UNIVERSIDADE ESTADUAL PAULISTA - UNESP

CÂMPUS DE JABOTICABAL

AJUSTE METABÓLICO E RESPOSTAS IMUNES DE PACUS JUVENIS ALIMENTADOS COM DIFERENTES NÍVEIS DE CARBOIDRATOS E SUBMETIDOS A JEJUM PROLONGADO

Rodrigo Yukihiro Gimbo
Zootecnista

2015

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Orientadora: Porfa. Dra. Elisabeth Criscuolo Urbinati

Tese apresentada à Faculdade de Ciências Agrárias e Veterinárias – Unesp, Câmpus de Jaboticabal, como parte das exigências para a obtenção do título de Doutor em Zootecnia.

2015

DADOS CURRICULARES

RODRIGO YUKIHIRO GIMBO – nascido na cidade de Taubaté-SP, no dia 29/07/1985. Em março de 2004 ingressou na UNESP, campus de Dracena, concluindo o curso de Zootecnia em janeiro de 2009. Durante a graduação, participou de diversos congressos, realizou estágios na área de produção animal e iniciação científica sob orientação do Prof. Dr. Leonardo Susumu Takahashi. Em março de 2009, iniciou o curso de mestrado em Zootecnia pela UNESP, Faculdade de Ciências Agrárias e Veterinária em Jaboticabal, sob orientação da Profa. Dra. Elisabeth Criscuolo Urbinati. Após conclusão do mestrado em 2011, ingressou no curso de doutorado em Zootecnia, na mesma instituição, e sob mesma orientação. No dia 13 de fevereiro de 2015, submeteu o presente estudo para avaliação pela banca examinadora com parte desta tese já publicada ou em processo de publicação.

Aos meus pais,

Francisco e Nelza

E a todos amigos de pós graduação,

Dedico

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Tabela de abreviaturas*

CHO	Carboidrato
CRH	Hormônio liberador de corticotropina
HPI	Hipotálamo-pituitária-interrenal
WG	Weight gain
SGR	Specific growth rate
PER	Protein eficiency rate
HK	Hexoquinase
GK	Glicoquinase
G6PDH	Glicose 6-fosfato desidrogenase
AST	Aspartato aminotransferase

*****As abreviações podem ser utilizadas em sua respectiva tradução em ingês

AJUSTE METABÓLICO E RESPOSTAS IMUNES DE PACUS JUVENIS ALIMENTADOS COM DIFERENTES NÍVEIS DE CARBOIDRATOS E SUBMETIDOS A JEJUM PROLONGADO

Resumo

Este trabalho está dividido em três artigos no formato para a submissão e publicação. No primeiro, testamos níveis de carboidratos (CHO) associados ao jejum e realimentação como estratégia de induzir as vias catabólicas e anabólicas dos diferentes nutrientes e auxiliar no entendimento da intolerância dos peixes aos CHOs; no segundo, avaliamos o uso da amônia sérica como indicador de dietas desbalanceadas; e, ainda, no terceiro artigo avaliamos como o jejum afeta a imunidade e a estratégia metabólica adotada pelos peixes para sustentar a resposta imune em condições de déficit energético. Os resultados indicam que o pacu pode tolerar altos níveis de CHO, uma vez que os valores de glicemia e glicogênio hepático foram semelhantes entre os peixes que ingeriram 25 e 45% CHO, além de suportar longos períodos de jejum (30 dias), sem comprometer a capacidade de resposta de ganho em peso. Entretanto, 25% CHO resultou em menor acúmulo de lipídeo visceral, associado com maior lipólise (mais ácidos graxos livres circulantes) e menores níveis de triglicerídeo e colesterol circulantes após 30 dias de alimentação. Após 30 dias de jejum, os peixes consumiram as reservas energéticas avaliadas, mas após um dia de re-alimentação, apenas as reservas de glicogênio e os níveis circulantes de triglicerídeo e colesterol normalizaram, enquanto as reservas lipídicas teciduais foram restauradas ao final dos 30 dias de realimentação. As atividades da hexoquinase (HK), glicoquinase (GK), glicose 6-fosfato desidrogenase (G6PDH) e aspartato aminotransferase (AST) acompanharam o perfil das variáveis metabólicas, reforçando que o pacu é capaz de usar altos níveis de CHO e ajustar seu metabolismo para suportar longos períodos de jejum. Como foram utilizadas duas dietas, uma balanceada em proteína e energia (45% CHO), e outra desbalanceada (25% CHO), testamos a determinação da concentração da amônia plasmática para validar o uso deste parâmetro como indicador de dietas desbalanceadas na alimentação de pacu. A atividade da AST foi usada para comprovar a ocorrência do catabolismo de proteínas após 30 dias ingerindo ambas as dietas. Ao final deste período, observamos o aumento da amônia plasmática e da atividade da AST nos peixes que ingeriram 25% CHO, indicando que a determinação da amônia plasmática é um eficaz indicador da utilização de dietas desbalanceadas. Por fim, para avaliar o custo metabólico da resposta imune em condição de déficit energético, dois grupos de peixes foram alimentado durante 30 dias ou submetido ao jejum pelo mesmo período. Após 30 dias, os peixes foram amostrados e inoculados com *A. hydrophila* e amostrados novamente após 3 e 24 horas. A atividade respiratória dos leucócitos foi menor nos peixes

submetidos ao jejum, entretanto, após a inoculação da bactéria ambos grupos foram capazes de elevar a atividade respiratória dos leucócitos, atingindo o mesmo nível. A atividade do sistema complemento, reduzida pelo jejum, aumentou em resposta à inoculação da bactéria. A concentração de lisozima foi mais elevada nos peixes em jejum antes e após 3 horas da inoculação da bactéria, e o grupo alimentado alcançou os mesmos níveis do grupo em jejum apenas após 24 horas da inoculação. Os peixes alimentados sustentaram a resposta imune num primeiro momento graças às reservas de glicogênio e enquanto os peixes em jejum dependeram principalmente das reservas lipídicas e num segundo momento, ambos os grupos dependeram de lipídeos para fornecer energia para os processos imunes. Assim, mostramos que construir uma resposta imune é um processo caro, entretanto, o pacu, mesmo em condição de déficit energético é capaz de mobilizar suas reservas de energéticas para sobreviver após uma infecção bacteriana.

Palavras chave: Imunidade de peixe, metabolismo de carboidrato, metabolitos sanguíneos, peixe tropical

METABOLIC ADJUST AND IMMUNE RESPONSE OF PACU JUVENILES FED WITH DIFFERENT CARBOHYDRATES LEVELS AND SUBMITTED TO LONG-TERM FASTING

Abstract

This study is divided in three papers formatted to be submitted and publishing. In the first one, we tested carbohydrate (CHO) levels associated to fasting and refeeding as strategy to induce catabolic and anabolic pathways of different nutrients and assist in understanding of CHO intolerance in fish; in the second, we evaluated the serum ammonia as indicator of unbalanced diets utilization; and also, in a third paper, we evaluated how fasting affect the fish immunity and the metabolic strategies adopted by fish to sustain the immune response under energy deficit conditions. The results indicate pacu use efficiently high CHO levels, once values of blood glucose and liver glycogen were similar between fish fed with 25 and 45% CHO, besides this, pacu tolerates long-term fasting (30 days), without compromise the ability of weight gain response. However, 25% CHO resulted in lower mesenteric fat accumulation and higher lipolysis (more current non-esterified fatty acids) and lower levels of triglycerides and cholesterol after 30 days feeding. After 30 days fasting, fish consumed the evaluated energy reserves, but after one day re-feeding, only reserves of liver glycogen, triglycerides and cholesterol normalized, while the tissue lipid reserves were reestablished at the end of 30 days re-feeding. The hexokinase (HK), glucokinase (GK), glucose 6-phosphate dehydrogenase (G6PDH) and aspartate aminotransferase (AST) follow the metabolic variables profile, reinforcing that pacu is able to use high CHO levels and adjust their metabolism to tolerate long-term fasting. As we used two diets with different protein/energy ratio, we tested the serum ammonia determination to validate the use of this parameter as indicator of unbalanced diets utilization in pacu. The AST activity was used to prove the occurrence of protein catabolism after feeding fish during 30 days with both diets. At the end of period, we observed increasing in serum ammonia and AST activity in fish fed with 25% CHO diet, indicating the serum ammonia determination is a effective indicator of unbalanced diet utilization. Lastly, to evaluate the metabolic cost of immune response under energy déficit, two fish groups were fed during 30 days or submitted to fasting by same period. After 30 days, fish were sampled and inoculated with A. hydrophila and than sampled again after 3 and 24 hour. The leukocytes respiratory activity was lower in fish submitted to fasting, however, after bacteria inoculation, both groups increased leukocytes respiratory

activity to the same level. The complement system activity was reduced in fish submitted to fasting, but increased in response to bacteria inoculation. Lysozyme concentration was elevated in fasted fish before and 3 hours after inoculation, and fed group reached the same activity of fasted fish only at 24 hours after inoculation. Fed fish sustained the immune response in a first moment due to glycogen reserves while fasted fish depended on lipid reserves and in a second moment, both groups used lipid to provide energy to immune process. Thus, we showed that build an immune response is an expensive process, however, the pacu, even under energy deficit condition is able to mobilize energy reserves to survive after bacterial infection

