conclusions

There were few differences among all parameters evaluated between 9.00 and 4.60, therefore 4.60 showed better protein spare effect of lipid. High lipid levels affect negatively tropical carnivorous fish

species which proves that requirement for cold water fish cannot be used as basis. The surubim hybrid studied showed high physiologic and metabolic capacity adaptation to different levels of macronutrients intakes for growth maintenance. In order to have a complete overview of feed nutrient utilization by a fish species, it is imperative to evaluate growth, nutrient utilization and metabolism response all together.

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CAPÍTULO III

Protein:Carbohydrate ratio effects on growth and metabolism of the hybrid surubim (*Pseudoplatystoma fasciatum* x *Leiarius marmoratus*)

A ser submetido ao Journal of Animal Science

CAPÍTULO III

Protein:Carbohydrate ratio effects on growth and metabolism of the hybrid surubim (*Pseudoplatystoma fasciatum* x *Leiarius marmoratus*)

Abstract – Carbohydrate levels in fish nutrition has been widely studied in order to increase its percentage inclusion in the feed, especially for carnivorous fish. However there is little information available about tropical carnivorous fish nutritional requirement and nutrient utilization. Therefore, the aim of the present study was to investigate the effect of protein:carbohydrate ratio on growth, nutrient utilization, digestive enzyme activities and intermediary metabolism of the hybrid surubim *Pseudoplatystoma corruscans* x *Leiarius marmoratus*. Twelve juvenile (±6.9g initial weight) were fed 5% of total biomass for 60 days, two times a day. Four isoenergetic experimental diets with different protein:carbohydrate levels were tested 0.52/ 0.81/ 1.02/ 1.21). Final body weight was higher for the two groups fed higher protein:carbohydrate ratio. There was no difference among treatments for feed protein efficiency, but energy efficiency was higher in the groups with lowe carbohydrate percentage. There was also no significant difference for whole body content among treatments. Total alkaline protease and amylase were higher for groups fed higher protein levels, although no correlation was found for digestive enzymes activities and final body weight. Liver aspartate aminotransferase was the metabolic response with significant difference among treatments. Results shows that fish growth parameters and energy efficiency were higher at 40 and 45% of feed protein, although fish had a metabolic adaptation to higher carbohydrate inclusion levels in the feed.

Key words – surubim; performance, alkaline protease; amylase, intermediary metabolism, energy

INTRODUCTION

Evaluation of protein:energy ratio for fish has been widely study to provide macronutrients values for best protein and energy efficiency of diets in order to improve growth as well decrease nitrogen excretion in the environment (Almeida et al., 2011; Chatzifotis et al., 2010; Melo et al. 2006). Studies that evaluate protein:carbohydrate ratio commonly analyze growth and nutrient utilization (Abdel Tawwab et al., 2010; Zhou et al., 2013 Figueiredo et al., 2014). Digestive enzyme activities and metabolic responses has also been done for tropical species such as pacu *Piaractus mesopotamicus*, tambaqui *Colossoma macropomum*, blunt snout bream *Megalobrama amblycephala* and for the cold water fishes as rainbow trout *Oncorhyncus mykiss* (Li et al., 2012; Saravanan et al., 2012; Almeida et al., 2011; Bicudo et al., 2009). Studies that show growth performance, nutrient utilization, digestibility, enzyme activities and metabolic responses all together, provide a more complete information of how fish body is responding to different protein:energy ratios and its whole physiological adaptation. Therefore producers, researchers and the fish feed industry can have the perspective of the effect on the fish of a long term feeding period of different diet.

Studies of carnivorous fish nutrient utilization are more common in cold water fishes such as salmon and rainbow trout. Tropical water carnivorous fish studies are less studied and there is a lack of information about it. Performance, whole body or carcass composition, nutrient utilization, metabolic profile and digestive enzyme activities fed different protein, lipid and carbohydrate levels have been studied for the south American catfish *Pseudoplatystoma corruscans* (Martino et al., 2005; Lundsted et al., 2004; Martino et al., 2002) and *Pseudoplatystoma reticulatum* (Cornelio et al., 2014), for the Mediterranean meagre (*Argyrosomos regius*) and for the Asian catfish *Silurus meridionalis* (Liu et al., 2013). Nutrient utilization answers are similar no matter the natural food habit is. When dietetic carbohydrate is over metabolic and growth demand of fish body, it is stored mainly as viscera fat and affects physiological and metabolic parameters levels as well as decrease growth. It is desirable that most part of protein from the diet is used for body growth and not as metabolic energy, although it is known that some does. Protein in excess is discharged from the body as nitrogen compound, mainly ammonia, which causes negative environment impact to the water body where fish is raised. Also in this situation the most expensive feed ingredients is being discharged.