Keywords: Fish immunity, blood metabolites, carbohydrate metabolism, tropical fish

CAPÍTULO 1 – Considerações gerais

1. Introdução e justificativa

A glicose possui função fundamental como fonte de energia para a maioria dos mamíferos, entretanto, sua importância em peixes aparenta ser limitada (Wilson, 1994; Hemre et al., 2002; Stone, 2003). Em algumas espécies de peixes, principalmente em espécies carnívoras é possível observar uma hiperglicemia pós-prandial prolongada após alimentação com dietas ricas em carboidratos (Cowey e Walton, 1989; Wilson, 1994; Moon, 2001). Já em outras espécies, estudos mostraram a capacidade dos carboidratos dietéticos em reduzir o catabolismo de proteínas (Cho e Kaushik, 1990; Wilson, 1994, Baldan, 2008).

Ao contrário dos animais terrestres, os peixes dependem da proteína dietética como fonte primária de energia. A proteína da dieta por sua vez é o item mais oneroso e pode contribuir significativamente para o custo da dieta. Assim, é desejável que a proteína seja utilizada para crescimento e não para suprimento da necessidade energética dos peixes (Kumar et al., 2010).

Existem algumas hipóteses para explicar a baixa utilização de glicose dietética pelos peixes. Dentre elas, podemos citar a capacidade dos aminoácidos dietéticos de estimularem mais a secreção de insulina que a glicose (Mommsen e Plisetskaya, 1991), o menor número relativo de receptores de insulina no músculo de peixe em comparação com ratos (Párrizas et al., 1994), a baixa capacidade de fosforilação da glicose (Cowey e Walton, 1989), o baixo número de transportadores de glicose em músculo de peixe (Wright et al., 1998) e uma inadequada regulação da homeostase da glicose em resposta a um desbalanço entre a glicólise e a gliconeogênese (Panserat et al., 2000).

Neste estudo, associamos o uso de carboidratos com o jejum e realimentação como estratégia para ativar as vias catabólicas e anabólicas dos diferentes nutrientes e auxiliar no entendimento da questão da tolerância/intolerância aos carboidratos. Entretanto, devido à pouca informação na literatura (Martin et al., 2010) sobre o efeito

do jejum na imunidade, procurou-se estudar também a estratégia metabólica adotada pelos peixes para sustentar a resposta imune em condição de déficit energético.

2. Revisão de literatura

2.1. Homeostase da glicose em peixes

Carboidratos (CHOs) são bastante usados em dietas de animais domésticos como fonte de energia. Apesar de não existir exigência de carboidratos em dietas para peixes, sua inclusão em níveis adequados pode assegurar melhor eficiência na utilização de outros nutrientes (Wilson, 1994).

A utilização de CHOs pode reduzir o catabolismo de proteínas para síntese de glicose (Suarez e Mommsen, 1987), além de melhorar a eficiência de retenção proteica e diminuir as perdas metabólicas de nitrogênio no ambiente (Cowey e Walton, 1989; Wilson, 1994). A melhora no crescimento e o efeito poupador de proteína podem estar relacionados ao fato da glicose ser um importante combustível metabólico para os tecidos glicose-dependentes, tais como células vermelhas e tecido nervoso, entre outros. Desta forma, CHOs presentes na dieta de peixes podem reduzir a atividade gliconeogênica, afastando aminoácidos da via oxidativa (Cowey et al., 1977).

Para que a glicose possa ser utilizada como fonte de energia, é necessário que esta molécula seja transportada para o citoplasma da célula. Com exceção das células presentes no epitélio gastrintestinal e nos túbulos renais (transporte ativo secundário, co-transporte de sódio e glicose), a glicose entra nas células por difusão facilitada mediada por proteínas transportadoras de glicose (GLUTs). Em mamíferos, já foram identificadas 14 isoformas de GLUT, cada uma expressa por genes diferentes e presentes em diferentes tecidos (Mueckler e Thorens, 2013).

O metabolismo da glicose em peixes é bastante estudado devido a sua importância como fonte de energia (Wilson, 1994) e à baixa capacidade dos tecidos periféricos em utilizarem carboidratos dietéticos, quando comparados com aves e mamíferos (Wilson, 1994; Henre et al., 2002; Enes et al., 2009). Apesar da baixa taxa de absorção de carboidratos, há evidências de que a glicose entra na célula por meio de GLUTs. Wright et al. (1998) observaram proteínas reagindo com anticorpo contra GLUT-1 (de mamífero) no coração e no cérebro de tilápia. Já Krasnov et al. (1999)

observaram aumento na absorção metabolismo de carboidratos em embriões transgênicos de truta arco-íris expressando genes de GLUT-1 humano. Em estudo com pacu (*Piaractus mesopotamicus*), Baldan (2008) identificou uma proteína com alta homologia com a GLUT-4 do músculo esquelético de rato, porém com intensidade de banda reduzida.

Em condições aeróbicas, a glicose é catabolizada na via glicolítica, ciclo do ácido cítrico e fosforilação oxidativa para a produção de ATP, ou pode seguir a via das pentoses fosfato para a produção de NADPH e ribose 5-fosfato, necessários para a biossíntese de lipídeos e nucleotídeos, respectivamente. O excesso de glicose pode ser armazenado na forma de glicogênio (glicogênese) ou convertido a lipídeo (Enes et al., 2009). Em condições de jejum, a necessidade de glicose utilizada no metabolismo pode ser obtida pela degradação do glicogênio (glicogenólise) ou pela síntese *de novo* de glicose através da gliconeogênese (Pilkis e Granner, 1992).

No fígado de vertebrados, a glicoquinase ou hexoquinase IV, uma das três enzimas que regulam a glicólise, atua na taxa de utilização de glicose para controle de sua homeostase, pela fosforilação da glicose a glicose-6-fosfato. Esta, por sua vez, atua como inibidor da hexoquinase quando em concentrações elevadas (Berg et al., 2002). Outro ponto de controle da via é a fosforilação da frutose 6-fosfato, catalisada pela fosfo-frutoquinase. Esta enzima pode ser inibida por altas concentrações de ATP, ou ativada pela frutose 1,6-bifosfato. O último ponto de controle da via é a conversão de fosfoenolpiruvato a piruvato, catalizada pela enzima piruvato-quinase, reação que ocorre a favor da formação de ATP, sendo a frutose 1,6-bifosfato um importante ativador da via (“feed-foward regulation”) (Berg et al., 2002). Estudos com peixes teleósteos, na maioria carnívoros, mostram que diferentes espécies, mesmo com o mesmo hábito alimentar, possuem capacidades distintas de aproveitamento de carboidrato, com variações qualitativas e quantitativas da atividade da glicoquinase, sugerindo que esta pode ser uma explicação para a tolerância/intolerância ao carboidrato (Panserat et al., 2000).

2.2. Jejum e realimentação

Por estarem sujeitos a longos períodos de privação alimentar durante parte do ciclo de vida, os peixes desenvolveram habilidades para suportar períodos de jejum, sem comprometer a capacidade de sobrevivência (Love, 1970).

O jejum envolve uma série de adaptações fisiológicas para promover o ajuste biológico do animal nesta condição e suas consequências finais são altamente dependentes da espécie considerada, da idade do peixe e de condições experimentais como temperatura da água, fotoperíodo, dieta pré-jejum, e duração do jejum (Love, 1970; Weatherley e Gill, 1987; Blasco et al., 1991, Souza et al., 2000). A estratégia de jejum seguida de realimentação é uma ferramenta para manipular as alterações bioquímicas e metabólicas (Baldan, 2008).

Em algumas espécies, a primeira reserva energética a ser mobilizada é o glicogênio (Hung, et al., 1997; Méton et al., 2003). Esta hidrólise, além de fornecer substrato energético, ajuda a manter a homeostase da glicemia durante os primeiros estágios do jejum. Paralelamente à mobilização de glicogênio, reservas de lipídeos são usadas para obter energia e o uso da proteína muscular como fonte de energia só é utilizada em situações extremas (Navarro e Gutiérrez, 1995). Por outro lado, algumas espécies tentam preservar as reservas de glicogênio, degradando proteína para gliconeogênese e mobilizando lipídeos como substrato energético (Sheridan e Mommsen, 1991; Gillis e Ballantyne, 1996).