Although nowadays hybrid fish are not commonly raised, in some countries it still does and studies with hybrids are published (Maciel, 2014; Ventura, 2013; Essa et al., 2011). The hybrid surubim *Pseudoplatystoma corruscans* x *Leiarius marmoratus* is being largely raised in Brazil due to its higher growth index, boneless and tender meat. Its production is over 800 tons per year which is consumed in Brazil and also exported (MAPA, 2011). Carnivorous fish feed has high percentage of protein inclusion during all phases compared to omnivorous fish species. *P. corruscans* and *L. marmoratus* are piscivorous species, but *L. marmoratus* can have herbivorous feeding behavior as well (Fishbase, 2014). Rarely are the information published about this promising hybrid species (Maciel et al., 2014; Oliveira et al., 2013; Souza et al., 2014; Ventura et al., 2013) and also in the recent literature there is little information about the whole effect on growth of dietary macronutrient and its utilization by carnivorous tropical fish.

The aim of this study was to evaluate the effect of protein:carbohydrate ratio on growth, nutrient utilization, whole body composition, digestive enzyme activity and intermediary metabolism of the surubim hybrid *Pseudoplatystoma corruscans* x *Leiarius marmoratus*.

MATERIAL AND METHODS

1. Experimental diets

Four diets containing different levels of protein and carbohydrate were made as experimental treatments. It was formulated in order to have different protein(CP):carbohydrate(CHO) ratio. The values were: (0.52) 28.20% CP/ 53.94% CHO; (0.81) 36.44% CP/ 44.88% CHO; (1.02) 40.71% CP/ 39.51% CHO; (1.21) 45.24% CP/ 37.61% CHO. Feed were formulated in an isoenergetic (17.57 $kJ.g^{-1}$) basis. The dietary formulation and proximate composition are presented in Table 1. Feed were made by a commercial fish feed industry, Poytara®- São Paulo – Brazil. The chemical composition of the ingredients was determined according to the AOAC (1990) for the analyses of moisture, ash, crude protein, crude fiber, energy and lipids (Table 1). Gross energy was determined in a calorimetric pump.

Table 1. Formulation and proximate analysis of experimental diets (% dry matter).

	0.52	0.81	1.02	1.21
Ingredients				
Fish meal 66%	20.28	23.92	27.05	30.17
Corn meal	50.00	40.00	30.00	20.00
Poultry by-product 67%	5.70	10.00	15.00	20.00
Soybean meal 34%	20.80	23.02	25.10	27.12
Vitamin and Mineral*	2.00	2.00	2.00	2.00
NaCl	0.25	0.25	0.25	0.25
Antioxidant	0.25	0.25	0.25	0.25
Vitamin C	0.001	0.001	0.001	0.001
Methionine	0.665	0.501	0.346	0.191
Proximate composition (%)				
Moisture	2.34	3.09	1.46	8.19
Crude protein	28.20	36.44	40.71	45.25
Ether extract	5.82	4.94	5.98	7.70
ENN**	48.36	37.42	32.94	29.87
Ash	9.70	10.61	12.34	12.50
Crude Fiber	5.58	7.46	6.57	7.74
Gross Energy ($kJ.g^{-1}$)	18.57	17.25	19.16	17.83

***** Rovimix fish:vit. A: 5.000.000 UI; vit. D3: 200.000 UI; vit. E: 5.000 UI; vit. K3: 1.000 mg; vit. B1: 1.500 mg; vit. B2: 1.500 mg; vit. B6: 1500 mg; vit. B12: 4.000 mg; vit. C: 15.000 mg; folic acid: 500 mg; pantotenic acid: 4.000 mg; B.H.T.: 12,25 g; biotin: 50 mg; inositol: 1.000 mg; nicotinamide: 7.000 mg; coline: 40 g; cobalt: 10 mg; cupper: 500 mg; iron: 5.000 mg; iode: 50 mg; manganese: 1.500 mg; selenium: 10 mg; zinc: 5.000 mg; q.s.q.: 1.000 g

** ENN% = 100-(crude protein% + crude lipid% + moisture% + ash% +fiber%)

2. Fish and Feeding

Fish were obtained from CODEVASF (Companhia de Desenvolvimento do Vale do São Francisco e do Parnaíba), a Brazilian Federal development company. The juvenile surubim hybrid arrived in the laboratory, were acclimated for 15 days and fed a commercial diet (45% crude protein) priore trial began. To initiate the trial all the fish were individually weighed (average 6.9g). After this procedure they were sorted in 1000L PVC tanks, with 12 fish per tank. Three replicate groups of fish were used for each diet. Tanks were built in an open water system. Water parameters were monitored weekly (average temperature was 28.3˚C; pH 7.0 and dissolved oxygen 4.5 $mg.l^{-1}$). Fish were fed twice a day, at 5% of each tank total biomass, (800h and 1700h) for 60 days. Tanks were cleaned daily by scrubbing and siphoning of accumulated wastes, algae or any dirt that possibly came from the water.

3. Sample collection

At the end of the trial fish were starved for 24h. All fish in each tank were individually weighed for performance parameters: final body weight (FBW), weight gain (WG), feed consumption (FC), apparent feed conversion ratio (AFC), protein efficiency ratio (PE), energy efficiency ratio (EE), condition factor (CF) and specific growth rate (SGR) and survival rate (SR). Fish were anesthetized and blood samples from 3 fish, randomly selected, from each tank were draw from the causal vein. Glucose (Glc) was immediately measured with an automatic monitor (Accu-Ckech®). Subsequently fish were euthanized with eugenol (8ml/100L).Two samples from each tank were used for carcass analyzes. In sequence blood samples were centrifuged at 1000 rpm for 5 minutes and the plasma stored in a freezer (-20˚C) for a short period until the beginning of the total protein (TP), triglycerides (TG), free amino acids (FAA), alanine aminotransferase (ALT) analyzes. Liver and total intestine samples were collected from the same fish and immediately stored in a freezer (-20˚C) for short period until the beginning of the glycogen (GG) and enzymes activities analyzes.

4. Performance

Performance was measured by the following equations:

WG (g) = [final body weight (g) – initial body weight (g)]

FC = feed consumed (g)/time

AFC = [feed consumed (g)/ weight gain (g)]

PE = [weight gain (g)/ total protein intake ($g.kg^{-1}$, dry weight)] x 100

EE = [weight gain (g)/ total energy intake ($KJ.kg^{-1}$, dry weight)] x 100

CF = {final weight (g) x [final total length (cm)]^3 } x 100

SGR = {ln [final weight (g)] – ln [initial weight (g)]/time x 100

SR (%) = 100 x (final fish number/ initial fish number)

Whole Body Composition

The analysis of fish samples for crude protein, lipids and ash content was determined according to the AOAC (1990). Gross energy was determined in a calorimetric pump (Table 1).

5. Intestine Digestive Enzyme Activities

After collected intestine were immediately stored in -20°C. At the laboratory it was homogenized in 0.01M Tris-HCl pH 8.0 buffer using a tissue homogenizer. The resulted preparation was centrifuged at 10,000xg for 10 min at 4°C. The supernatants (crude enzyme extract) were used for analysis assays (Santos et al., 2013).

5.1 Total alkaline protease activity

The total enzymatic activity of proteases present in crude extracts was performed using 1% azocasein as substrate, prepared in 10 mM Tris-HCl, pH 8 0. Aliquots containing 30 μL of the crude extract were incubated with 50 μL of substrate solution for 1 hour at 25 °C. Then, 240μL of 10% trichloroacetic acid was added to stop the reaction. After 15 minutes the mixture was centrifuged at 8,000 xg for 5 minutes. The supernatant was collected and 70 μL of it was mixed in 130 μL 1M sodium hydroxide solution (revealing solution) in microplates. The absorbance was measured on a microplate reader (x-Mark Bio-

Rad) at a wavelength of 450 nm. A negative control (blank) was performed, replacing the enzyme extract by a solution of 10 mM Tris-HCl, pH 8.0 with added 0.15 M NaCl. The activities were carried out in triplicate and one unit (U) of enzyme activity was defined as the amount of enzyme required to hydrolyze azocasein and produce a change of 0.001 units of absorbance per minute.

5.2 Trypsin Activity (E.C. 3.4.21.4)

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

5.3 α-Amylase Activity (E.C. 3.2.1.1)

Alpha-amylase activity from the intestine of *Pseudoplatystoma* sp. was based on the method of Bernfeld (1995) using 2% (w/v) starch solution as substrate. The enzymatic preparations were in 0.2 M citrate-phosphate buffer, pH at 7.0 at 37 °C at 30 minutes. Then the assay was by addition of 3,5-dinitrosalicylic acid (DNSA) at 100°C for 10 minutes. The absorbance was measured at 570nm using a microplate reader (Bio-Rad 680). Both a substrate free control and an enzyme free control were run. The amount of maltose released from this assay was determined from the standard curve using commercial maltose. One milliunit of specific activity was defined as the amount of enzyme needed to release 1µg maltose per minute per milligram of soluble protein in enzyme solution at 37°C ($mU.min^{-1}.mg^{-1}$ Protein).

5.4 Stomach Pepsin

Acid protease activity was performed according to Pavlisko et al. (1997) using 2% (w/v) hemoglobin (prepared in 0.1 M Glycine-HCl, pH 2.5) as specific substrate. The enzymatic reaction was prepared in triplicate using microcentrifuge tubes to which were added 50 µl of sample, 350 µl of buffer 0.1M

Glycine-HCl, pH 2.5 and 100 µl of substrate. After 30 minutes at 37°C, 500 µl of 10% (w/v) trichloroacetic acid was added to stop the reaction and mixture was centrifuged at 10,000 xg for 15 minutes. The absorbance at 280 nm of the supernatant was measured with a microplate reader. One unit (U) of enzyme activity was defined as the amount of enzyme able to produce a change of 0,001 unit of absorbance per minute.