Os precursores gliconeogênicos são moléculas de não-carboidratos que podem ser usadas para produzir glicose. Estão inclusos nesta lista todos os intermediários da via glicolítica, do ciclo do ácido cítrico, além do glicerol, lactato e α-ceto ácidos, provenientes da deaminação dos aminoácidos não essenciais (Berg et al., 2002). Na via glicolítica, existem sete reações reversíveis, usadas para a síntese de glicose a partir do piruvato ou lactato. Entretanto, três reações são irreversíveis e devem ser contornadas por quatro reações alternativas que são energeticamente a favor da síntese de glicose. Assim, o piruvato é, primeiro, carboxilado pela enzima piruvato carboxilase, formando oxalacetato e posteriormente é convertido a fosfoenol piruvato pela fosfoenol piruvato carboxiquinase. Outra enzima importante é a frutose 1,6-bifosfatase que hidrolisa a frutose 1,6-bifosfato, formando frutose 6-fosfato. Por último, ocorre a hidrólise da glicose 6-fosfato pela glicose 6-fosfatase. Em conjunto,

essas enzimas fornecem uma via energeticamente favorável para a formação de glicose na forma livre.

Por outro lado, a realimentação dispara uma variedade de respostas que depende de fatores como espécie, idade, condições ambientais, período de jejum e o histórico alimentar anterior ao jejum (Navarro e Gutiérrez, 1995). No geral, os peixes realimentados mostram rápida recuperação do peso, conhecido como ganho compensatório, sustentado pela rápida recuperação do perfil metabólico (Soengas et al., 1996; Méton, et al., 2003; Morales et al., 2004).

Com relação à atividade de enzimas que regulam as vias glicolítica e gliconeogênica, o jejum (Caseras et al., 2000; Kirchner et al., 2003a; Metón et al., 2004; Kirchner et al., 2005; Soengas et al., 2006), assim como a restrição no fornecimento de energia na dieta (Caseras et al., 2000), reduzem de forma significativa a atividade e a expressão gênica da glicoquinase (hexoquinase IV) em truta arco-íris e “European seabream” (Caseras et al., 2000). Além disso, é possível observar o aumento da expressão desta enzima algumas horas após a realimentação (Soengas et al., 2006). Comportamento semelhante foi observado por Fideu et al. (1983), Bonamusa et al. (1992), Metón et al. (2003) e Kirchner et al. (2003b), sendo que a atividade da piruvato quinase no fígado de truta arco-íris reduziu após o jejum, e foi restaurada apenas após 20 dias de realimentação. De acordo com Caseras et al. (2000), este atraso na expressão gênica após a realimentação pode contribuir para explicar a hiperglicemia prolongada após a ingestão de alimentos.

Curtos períodos de privação alimentar (2 a 9 dias) não foram suficientes para produzir mudanças significativas na atividade e a expressão da glicoquinase (GK) (Pérez-Giménez et al., 2007) e na atividade da piruvato quinase (PK) (Metón et al., 2003). Já as enzimas gliconeogênicas fosfoenol piruvato caboxiquinase (PEPCK) (Kirchner et al., 2003), frutose 1,6-bifosfatase (FBFase) (Morata et al., 1982; Metóm et al., 1999; Metóm et al., 2003; Kirchner et al., 2003) e glicose 6-fosfatase (G6Fase) (Morata et al., 1982; Caseras et al., 2002; Metóm et al., 2003; Kirchner et al., 2003) possuem atividade e expressão gênicas aumentadas após jejum prolongado quando comparados com peixes continuamente alimentados.

2.3. Sistema imune em peixes

O sistema imune dos peixes, como nos demais vertebrados, apresenta respostas imunes inatas ou não especificas que funcionam como uma primeira barreira contra microrganismos, e respostas imunes específicas, mais lentas, dependentes do reconhecimento de antígenos, produção de anticorpos específicos e formação de memória imunológica (Bernstein et al., 1998).

A imunidade inata tem origem em células e moléculas presentes em tecidos e fluidos corporais que desempenham ação protetora. Algumas proteínas foram identificadas em peixes, a exemplo da mucotripsina, transferrina, lisozima, proteínas do sistema complemento e lectinas (Dalmo et al., 1997). A imunidade inata celular é aquela gerada pela ação de monócitos, macrófagos e granulócitos (neutrófilos, eosinófilos e basófilos) e células citotóxicas (“natural killers”) (Secombes, 1996), que atuam no reconhecimento e na eliminação de patógenos, funções essenciais nesta etapa de defesa do organismo (Zaccone et al., 2009). A inflamação em peixe inicia-se como nos mamíferos, com o aumento na liberação de neutrófilos, resultando em um aumento do número das células circulantes, podendo seu perfil ser usado como indicativo de infecção (Kindt et al., 2006). Os fagócitos também desempenham um papel importante em função de sua atividade respiratória. Durante a fagocitose, ocorre a produção de espécies reativas de oxigênio que desempenham função bactericida (Afonso et al., 1998).

Muitas moléculas e células que atuam na resposta imune inata podem ser utilizadas como indicadores no monitoramento da saúde dos peixes (Robertsen, 1999). Composto por várias proteínas solúveis, o sistema complemento desempenha um papel importante na imunidade não específica atuando nos processos biológicos de fagocitose, opsonização, quimiotaxia de leucócitos e inativação de toxinas liberadas por bactérias (Secombes, 1996; Claire et al., 2002; Boshra e Sunyer, 2006, Nakao et al., 2011). As proteínas do sistema complemento também estão envolvidas nos mecanismos de recrutamento de células fagocíticas em reações inflamatórias e na exposição de antígenos aos linfócitos, atividades relacionadas com a via clássica do sistema (Claire et al., 2002; Boshra e Sunyer, 2006). O sistema complemento, mais estudado e conhecido em mamíferos, é composto por 30 proteínas plasmáticas e de membrana, que são ativadas em três vias de reações distintas as quais convergem em C3, uma convertase, pivô central do sistema. A ação das três vias, clássica,

alternativa e das lectinas forma o complexo de ataque à membrana (CAM), responsável pela atividade lítica em patógenos (Nonaka e Smith, 2000; Nakao et al., 2011). O sistema complemento dos peixes é funcionalmente similar ao de mamíferos, sendo que as principais vias descritas em mamíferos, clássica, alternativa e lítica, foram identificadas em nível funcional em peixes ósseos e cartilaginosos (Nonaka e Smith, 2000). Biller-Takahashi et al. (2012) observaram aumento da atividade lítica da via alternativa das proteínas no sistema complemento em pacus após desafio com *Aeromonas hydrophila*.

A lisozima é uma molécula importante na defesa do organismo contra patógenos. É uma enzima produzida pelos leucócitos e apresenta atividade lítica sobre membranas de diversas espécies de bactérias, tanto em bactérias Gram positivas quanto negativas. Em peixes, a enzima encontra-se amplamente distribuída sobre a pele, muco, brânquias, trato intestinal, soro, tecidos linfóides e outros fluidos corporais. Variações nos níveis séricos de lisozima podem ocorrer devido à sazonalidade, sexo, maturação sexual, alimentação, temperatura da água, estresse e infecções (Hernández e Tort, 2003). A mensuração da concentração sérica de lisozima pode ter valor diagnóstico na determinação da condição imunológica e resistência a doenças (Saurabh e Sahoo, 2008). Embora ambas as respostas, inatas e específicas, tenham papel fundamental na defesa contra patógenos, acredita-se que, para os peixes, as respostas inatas sejam mais importantes quando comparados com mamíferos (Saurabh e Sahoo, 2008; Urbinati et al., 2014).

O entendimento da biologia dos peixes, em particular da resposta imune, é importante para um manejo sanitário apropriado. O estudo da resposta inata nestes animais tem gerado interesse crescente nos últimos anos e pode ser considerado um fator chave na defesa primária e na organização da imunidade adquirida (Whyte, 2007).

2.4. Modulação do sistema imune pelo estresse

A ativação da resposta de estresse pode provocar tanto ativação como inibição do sistema imune. Em um primeiro momento, ocorre ativação, principalmente provocada por catecolaminas e pelo hormônio liberador de corticotropina (CRH) e, posteriormente, ocorre inibição, provocada pela liberação dos hormônios do eixo hipotálamo-pituitária-interrenal (HPI), particularmente relacionada à ação do cortisol

(Tort, 2011). Em situações de estresse, o aumento das catecolaminas atua nos tecidos hematopoiéticos, aumentando a libração de eritrócitos na circulação, e o aumento de cortisol diminui a produção de leucócitos. Da mesma forma, monócitos e linfócitos circulantes podem ser diretamente afetados pelos hormônios (Ellis, 1981).