6. Metabolic intermediates

Metabolite concentration was carried out in plasma and liver tissue extracts free of protein.
Glucose was measured at the end of the trial, in the fish blood using an automatic glucose reader Accuchek®, Brazil. Total protein was determined by Biureto method (Hiller, 1948). Twenty (20) microliters of plasma was incubated for 10 minutes at 37 degrees Celsius. The reading was at 545 nm and the measure unit was expressed in $g.dL^{-1}$. Plasma triglycerides were analyzed by enzymatic colorimetric method using a commercial kit by Labtest®, Brazil. It was used 10 microliters of plasma samples containing reagent in 1ml of buffer pH 7.0, incubated for 10 minutes at 37 degrees. The samples were read at 500nm and the units of measurement were in $mg.dL^{-1}$. Free amino acids were determined in neutral extracts at 570nm (Copley 1941). Alanin aminotransferase were analyzed by kinetic method using a commercial kit by Labtest®.

In the liver, glycogen was assayed in alcoholic precipitates from tissues alkaline homogenates (Bidinotto et al. 1997). Aspartate aminotransferase were analyzed by kinetic method using a commercial kit by Labtest®. Glutamate dehydrogenase followed Hochachka et al. (1978). Reaction solution was formed by imidazole-HCl pH 7,7-50 mM buffer solution, ammonium acetate 250 mM, NADH 0,1 mM, ADP 1 mM, NADP 0,5 mM, 2-cetoglutarate 5 mM and enzyme crude solution. Specific enzyme activity was expressed in µmol per minute (mU) per mg of protein (mU/mg protein).

Statistic

All data were analyzed by one-way analysis of variance (ANOVA). Significant differences between means were determined by the Tukey´s multiple range test. Linear, polynomial and exponential models were tested for all regressions, and the best model was selected based on R^2 The probability level of

0.05 was used for rejection of the null. Data of digestive enzymes activities and fish final weight were analyzed by Pearson´s Correlation Test in order to verify correlation among those parameters.

RESULTS

Performance was influence by the different experimental diets, during the trial period (Table 2). Better results were found at 1.02 and 1.21 protein:carbohydrate levels fish groups. Individual final body weight was statistically lower at 0.52 compared to the others. The same response was found for individual weight gain. Although higher for groups fed higher protein levels, there was no statically difference ($P>0.05$) among treatment for feed consumption. Apparent feed conversion was lower for group treatments fed higher protein increase but without significant difference ($P>0.05$). Protein efficiency ratio also did not show statistically difference ($P>0.05$) among the experimental groups. Conversely, energy efficiency was lower for groups with higher carbohydrate levels than groups with lower carbohydrate levels. Condition factor is also a performance parameters that did not show statistically difference ($P>0.05$) among experimental treatments. In the opposite, specific growth rate was lower at group with higher carbohydrate levels and lower protein level and increased as carbohydrate level decreased and protein level increased.

Table 2. Growth parameters and whole body composition of hybrid surubim fed with diferent protein:carbohydrate ratio feed. Average per experimental unit (mean±S.D*)

	Protein:carbohydrate ratio			
	0.52	0.81	1.02	1.21
Final Body Weight (g)	44.34±1.77^{B}	48.61±4.96^{AB}	70.79±16.36^{A}	70.05±8.11^{A}
Weight Gain (g)	37.83±1.71^{B}	42.60±3.85^{AB}	64.37±15.28^{A}	63.83±7.27^{A}
FC (g)**	75.32±2.68	81.90±12.12	114.87±24.39	110.64±14.72
AFC**	1.99±0.03	1.92±0.19	1.79±0.07	1.73±0.03
FL** (cm)	17.34±0.57^{B}	18.59±0.43^{AB}	20.50±1.15^{A}	20.41±0.98^{A}
Protein Efficiency	13.51±0.61	11.83±1.07	16.09±3.82	14.18±1.62
Energy Efficiency	0.84±0.04^{C}	1.03±0.09^{BC}	1.40±0.33^{AB}	1.55±0.18^{A}
Condition Factor	0.85±0.08	0.76±0.05	0.81±0.05	0.82±0.03
SGR(%)**	0.97±0.08^{C}	1.12±0.01^{B}	1.24±0.04^{AB}	1.28±0.05^{A}
Whole body (%)				
Dry matter	93.29±0.45	94.25±0.41	94.30±0.62	94.49±2.97
Ash	12.14±0.49^{a}	11.57±0.71^{ab}	12.11±0.79^{a}	10.63±2.31^{b}
Lipid	24.78±1.15	24.92±1.99	24.95±4.52	25.75±2.95
Crude protein	8.52±0.34	8.22±0.32	8.57±0.30	8.08±0.50

*Different letters in the same line indicates statistical difference (*P<0.05*) between treatments (Tukey).

** SGR = Specifc Growth Rate; FC = feed consumption; AFC = apparent feed convertion ; FL = final length

After the trial period of 60 days, dry matter, crude protein and crude energy in the carcass, did not show statically difference (P>0.05) among treatments (Table 2). For carcass composition, ash was the only parameters that showed difference (P>0.05). For this parameter 1.21 protein:carbohydrate treatment showed lower ash level than the other treatments.

Digestive enzymes activities showed changes in the different experimental treatments (Table 3). Stomach pepsin was not different among treatment. Total alkaline protease activity ($U.mg^{-1}$) was significantly higher in the treatments with higher crude protein level than in others. Although a

protease, trypsin did not follow the same pattern. All values for trypsin were statistically the same but at 1.02 protein:carbohydrate ratio treatment. Following the pattern of total alkaline protease, amylase was higher in the treatments with higher protein level and not in the treatments with higher carbohydrate levels. Pearson´s correlation test was done in order to evaluate the correlation among final weight and digestive enzymes activity, but no correlation was found.

Table 3. Digestive enzymes activities (U.mg^{-1} protein) from surubim (*Pseusoplatystoma fasciatum* x *Leiarius marmoratus*) intestine, fed different protein:carbohydrate levels. (mean±S.D)

	Protein:carbohydrate ratio			
	0.52	0.52	0.52	0.52
Stomach				
Pepsin	110.20±21.17	62.25±16.53	83.33±18.53	92.85±23.08
Intestine				
Total Alkaline Protease	0.100±0.02^{AB}	0.065±0.03^{B}	0.117±0.03^{A}	0.128±0.04^{A}
Trypsin	64.382±9.11^{A}	73.117±35.29^{A}	35.244±15.81^{B}	72.216±21.38^{A}
Amylase	1.408±0.76^{B}	1.597±0.38^{B}	2.41±0.64^{A}	2.42±0.75^{A}

*Different letters in the same line indicates statistical difference ($P<0.05$) between treatments (Tukey).

Responses of the intermediary metabolism showed that in the blood, glucose, total plasma protein, triglycerides, free amino acids and ALT did not show any significant difference among the treatments (Table 4). Liver glycogen showed significant difference only in 1.02 protein:carbohydrate ratio treatment. As it also showed difference for AST in the liver, but GDH did not show any statistical difference among treatments.

Table 4. Hybrid surubim juvenile fed with diferent protein:carbohydrate ratio feed blood, plasma and liver metabolic responses. (mean±S.D*)

	Protein:carbohydrate ratio			
	0.52	0.52	0.52	0.52
Blood				
Glucose (mg.dL^{-1})	86.92±33.32	88.67±33.18	84.42±18.17	112.17±16.73
Plasma				
Total Protein (mg.dL^{-1})	16.74±4.82	17.04±4.93	21.10±4.25	18.51±3.52
Triglycerides (mg.dL^{-1})	6.19±3.61	4.88±2.28	7.75±4.96	8.14±2.90
Free Aminoacids (UI.mg protein $^{-1}$)	16.35±3.81	16.51±2.70	20.06±5.57	19.47±2.26
Alanin Aminotransferase (UI.mg protein $^{-1}$)	67.55±10.11	68.67±5.76	70.56±5.10	71.46±6.45
Liver				
Glycogen (umols glicose.g^{-1})	146.06±49.97^{A}	194.23±55.96^{A}	70.77±28.79^{B}	187.06±47.15^{A}
Aspartato Aminotransferase (UI.mg protein $^{-1}$)	97.22±21.77^{AB}	71.76±53.72^{AB}	37.48±17.74^{B}	120.30±42.81^{A}
Glutamato Desidrogenase (UI.mg protein $^{-1}$)	36.96±18.53	35.31±23.28	26.59±13.03	49.08±45.70

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

DISCUSSION

In the present study it was observed that the percentage of protein and carbohydrate inclusion, affected growth performance of fish evaluated. Higher protein and lower carbohydrate percentage in the feed, led to higher growth performance. Same result was found for the tropical carnivorous siluriforme, *Lophiosilurus alexandri*, fed different CP:CHO ratio (1.24/ 0.84/ 0.51/ 0.33) (Figueiredo et al., 2014). Low percentage of protein in the feed might not have a good palatability for fish and as consequence feed intake and growth performance are low (Giri et al., 2011). Higher growth parameters were also found for catfish (*Horabagrus brachysoma*) and tilapia when fed diets with high levels of protein (Abdel-Tawwab et al., 2010; Giri et al., 2011; Li et al.,2013; Xiong et al., 2014). On the opposite, Teixeira et al. (2013) fed 170g juvenile hybrid surubim (*Pseudoplatystoma* spp.) with increasing protein levels (36-50%) observed higher growth performance at lower protein levels. Therefore, increasing protein level did not lead to an improvement of nutrient utilization. Higher protein inclusion (45%) on the feed also resulted in lower protein efficiency ratio in tilapia juvenile (Abdel-Tawwab et al., 2010). Isonitrogenous diets with different carbohydrate and lipid ratio, also promote differences in growth parameters. This was observed in several fish species with different feeding behavior such as S. *meridionalis* (Luo et al., 2010), hybrid surubim (Pietro-Guevara et al., 2015), *Megalobrama amblycephala* (Zhou et al., 2013), carnivorous golden pompano, *Trachinotus ovatus* (Zhou et al., 2015), omnivorous tropical *Piaractus brachypomus* (Vásques-Torres & Arias-Castellanos, 2013), grass carp juvenile *Ctenopharyngodon idella* (Gao et al., 2010). In the present study it was observed that the hybrid surubim were able to have better growth performance when fed up to 395 $g.kg^{-1}$ of carbohydrate diet. This is similar to another catfish *Horabagrus brachysoma* – 374 $g.kg^{-1}$ (Giri et al., 2011). Different CHO:LP ratio also affects fish physiological condition, as non-specific immune response, oxidative status and liver histology, as it was shown for juvenile yellow catfish *Pelteobagrus fulvidraco* (Wang et al., 2014). Those parameters can also be used to determined nutritional optimum conditions.