Em caso de estresse agudo, foi observado aumento no número de receptores de glicocorticoides nos leucócitos do rim cefálico (Maule e Schreck, 1991). Apesar de poucas informações em peixes, alguns estudos mostram alterações em número e padrão de distribuição de células brancas decorrentes de estresse, pela necessidade de mobilização de células para locais afetados e aumento da eficiência de defesa (Wojtaszek et al., 2002; Dhabhar, 2002). A fase aguda do estresse favorece a mobilização de células de defesa e a distribuição dos diferentes tipos celulares, de acordo com a necessidade (Dhabhar, 2002). Seguindo o aumento inicial de leucócitos, e com a permanência da ativação do eixo HPI, ocorre uma diminuição geral do número de células circulantes, reflexo da migração e permanência das mesmas nos órgãos afetados (Tort, 2011).

A redução da quantidade e atividade dos componentes do sistema imune pode diminuir a resistência dos peixes às doenças e facilitar a infecção por microrganismos patogênicos oportunistas, como a *Aeromonas hydrophila* é um bastonete ou coco-bastonete Gram negativo aeróbio ou anaeróbio facultativo, agente etiológico da enfermidade conhecida como septicemia hemorrágica. Está presente em praticamente todos os ambientes aquáticos, assim como na pele e no trato intestinal dos peixes de água doce (Holliman, 1993). A ampla distribuição da bactéria e sua adaptação a mudanças ambientais deve-se a ampla variedade de enzimas secretadas por suas cepas (Pemberton et al., 1997). A manifestação da septicemia hemorrágica está normalmente relacionada a situações estressantes como a ocorrência de parasitoses (Martins et al., 2000), condições inapropriadas da água, tais como grande quantidade de matéria orgânica, baixa concentração de oxigênio dissolvido, oscilações térmicas e outras formas de fragilidade dos hospedeiros (Austin e Austin, 2007).

2.5. Estresse nutricional

A nutrição e o manejo alimentar dos peixes têm como principal objetivo melhorar a eficiência produtiva, entretanto, se inadequadas estas ferramentas podem

se tornar fatores estressores durante a criação. Devido ao grande número de espécies de peixe criadas no Brasil, o mercado de ração oferece aos produtores dietas formuladas de acordo com o hábito alimentar do peixe (carnívoro ou onívoro), mas que podem não suprir as exigências nutricionais de algumas espécies ou até induzir os animais a um quadro de estresse metabólico em função do excesso ou deficiência de algum nutriente na dieta. Pouco se sabe como dietas desbalanceadas podem afetar a resposta de estresse (Serra et al., in press).

O manejo alimentar também pode levar os peixes a um quadro de estresse. Tanto o jejum e a restrição alimentar quanto alterações na frequência de alimentação dos peixes são muito utilizados em piscicultura para melhorar o desempenho produtivo (ALI et al., 2003) e a qualidade de água. Entretanto, estes manejos podem alterar vias metabólicas, e a regulação hormonal do metabolismo de peixes ocorre por processos complexos que envolve diversos fatores, incluindo o cortisol (MOMMSEN et al.,1999).

Os efeitos da restrição de alimentos na liberação de cortisol são variáveis. Em ”Artic charr” *Salvelinus alpinus* submetidos a restrição alimentar por 141 dias (JØRGENSEN et al., 1999) e em “longjaw mudsucker” *Gillichthys mirabilis* após 20 dias de jejum, os níveis de cortisol aumentaram, e houve normalização dos níveis do hormônio com o retorno da alimentação (KELLEY et al., 2001). Já em bagre do canal, houve diminuição dos níveis de cortisol após 21 dias de jejum (SMALL, 2005). Em truta arco íris, o jejum não mostrou ser um estressor nutricional, e a mobilização de energia durante o jejum, no inverno, pode ser alcançada sem o envolvimento do cortisol (POTTINGER et al., 2003). Apesar dos resultados contraditórios, o cortisol possui importância na mobilização de reservas energéticas dos peixes em jejum, estimulando a glicogenólise e a gliconeogênese hepáticas (MOMMSEN et al., 1999), e podem ser dependentes da espécie, fatores ambientais e intensidade da restrição. Outro indicador de estresse que se modificou com a restrição alimentar foi a expressão gênica de proteínas de choque térmico HSP70 e HSP90, em larvas de truta arco íris submetidas a privação alimentar por sete dias (CARA et al., 2005).

2.6. Pacu (*Piaractus mesopotamicus*)

O pacu é encontrado nas bacias dos rios Paraná, Paraguai e Uruguai (Godoy, 1975). Dentre as espécies nativas exploradas comercialmente, ele é um dos peixes

mais estudados nas regiões Sul, Sudeste e Centro-oeste do Brasil (Urbinati et al., 2013). Esta espécie é capaz de alimentar-se tanto de pequenos animais como também de folhas, caules, flores, frutos e sementes, mostrando que ao contrário da maioria das espécies de peixes, principalmente espécies carnívoras, é capaz de tolerar altos níveis de carboidrato dietético (Urbinati et al., 2013). Pacus alimentados com altos níveis de carboidratos mostraram boa capacidade de aproveitar este ingrediente, sendo observadas melhores taxas de crescimento (Figueiredo-Garutti, 1996) e melhor conversão alimentar (Baldan, 2008). Por apresentar estas características, o pacu mostra-se um excelente modelo biológico em estudos de tolerância a carboidratos em peixes.

3. Objetivos gerais

O objetivo deste estudo foi avaliar a capacidade de aproveitamento de carboidratos dietéticos em juvenis de e verificar a relação do jejum com as respostas imunes inatas.

3.1. Objetivos específicos

Avaliar o desempenho de juvenis de pacu alimentados com diferentes níveis de carboidratos e submetidos à restrição alimentar, seguido de realimentação.

Estudar as respostas metabólicas sanguíneas (glicemia, ácidos graxos, triglicerídeos, colesterol e proteínas totais) e teciduais (lipídeo e glicogênio) dos peixes.

Verificar o uso da amônia plasmática como indicador da utilização de dietas desbalanceadas em pacu.

Avaliar as respostas imunes inatas (atividade respiratória de leucócitos, concentração de lisozima e atividade do sistema complemento) do pacu após período prolongado de restrição alimentar.

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CAPÍTULO 2 – Juvenile pacu fish efficiently utilize high levels of dietary carbohydrates for growth under different feeding strategies

Abstract

This study evaluated metabolic patterns in pacu fed diets containing 25% and 45% carbohydrates (CHO). Fish were fed for 30 days, fasted for 30 days and then were re-fed for 30 days with the same initial diet. Fish were collected and analyzed at days 30, 60, 62 and 90 of the experimental period to assess weight gain (WG), specific growth rate (SGR) and protein efficiency rate (PER), as well as blood glucose, triglycerides, cholesterol, non-esterified fatty acids (NEFA), total protein, liver glycogen, liver fat, mesenteric fat (MFI) and activity of the metabolic liver enzymes of glycolysis (hexokinase, HK; glucokinase, GK), lipogenesis/pentose phosphate pathway (glucose 6 phosphate desidrogenase, G6PDH) and amino acid metabolism (aspartate aminotransferase, AST). Continuously-fed fish on the 45% CHO diet showed better growth performance during the entire experimental period. Fasted fish lost weight at day 60 but, regardless of diet, recovered growth potential. In the initial sample (day 30), levels of blood glucose and liver glycogen did not vary with dietary CHO level. The lack of hyperglycemia in fish fed 45% CHO can be explained by the observed elevation in HK and GK activities, at day 30. Indeed, after fasting, in the acute response to re-feeding, these fish also reacted with a greater elevation of GK. During fasting, fish previously fed either diet reduced G6PDH activity and MFI, reduced blood triglycerides and increased cholesterol levels. However, only in fish fed 25% CHO did fasting induce protein catabolism for energy maintenance, as evidenced by an elevation in AST activity at day 60. Metabolic responses and enzymatic activity demonstrate that pacu modulates energy metabolism according to diet and feeding strategy to accumulate or mobilize reserves.

Keyword: metabolism, enzyme activity, growth performance

Introduction

Protein sources constitute some of the most expensive components of fish diets. Therefore, excess dietary protein, unbalances in the protein to energy ratio, and alterations to dietary amino acids profiles may have a drastic impact on fish farming costs (Kim et al., 2014). Moreover, improper feeding of fish may increase nitrogen

excretion to the environment (Cho and Kaushik, 1990; Einen and Roem, 1997, Mente et al., 2003). Thus, optimizing the conversion of feed protein into fish growth by manipulating dietary carbohydrates (CHO) and lipids improves productivity and reduces environmental impacts of farming (Kaushik and Médale, 1994, Kumar et al., 2010). Dietary CHOs represent the cheapest energy source. However, most teleosts do not tolerate high CHO concentrations, and maximum dietary levels depend on fish species (Wilson, 1994; Hemre et al., 2002; Polakof et al., 2010), Formulated diets usually contain less than 20% CHO for carnivorous fish, and between 30% to 40% for omnivorous fish (Wilson, 1994).

Fish adjust to different nutritional conditions by changing their metabolic profile (Walton and Cowey, 1982; Metón et al., 1999; Lundstedt et al., 2004). In general, protein-rich diets stimulate the proteolytic and gluconeogenic pathways. On the other hand, the partial replacement of proteins by CHO stimulates glycolysis, glucogenesis and lipogenesis, reducing protein catabolism and gluconeogenesis (Pérez-Jiménez et al., 2009). Until now, the reasons for glucose intolerance in fish are not fully clear (Enes et al., 2009; Pérez-Jiménez et al., in press).

The manipulation of dietary CHO and of feeding strategies (e.g., feeding, fasting, re-feeding) provides a tool for the study of metabolic changes associated with glucose intolerance in fish (Wilson, 1994; Moon, 2001; Hemre et al., 2002; Fu and Xie, 2004; Polakof et al., 2010; Pérez-Jiménez et al., 2012). However, most previous studies focused on carnivorous species that do not use dietary CHO efficiently (Wilson, 1994; Fu and Xie, 2004). Usually, feeding or re-feeding these species results in a prolonged postprandial hyperglycemia. On the other hand, carnivorous fish are better adapted to fasting, because of their low feeding frequency under natural conditions, whereas omnivorous fish ingest food continuously (Bond, 1996). In this sense, the study of dietary CHO manipulation in fish with different natural habits may shed light on this important tolerance mechanism. The pacu (*Piaractus mesopotamicus*), natural to South America, represents one of the most commonly farmed tropical fish. In its natural habitat, it mostly feeds on roots, seeds and fruit, thus, it is highly adapted to dietary CHO.

In this study, we investigated the metabolic adjustments of pacu to feeding, fasting and re-feeding with two CHO levels. We assessed fish growth, and the acute

and chronic metabolic responses to re-feeding, including changes to blood metabolic parameters, tissue energy reserves and to the activity of metabolic enzymes.

Material and methods

Fish and experimental conditions

We used 300 fish (15.8± 1.2 g), obtained from a fish farm and initially held in 20 100 L polietilene tanks (15 fish $tank^{-1}$) for one week acclimatization, being fed with a commercial diet. During this period, we observed the appropriate feeding rate (4%) to be used during experiment. Water temperature (29.1 $\pm$ 0.3°C) and dissolved oxygen (> 5.0 mg L^{-1}) were monitored. Photoperiod was 12 h light: 12 h dark.

Experimental design and sampling

Fish were distributed in four groups: (1) Fed with a 25 % CHO diet during the 90-days trial; (2) fed with a 45 % CHO diet during the 90-day trial; (3) fed with a 25 % CHO diet for 30 days, followed by a 30-day fasting period and a 30-day re-feeding period with the same diet; (4) fed with 45 % CHO diet for 30 days, followed by a 30-day fasting period and a 30-day re-feeding period with the same diet. Diets were offered twice a day (9:00 AM and 5:00 PM) at 4% of body weight, calculated at each 15 days. At 30 (initial sampling), 60, 62 and 90 days of trial, 10 fish from each treatment (two fish per tank) were anaesthetized (benzocaine, 0.1 g L^{-1}) for blood collection and body measurements. The blood, drawn from the caudal vessel, was dispensed in microtubes containing anticoagulant (plasma) and microtubes without anticoagulant (serum). Plasma was separated by immediate centrifugation of whole blood (3000 rpm during 10 minutes at 4 ºC), and serum after blood remain at room temperature, for 3 h, to clot.

Following blood sampling, fish were euthanized (benzocaine, 0.4 g L^{-1}) and mesenteric fat, liver and white muscle from dorsal portion were removed. Mesenteric fat and liver were weighed to calculate the mesenteric fat and hepatosomatc index (MFI or HSI) [(tissue weight / body weight) x 100]. White muscle was processed to determine lipid concentration, a portion of liver was used to determine lipid and glycogen concentration and other portion of liver was used to enzymes activity assays.

Specific procedures

Experimental diets

Two isonitrogenous and isolipidic experimental diets were formulated varying to contain either 25 or 45 % carbohydrate (Table 1). Dry ingredients were weighed and mixed until to obtain a homogenous mixture. Water (40 %) was added to the mixture and then pelletized. After dried, the diets were stored at -20ºC until needed.

Table 1. Formulation and nutrient composition of experimental diets.

Ingredients	Experimental diets	
	25 % CHO	45 % CHO
Fish meal	18.75	13.00
Soybean meal	9.03	14.00
Viscera meal	13.00	15.98
Corn	11.00	14.00
Rice meal	16.60	3.00
Wheat meal	9.00	9.00
Corn starch	2.00	26.00
Soybean oil	0.40	3.00
Bicalcic phosphate	0.50	0.50
Premix[1]	0.50	0.50
BHT	0.02	0.02
Caulim	19.20	1.00
Proximate composition (% dry matter)		
Digestive protein (%)	21.70	21.80
Digestive energy (kcal kg^{-1})	2,438.20	3,339.40
Fat (%)	7.50	7.50
Carbohydrate (%)	25.00	44.50

[1]Premix: vitamin A 860,000 UI; vitamin D3 240,000 UI; vitamin E 10,500 UI; vitamin K3 1,400 mg; vitamin B1 2,100 mg; vitamin B2 2,150 mg; vitamin B6 2,100 mg; vitamin B12 2,200 mcg; Niacin 10,000mg; calcium pantotenate 5,600 mg; folic acid 580 mg; biotin 17mg; vitamin C 18,000 mg; metionin 100,000 mg; colin 60,000 mg; cooper 1,800 mg; manganese 5,000 mg; zinc 8,000 mg; iodine 90 mg; cobalt 55 mg; selenium 30 mg.

Growth performance

Data from 30, 60 and 90 days of experiment were used to calculate the growth performance, as follow: weight gain (g) (WG) = final body weight – initial body weight; specific growth rate (%) (SGR) = ((ln final body weight – ln initial body weight)/days) x100) and protein efficiency rate (%) (PER) = (weight gain/consumed protein) x 100.

Metabolic assays

Plasma was used to determine blood glucose and triglycerides concentrations (kit Labtest, Sao Paulo, Brazil, code 84 and 87, respectively) and serum to determine cholesterol and total protein (kit Labtest, Sao Paulo, Brazil, code 76 and 99). Liver glycogen level was measured according to Moon et al. (1989) and liver and muscle lipid levels following Bligh and Dyer (1959).

Enzyme activity assays

The enzymes activity was determined in liver. Tissue samples were homogenized according proposed by Pérez-Giménez et al. (2009) and enzimes activity were performed as follow:

Hexokinase (HK, 2.7.1.1) activity was determined as previously described by Vijayan et al. (1990). The reaction mixture contained 50 mM imidazole–HCl buffer (pH 7.4), 2.5 mM ATP, 5 mM MgCl 2 , 0.4 mM NADP, 2 units mL^{-1} G6PDH and 1 mM D-glucose.

Glucokinase (GK, 2.7.1.2) activity was determined as previously described by Vijayan et al. (1990). The reaction mixture contained 50 mM imidazole–HCl buffer (pH 7.4), 2.5 mM ATP, 5 mM MgCl 2 , 0.4 mM NADP, 2 units mL^{-1} G6PDH and 100 mM D-glucose.

Glucose 6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) activity was measured as previously described by Morales et al. (1990), using a reaction mixture containing 50 mM imidazole–HCl buffer (pH 7.4), 5 mM MgCl 2 , 2 mM NADP and 1 mM glucose-6-phosphate.

Aspartate aminotransferase (AST, EC 2.6.11) activity was determined as described by Singer et al. (1990). The reaction mixture contained 50 mM imidazole–HCl buffer (pH 7.4), 10 mM α-ketoglutarate, 0.3 mM NADH, 0.05 mM pyridoxal phosphate, 3 units mL^{-1} MDH and 25 mM L-aspartate.

The enzymatic reactions were initiated by the addition of the tissue extract, following adaptations of Moura (unpublished data) to HK, GK and G6PDH in pacu. All enzyme activities are expressed as milliunits per milligram of soluble protein (specific activity). One unit of enzyme activity was defined as the amount of enzyme required to transform 1 μmol of substrate per min under the above assay conditions. Soluble

protein concentration was determined according to Bradford (1976), with bovine serum albumin used as the standard.

Statistical analysis

The experiment was conducted in an entirely randomized design and results were analyzed by a three-way ANOVA. Two feeding strategies (fasted and fed) x two CHO levels (25 and 45 % CHO) x three or four periods of sampling factorial, followed by Tukey's post-hoc tests, after being tested for normality (Cramer Von Mises) and homoscedasticity tests (Brown-Forsythe). $P < 0.05$ was used as the level of statistical significance. Values in figures are means ± standard error (S.E.) of the mean for growth performance and means ± standard deviation (S.D.) of the mean for other variables.