Different main macronutrients (protein, lipid and carbohydrate) inclusion level affects feed intake and growth performance and is positively correlated with trophic level (Schrama et al., 2012; Li et al., 2013). As well as nutrients form also affects growth performance and metabolism, as it was recorded for bagrid catfish (Hamid et al., 2011) and tilapia (Chen et al., 2013) fed different carbohydrate sources. In this study, although no oil was added to the formula, the ratio carbohydrate:lipid was different. This fact may also affect digestibility and nutrients use for growth (Schrama et al., 2012).

Lower feed energy efficiency found in groups fed higher carbohydrate content might contribute to the response of a dubious question about this hybrid surubim feeding behavior. Hybrid surubim *Pseudoplatystoma* spp. (150g final weight) also did not show better energy efficiency ratio when fed different energy levels and source (Teixeira et al., 2013). The results above show that, despite differences in natural eating behavior, the main influence of nutrients follow a same pattern in fish growth performance.

Ash was the only parameter of whole body composition significantly affected ($p>0.05$) by treatments in the present study. This is in accordance to the catfish *Horabagrus brachysoma* carcass composition also affected by different protein:carbohydrate ratio (Giri et al., 2011) but not for tilapia fed different energy sources (Schrama et al., 2012). Whole body protein, lipid and energy content of hybrid surubim did not vary among treatments ($p>0.05$). This is similar to what was found for Nile tilapia fed different CP:CHO ratio (Xiong et al., 2014) and for golden pompano, *Trachinotus ovatus*, fed isonitrogenous diets whole body protein (Zhou et al., 2015). It indicates that carbohydrate levels did not induce lipid accumulation in the fish body as a result of *de novo* synthesis of fatty acids (Zhou et al., 2015). Also no difference was found in protein and ash carcass content, but in lipid and energy value for the same hybrid surubim fed different CHO:LP diets with fixed protein percentage (Pietro-Guevara et al., 2015), for southern catfish S. *meridionalis* fed high carbohydrate and lipid diets (Luo et al., 2010) and for tilapia juvenile fed different protein and energy content (Li et al., 2013). Difference found for the same fish hybrid might be due to feed protein content and also fish size used at the trials. The opposite result was found for 170g juvenile surubim *Pseudoplatystoma* spp. fed increased protein levels (36-52%), where protein content increased and lipid content decreased, both linear (Teixeira et al., 2013). Grass carp and golden pompano, *Trachinotus ovatus*, fed increasing levels of lipid, showed increased levels of body lipid (Gao et al., 2010; Zhou et al., 2015). As in the present study, energy content of whole body composition did not show difference among treatment, which means that fish, did not reach its growth potential. The same was noticed for *Piaractus brachypomus*, fed with different levels of CHO:LP (Vásques-Torres & Arias-Castellanos, 2013) and for *Megalobrama amblycephala* fed different CP:CHO (Zhou et al., 2013).

There was significant difference in all digestive enzymes activities evaluated in surubim intestine from the present study. Digestion and absorption of nutrients for fish growth depends on digestive enzymes activities. However no correlation was observed between enzymes activities and fish final weight,

within each treatment. Saponins increased levels in diets for common carp resulted in lower growth parameters, but higher intestine amylase and trypsin activity (Serrano, 2013). Similar results were found for blunt snout bream *Megalobrama ambychephala* fed different CP:LP ratios, where higher growth treatment had lower amylase activity and no influence on total alkaline protease (Li et al., 2012). These findings contribute to the fact that there is no relation between fish growth and digestive enzymes activities. Total alkaline protease and amylase were higher in the treatments with 40 and 45% CP and lower carbohydrate percentage. The opposite was found for the surubim *Pseudoplatystoma corruscans* also fed different CP:CHO, where higher protein feed levels treatments showed lower total alkaline protease activity and no difference was observed for amylase activity (Lundstead at el., 2004). Total alkaline protease was also changed in tilapia fed different percentage of shrimp protein hydrolysate, although no difference was observed in growth parameters (Santos et al., 2013). Trypsin showed an awkward result with significant and lower result only for the treatment with 40%CP. In Atlantic salmon, high trypsin values have been related to gastrointestinal damage caused by a harmful feed ingredient (Hansen et al., 2010; Penn et al., 2011; Chikwati et al., 2013), which could be hard to relate in the present study. Zhong et al. (2011) affirm that trypsin activity, mRNA level and mRNA expression is affected by the concentration of an ingredient on *Fugu obscurus*. The carnivorous golden pompano, *Trachinotus ovatus*, fed fixed CP percentage and different CHO:LP ratio, did not show difference among total alkaline protease activity, but amylase activity was higher at intermediary carbohydrate inclusion (11-22%) (Zhou et al., 2015). Digestive enzymes activities are influenced by feed nutrients, ingredients and fish species, even within the same natural feeding behavior (Gominho-Rosa et al., 2015).