Bioethical statement

The experimental procedures were approved by the Comissão de Ética no Uso de Animais (CEUA – Protocol 002112/12) and performed in accordance with the Guidelines of the Ethical Principles in Animal Experimentation, adopted by the Colégio Brasileiro de Experimentação (COBEA).

Results

Fish performance

To evaluate growth performance of pacu fed with different CHO levels followed by long-term fasting and re-feeding, we collected and analyzed fish at days 30, 60 and 90 of the experimental period. Feeding strategy and CHO only had independent effects on all tested variables. Inclusion of 45% CHO resulted in higher values ($P<0.05$) of WG, SGR and PER at the initial 30 day sampling (Fig. 1). At day 60 – after 30 days of fasting – no differences were observed between diets within the same feeding strategy. At this sampling, both fasted fish lost weight. At day 90 – after 30 days of re-feeding – fish continuously-fed with 45% CHO had higher WG, whereas no other differences were detected among groups.

Metabolic responses

To evaluate the metabolic adjustment of pacu fed with different CHO levels followed by long-term fasting and re-feeding, we collected and analyzed fish at days 30, 60, 62 and 90 of the experimental period. At day 62, we aimed to evaluate acute

changes to the metabolic profile, soon after reestablishing the feeding protocol, as opposed to the chronic effects of re-feeding observed at day 90.

Blood glucose did not differ ($P>0.05$) between diets after 30 days of feeding (Fig. 2 A). At day 60, blood glucose was reduced in all animals in comparison to the initial sampling. However, this reduction was significantly greater in fasted fish in comparison to continuously-fed fish, independently of dietary CHO. After one day of re-feeding (day 62), both fasted fish groups showed increased blood glucose levels that were similar to those of continuously-fed fish. At the final sampling, blood glucose of all treatment groups returned to the levels observed at day 30.

Fish fed 45% CHO showed higher initial sampling levels of triglycerides than fish fed 25% CHO (Fig. 2 B). After 30 days of fasting, both fasted fish groups had lower triglycerides levels than continuously-fed fish, but there were no differences between diets. Continuously-fed fish maintained the increased triglyceride levels unchanged until the end of the experimental period. On the other hand, regarding fasted fish, at day 62, only in fish re-fed with 45% CHO did triglycerides return to the same levels of fed fish. At day 90, triglyceride levels in previously-fasted fish on both diets had returned to the levels observed in continuously-fed fish.

At the initial sampling, cholesterol levels were the lowest observed throughout the experiment, and fish fed a 25% CHO diet had lower levels than fish fed a 45% CHO diet (Fig. 2 C). At day 60, we observed a general increase in cholesterol levels for fish in both diets. However, fasted fish had higher cholesterol levels than those observed in fed fish, but levels were the same for all groups at day 62. At day 90, cholesterol levels were lower for all fish, but only returned to the initial levels in previously-fasted animals. NEFA levels only differed between diets at the initial sampling, when fish fed 25% CHO had higher NEFA levels than fish fed 45% CHO (Fig 2 D). Total protein did not differ among treatments or time points (Data not showed).

Liver glycogen did not differ ($P>0.05$) between diets after 30 days of feeding (Fig. 3 A). At day 60, liver glycogen was reduced in all animals in comparison to the initial sampling. However, this reduction was significantly greater in fasted fish in comparison to continuously-fed fish, independently of dietary CHO. After one day of re-feeding (day 62), both fasted fish groups showed increased liver glycogen levels

that were similar to those of continuously-fed fish. At the final sampling, liver glycogen levels remained similar to those observed at day 62.

Liver lipid levels did not differ among treatments when comparing the initial to the final samples. At days 60 and 62, liver lipids were not statistically analyzed due to the low amount of tissue obtained after fasting, but the fasted group values were obtained from a pool of all samples from each treatment (Fig. 3 B).

At day 30, HSI was the highest observed throughout the experiment for all groups, however, HSI of fish fed 45% CHO was higher than seen in fish fed 25% CHO (Fig. 3 C). At day 60, fasted fish on both diets had decreased HSI compared to continuously-fed fish and compared to fish sampled at the first time point. These values remained unaltered one day after re-feeding started. At the end of 90 days, fish re-fed with 45% CHO had higher HIS levels than those observed in continuously-fed fish in the same diet group, and fish re-fed with 25% CHO.

At the initial sampling, MFI was higher in fish fed a 45% CHO diet. At days 60, 62 and 90, MFI had the same profile: fasted fish had a lower index than fed fish. Fish re-fed with 25% CHO had reduced MFI at day 62 (Fig 3 D). Comparison among sampling times showed a tendency towards increasing MFI in continuously-fed fish, but not fasted animals.

Enzyme activity

At the initial sampling, HK activity was higher in fish fed 45% CHO than in those fed 25% CHO (Fig. 4 A). At day 60, both fed fish groups kept the same profile observed at the initial sampling, but fasted fish had significantly reduced HK activity. No alterations to this profile were observed 1 day after re-feeding started, except for a recovery of HK activity in fish re-fed a 45% CHO diet, albeit to a level that was lower than observed in continuously fed fish. At the end of the experiment, we observed increased HK activity in fish re-fed a 45% CHO diet, in comparison to continuously fed fish. In continuously fed fish, HK activity tended to decrease with time, but statistical significance was only observed at the 90-day time point.

Fish fed 45% CHO had higher GK at day 30 (Fig 4 B). At day 60, both fasted fish groups reduced GK activity, but only fish previously fed 45% CHO differed significantly from continuously-fed fish on the same diet. At day 62, only fish re-fed

45% CHO increased GK activity to values higher than those seen in continuously-fed fish. This pattern was only observed in fish re-fed 25% CHO at day 90.

Dietary CHO did not affect G6PDH activity at the initial sampling, and activity remained constant for continuously-fed fish (Fig. 4 C). At day 60, both fasted fish groups had reduced G6PDH activity when compared to continuously-fed fish and with previous sampling time. This pattern remained in at day 62 but disappeared at day 90, when all groups had similar levels of G6PDH activity.

AST activity was higher in fish continuously feeding on 25% CHO at the initial sampling and at day 60 compared to fish feeding on 45% CHO. Values for continuously-fed animals were constant throughout the experiment (Fig 4 D). Fish previously fed 25% CHO showed increased AST activity in response to fasting, and both re-fed groups had reduced AST activity at day 62 compared to continuously-fed fish. At the end of the experiment, fish in all treatment groups had the same levels of AST activity.

Discussion

Dietary manipulation allows for the assessment of glucose intolerance mechanisms in fish (Wilson, 1994; Moon, 2001; Hemre et al., 2002; Fu and Xie, 2004; Polakof et al., 2010; Pérez-Jiménez et al., 2012). To date, however, most studies have focused on carnivorous species that are naturally less adapted to dietary CHO. In this study, we assessed metabolic changes in the fruit-eating fish pacu. We observed that pacu efficiently used high levels of dietary CHO for growth under different feeding strategies.

The inclusion of 45% CHO in diets increased the growth performance of pacu juveniles. Higher WG values for fish fed this higher CHO diet compared to the 25% CHO diet were associated with better SGR and PER at day 30. At day 90, differences between fish fed 25% and 45% CHO diets were intensified. Two characids economically important in the Amazon region of South America, the black pacu, *Colossoma macropomum*, and red pacu, *Piaractus brachypomus*, both omnivorous species, were fed diets containing Amazonian carbohydrate-rich plant feedstuffs (yucca, *Manihot sculenta*, plantain, *Musa paradisiaca*, or pijuayo, *Bactris gasipaes*) as alternative energy sources for practical diets. The diets contained around 45% CHO. However, differently from our study, the energy in the test feedstuffs was used partly

for liver glycogen synthesis rather than for growth because no significant differences in body weight was observed among fish fed the test diets (Lochmann et al., 2009).

The 25% CHO diet was intentionally formulated to have a lower amount of energy than that provided by the 45% CHO diet, because dietary CHO but not lipids were altered, resulting in diets with different protein to energy ratios. Some benefits of diets with high-energy content have been reported, such as better growth, food utilization and protein retention besides reduced nutrient excretion (Einen and Roem, 1997). The increased AST activity in fish fed 25% CHO, observed in this study, suggests that the imbalance between energy and protein may have resulted in protein catabolism for energetic purposes increasing nitrogen excretion, as previously reported (Cho and Kaushik, 1990). When insufficient energy is available in a diet from non-protein sources, protein may be catabolized to meet the energy requirements at the cost of nutrient supply somatic growth (Capuzzo & Lancaster 1979). In a previous study, Peragón et al. (1999) demonstrated that absence of carbohydrates in diet of rainbow trout (*Oncorhynchus mykiss*) reduces significant growth and daily weight gain due to muscle mass loss.