In the present study, no blood or plasma metabolic responses were statistically different among treatments, only liver metabolic parameters. Blood glucose, ALT, total protein and liver AST of Nile tilapia fed different levels of protein (25, 35, 45%) and carbohydrate (56, 47, 35%), increased linearly with increasing of dietetic protein and fish weight (Abdel-Tawwab et al., 2010). Higher AST levels with increase in dietetic protein levels, as found in the present study, indicate amino acids transamination for energetic use, nitrogen compounds excretion and a metabolic protein catabolism increase (Melo et al., 2006; Souza et al., 2014). It can also be related to health and condition of fish, as it indicates liver damage (Wang et al., 2014). The tropical carnivorous *Lophiosilurus alexandri*, fed higher CP:CHO ratios levels showed higher plasma free amino acids, but lower blood glucose levels

(Figueiredo et al., 2014). High plasma protein and/or total free amino acids can indicate that there is feed protein in excess or that fish is breaking down muscular protein in order to maintain growth metabolism (Mach & Nortvedt, 2011). This fact could not be shown in the present study. There was no difference among treatments blood glucose, even that carbohydrate inclusion percentage were so different. Blood glucose was higher in the southern catfish S. *meridionalis* and golden pompano, *Trachinotus ovatus*, fed diets with higher carbohydrate level (Luo et al., 2010; Zhou et al., 2015). Skyba-Cassy et al. (2013), after a study with trout, postulated two hypotheses that can answer the results found in the present study: (1) an atypical regulation of hepatic glucose metabolism in fish fed high levels of carbohydrates; (2) an endogenous production of glucose through gluconeogenesis. Grass carp fed different CHO:LP ratio (1.7 – 202.5) and golden pompano, *Trachinotus ovatus*, showed higher triglycerides levels at higher CHO:LP ratios (Gao et al., 2010; Zhou et al., 2015). Tilapia fed reduced protein/starch (25,2%/26,82%) diet had increased plasma triglyceride content (Xiong et al., 2014). Also *Megalobrama amblycephalos* fed different CP:CHO ratio, showed higher plasma triglycerides and glucose at higher carbohydrate diets (Zhou et al., 2013). These responses were not observed in the present study, maybe due to low feed lipid level. Dietary energy levels influences triglycerides plasma levels, since it can be related to active lipid transport (Gao et al., 2010; Zhou et al., 2015). Macronutrient levels are more likely to change metabolic parameters values than macronutrient source. Different sources of animal protein for Atlantic salmom did not change plasma total protein, triglycerides, glucose and ALT (Hansen et al., 2010). There was no difference on liver glycogen between fish fed higher and lower carbohydrate content. This result could mean that in any treatment there was not energy enough derived from digestible carbohydrate in order to be stored by the liver (Zhou et al., 2015). Higher liver glycogen content was found in the same surubim hybrid (350g final weight average) fed diets with lower CHO:LP ratio (Pietro-Guevara et al., 2015), the southern catfish S. *meridionalis* (Luo et al., 2010) and Wuchang bream *Megalobrama amblycephalo* (Zhou et al., 2013) fed diets with higher carbohydrate level. Factorial experimental designs can demonstrate better the effects and interactions of different macronutrient in fish feed.

Conclusion

Fish growth parameters and energy efficiency were higher at 1.02 and 1.21 protein:carbohydrate ratio diets. Therefore, there is no parameter analyzed indicating that a negative effect of carbohydrate in excess caused damage in the hybrid surubim studied. These results also demonstrate that, although growth was significant lower at low protein:carbohydrate ratio, fish were able to adapt its metabolism in order to improve nutrients utilization.

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4. CONSIDERAÇÕES FINAIS

Neste estudo pode-se observar que os macronutrientes dietéticos avaliados exercem influencia sobre o desempenho e metabolismo do surubim híbrido. As fontes de energia seja lipídio, carboidrato ou proteína, afetam o metabolismo e os animais conseguiram manter o crescimento mediante um processo de adaptação fisiológica.

O surubim híbrido avaliado mostrou melhores respostas metabólicas quando alimentado com fonte de carboidrato amiláceo. No entanto estudos complementares avaliando outras fontes devem ser realizados.

Altas taxas de inclusão de lipídios na dieta para o surubim avaliado afetam negativamente o desempenho e o metabolismo de forma superior a altas taxas de inclusão de carboidratos. No entanto um delineamento experimental fatorial é o mais indicado para responder esta hipótese.

Neste estudo foram realizadas análises estatísticas com o objetivo de correlacionar o efeito de macro nutrientes dietéticos com as atividades das enzimas digestivas (protease alcalina total, amilase, lipase e tripsina) e o peso final, no entanto nenhuma correlação foi encontrada.

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ANEXO I

NORMAS DAS REVISTAS

AQUACULTURE

Types of paper

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.

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

Physiology:
Fish: A. P. (Tony) Farrell
Invertebrates: J. Benzie

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

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

Relevant physiological topics include, but are not limited to:

- Reproductive and endocrine physiology, including control of development and sex differentiation, induced ovulation and spermiation, gamete quality, storage and cryopreservation, physiology of gynogenetic, and triploid and transgenic organisms
- Cardiorespiratory, muscle and exercise physiology
- Osmoregulatory physiology
- Digestive physiology, including endocrine and environmental regulation of growth
- Larval physiology and ontogeny, including metamorphosis, smolting and molting
- Performance under variable culture conditions, including temperature, water quality, rearing density, and stress and disease physiology
- Physiology of harvest and handling techniques

Genetics:

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. Submitted manuscripts must have a valid hypothesis or objective, clearly state the relevance to aquaculture, have proper experimental design with appropriate sample size and controls and utilize appropriate statistical analysis.

Relevant genetics topics include, but are not limited to:

- Breeding programs using classic selection procedures, markers or combining marker assisted selection with classic selection
- Applications of crossbreeding and interspecific hybridization
- Evaluation of commercially important phenotypes among cultured strains, populations or stocks
- Applications of biotechnology and genetic manipulation methods
- Development of linkage maps, identification of QTL or association of commercially important traits with specific gene(s). Where appropriate, linkage maps should include co-dominant markers, such as microsatellite DNA and SNP markers, to enable application to other populations and facilitate comparative mapping.

Aquaculture will NOT accept manuscripts dealing with the application of well-described techniques to yet another species, unless the application solves a specific biological problem

important to aquaculture production; or 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.
Aquaculture will not accept manuscripts focusing mainly on population genetics studies that are based on RAPD and AFLP markers, since the dominance and multilocus nature of the fingerprints are not suitable for making inferences about population genetic diversity and structure.

Sustainability and Society:
D.C. Little

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

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

Disease
B. Austin

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

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The methods section must include a subsection that describes the methods used for statistical analysis (see the section on statistical analysis in the Appendix) and the sample size must be justified by the results of appropriate calculations and related to the study outcomes.

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While it is generally desirable that the presentation of the results and the discussion of their significance should be presented separately, there may be occasions when combining these sections may be beneficial. Authors may also find that additional or alternative sections such as 'conclusions' may be useful. The discussion should be **no longer than five manuscript pages**.

Acknowledgments

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References

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Examples of correct forms of references are given below.

Journal articles

1. Rebello SA, Koh H, Chen C *et al.* (2014) Amount, type, and sources of carbohydrates in relation to ischemic heart disease mortality in a Chinese population: a prospective cohort study. *Am J Clin Nutr* **100**, 53-64.
2. Villar J, Ismail LC, Victora CG *et al.* (2014) International standards for newborn weight, length, and head circumference by gestational age and sex: the Newborn Cross-Sectional Study of the INTERGROWTH-21st Project.*Lancet* **384**, 857-868.
3. Alonso VR & Guarner F (2013) Linking the gut microbiota to human health. *Br J Nutr* **109**, Suppl. 2, S21–S26.
4. Bauserman M, Lokangaka A, Gado J et al. A cluster-randomized trial determining the efficacy of caterpillar cereal as a locally available and sustainable complementary food to prevent stunting and anaemia. *Public Health Nutr*. Published online: 29 January 2015. doi: 10.1017/S1368980014003334.

Books and monographs

1. Bradbury J (2002) Dietary intervention in edentulous patients. PhD Thesis, University of Newcastle.
2. Ailhaud G & Hauner H (2004) Development of white adipose tissue. In *Handbook of Obesity. Etiology and Pathophysiology*, 2nd ed., pp. 481–514 [GA Bray and C Bouchard, editors]. New York: Marcel Dekker.
3. Bruinsma J (editor) (2003) *World Agriculture towards 2015/2030: An FAO Perspective*. London: Earthscan Publications.
4. World Health Organization (2003) *Diet, Nutrition and the Prevention of Chronic Diseases*. Joint WHO/FAO Expert Consultation. WHO Technical Report Series no. 916. Geneva: WHO.
5. Keiding L (1997) *Astma, Allergi og Anden Overfølsomhed i Danmark – Og Udviklingen 1987–199I (Asthma, Allergy and Other Hypersensitivities in Denmark, 1987–1991)*. Copenhagen, Denmark: Dansk Institut for Klinisk Epidemiologi.

Sources from the internet

1. Nationmaster (2005) HIV AIDS – Adult prevalence rate. http://www.nationmaster.com/graph-T/hea_hiv_aid_adu_pre_rat (accessed June 2013).

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