At day 60, fasted fish lost weight. Weight loss results from energy mobilization from different body reserves to maintain vital processes during fasting (Pérez-Jiménez et al., 2012). In our study, pacu used glycogen and mesenteric fat, however, pacu juveniles did not lose the ability to re-grow. Re-fed fish grew although they had lower WG compared to continuously-fed fish. Fasted fish weighed 38.9 ± 6.0 g at the end of fasting against 78.9 ± 23.4 g of continuously-fed fish, but both groups had similar SGRs at day 90, the final sampling.

We did not observe an effect of dietary CHO levels on blood glucose. However, at day 30, HK and GK activity in fish fed 45% CHO was higher. Soon after glucose enters cells, it is phosphorylated to glucose 6-phosphate by HK and GK and enters one of three pathways; glycolysis, glycogenesis or pentose-phosphate. At day 30, all fish groups had similar glycogen levels. In the common carp (*Cyprinus carpio*), an omnivorous fish, the hepatic glycogen contents were higher in the fish fed digestible starch than in those on a CHO free diet and the activity of HK remained almost constant after ingestion of the carbohydrate diets, while the activity in fish on the CHO free diet declined 24 h after feeding (Capilla et al., 2004). Together, our findings indicate that,

with high CHO levels, fish may have increased energy generation through the glycolysis and pentose-phosphate pathways. In this mode, protein sources are spared and directed towards growth, whereas NADPH is directed towards lipid synthesis, as supported by the higher MFI observed in these fish. At day 60, immediately after fasting, HK and GK were similarly reduced regardless of diet, evidently as a result of reduced substrate. HK did not change in the acute response to re-feeding (day 62), but GK showed a large increase in fish fed the 45% CHO diet. In gilthead sea bream juveniles, dietary glucose was more effective in inducing the response of GK than starch. These higher GK activity may be related to the higher glycaemia observed in fish fed the glucose diet, which may increase glucose uptake by the liver. Accordingly, both HSI and liver glycogen content were far higher in fish fed the glucose diet than the starch diet (Enes et al., 2008).

Thus, reduced blood glucose and liver glycogen levels, and the prompt recovery of these values after one day of re-feeding indicate the importance of glucose homeostasis and liver glycogen as important energy reserves in pacu juveniles.

The higher triglyceride and cholesterol levels observed at day 30 in fish fed 45% CHO seem to reflect nutritional status. A previous study with the same species reported unchanged triglycerides with different dietary CHO levels (Abimorad et al., 2007). Differently from our study, these authors used *ad libitum* feeding and observed a tendency towards reduced consumption with increasing CHO concentrations. In our study, we controlled feeding and differences in CHO intake. Thus, fish indeed consumed more CHO, and excess CHO may have been converted to triglycerides in the liver and stored in adipocytes, as previously reported (White, 2009). Values of MFI at day 30 support this view.

After fasting, triglycerides and cholesterol levels had different profiles. Fasted fish on both diets had reduced triglycerides and increased cholesterol levels. Under long-term fasting, glycerol derived from the hydrolysis of triglycerides is actively used as a substrate for the gluconeogenic process (Pérez-Jiménez et al., 2012). The reduction of MFI in fasted fish reflects the important role of lipid reserves during long-term fasting. In previous studies with pacu, cholesterol levels either reduced (Takahashi et al., 2011) or increased (Fávero et al., unpublished data) after fasting. In our study, increased cholesterol levels may have resulted from stimulation, during

prolonged fasting, of the cholesterol synthesis pathway by acetyl Co-A provided from the β-oxidation of fatty acids (Maita et al., 2006). According to Fávero et al. (unpublished data), the increase in cholesterol levels in fasted fish may be associated with the stress induced by food deprivation, because cholesterol represents a precursor of cortisol. The importance of cortisol in the regulation of energy metabolism and immune response in fasted pacu has been described (Gimbo et al., 2015).

In fasted fish, lipid synthesis was reduced as evidenced by a decrease in G6PDH activity. This reduction, observed at days 60 and 62 after fasting, correlates well with reduced MFI. Low G6PDH activity, in turn, may be associated with reduced HK activity in the absence of glucose during fasting. Glucose 6-phosphate, resulting from HK activity, may follow the pentose-phosphate pathway, involving G6PDH, a key enzyme catalyzing the first step of the pathway which generates NADPH for anabolic pathways, including lipid synthesis, and protection systems in various organisms, including fish (Hu et al., 2013).. The lack of alterations in HK and G6PDH activities between days 60 and 62 with ensuing normalization at day 90 may reflect the time lapse necessary for up-regulation of gene transcription and mRNA translation.

During prolonged fasting, fish previously fed 25% CHO used glycogen, mesenteric fat and protein as energy sources. Total serum protein did not differ between fasted and fed fish, but increased AST values indicate increased protein catabolism in fish fed 25% CHO. Unchanged AST activity in fish fed 45% CHO suggests prevention of protein catabolism during long-term fasting. In *Dentex dentex*, plasma protein concentrations decreased after prolonged fasting for 5 weeks (Pérez-Jiménez, et al., 2012), and similar results were observed in carp (Shimeno et al., 1997). However, even with elevated protein catabolism, the unchanged total serum protein observed in this study indicates that a protein protection mechanism may exist in pacu. One day after re-feeding started, previously fasted fish on both diets had lower AST activity than continuously-fed fish. This response reduces protein catabolism in order to restore blood metabolites, energy reserves and to promote a return to normal growth conditions.

The hormonal control of metabolic pathways by insulin is well known. In salmonids, plasma insulin titers increased after a high carbohydrate meal or a glucose load (Mommsen and Plisetskaya, 1991). This rise in insulin may affect metabolism in

hepatocytes because of the anabolic and anti-catabolic effects of this hormone in fish. Although we did not measure insulin, our results suggest an effect of this hormone as metabolic mediator in pacu. High CHO levels, supposedly resulting in high insulin levels, increased HK activity and decreased AST activity. During fasting, a period of low insulin, AST activity increased, whereas glycogen reserves, MFI, HK and G6PDH activity decreased.

In conclusion, we show that pacu efficiently utilizes high dietary CHO levels reducing protein catabolism to promote growth. This fish endures prolonged fasting, adjusting enzyme activities to spare energy during food deprivation, and to quickly replenish energy reserves after the reestablishment of an appropriate feeding schedule.

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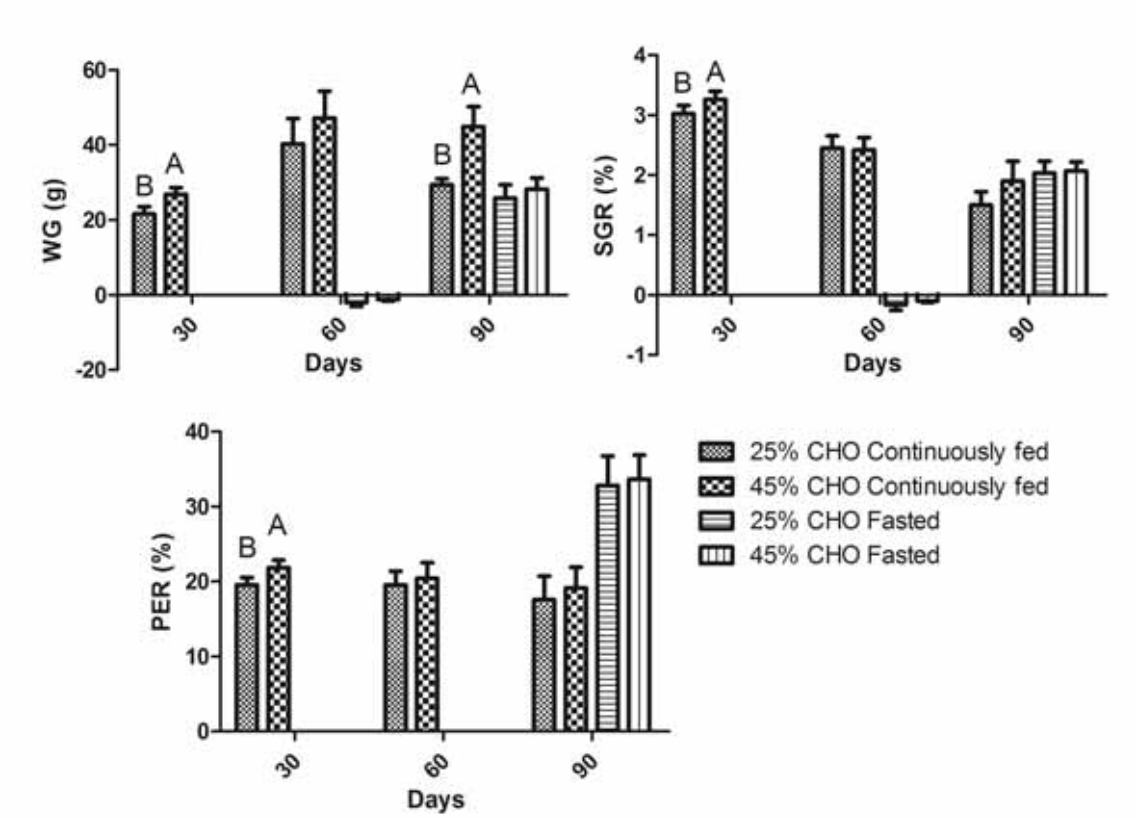

Figure 1. **Effects of carbohydrates (CHO) and feeding strategy on weight gain (WG), specific growth rate (SGR) and protein efficiency rate (PER)**. Pacu fish fed diets containing 25% or 45% carbohydrates (CHO) were submitted to a sequence of feeding, fasting and re-feeding periods of 30 days each (Fasted), or were continuously fed. Fish were collected and analyzed after each period – days 30, 60 and 90 of the experimental protocol. Uppercase letters indicate differences between fish continuously fed and lowercase letters indicate differences between fasted fish analyzed by Tukey test ($P<0.05$).

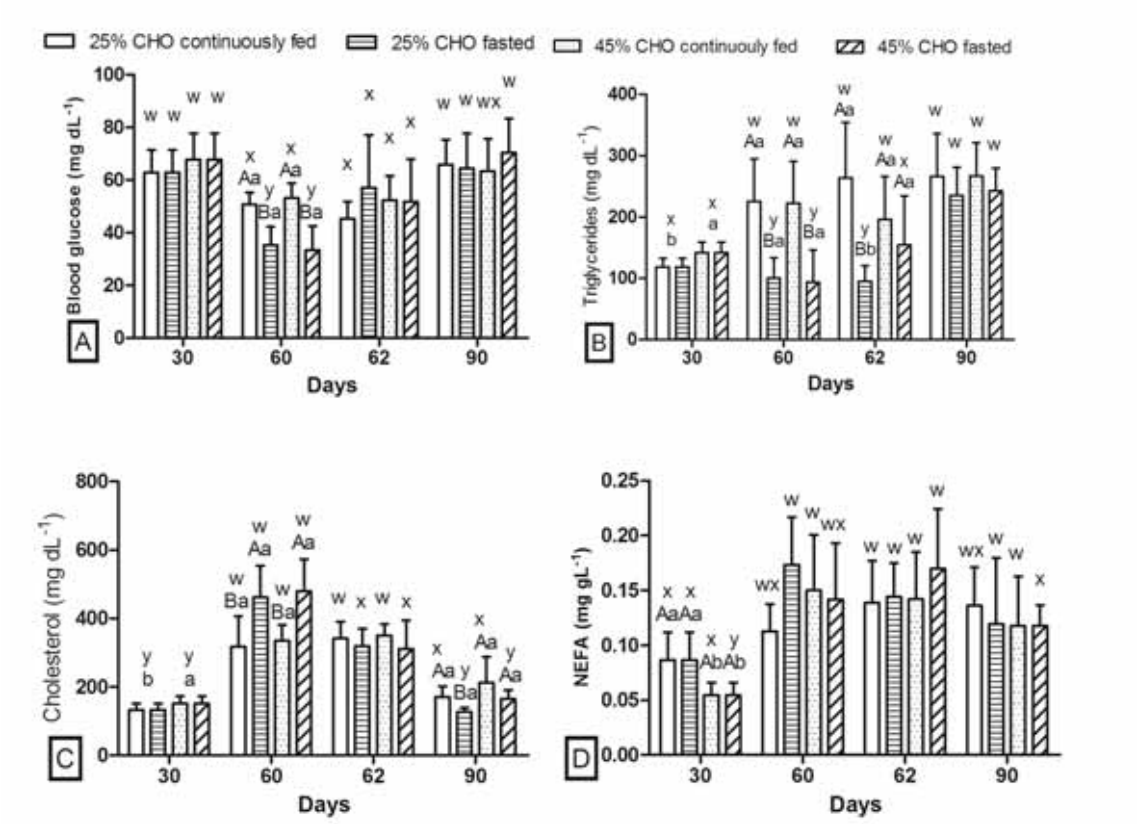

Figure 2. Effects of carbohydrates (CHO) and feeding strategy on blood glucose, triglycerides, cholesterol and non-esterified fatty acids (NEFA) levels. Pacu fish fed diets containing 25% or 45% carbohydrates (CHO) were submitted to a sequence of feeding, fasting and re-feeding periods of 30 days each (Fasted), or were continuously fed. Fish were collected and analyzed after each period – days 30, 60 and 90 of the experimental protocol, and at day 62 for the evaluation of the acute response to re-feeding. Uppercase letters indicate differences between feeding strategies, lowercase letters (a, b) indicate differences between diets, and lowercase letters (w, x, y) indicate differences between time points analyzed by Tukey test ($P<0.05$).

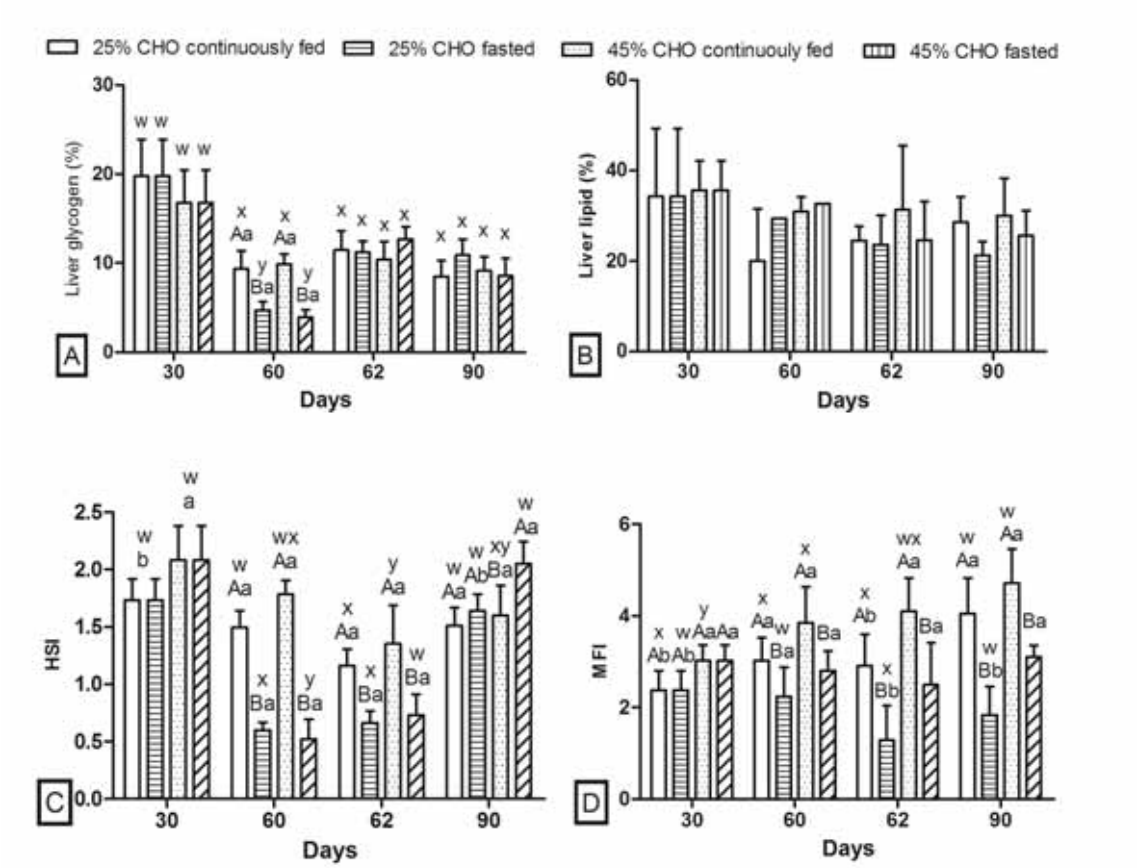

Figure 3. Effects of carbohydrates (CHO) and feeding strategy on liver glycogen, liver lipids, hepatossomatic index (HSI) and mesenteric fat index (MFI). Pacu fish fed diets containing 25% or 45% carbohydrates (CHO) were submitted to a sequence of feeding, fasting and re-feeding periods of 30 days each (Fasted), or were continuously fed. Fish were collected and analyzed after each period – days 30, 60 and 90 of the experimental protocol, and at day 62 for the evaluation of the acute response to re-feeding. Uppercase letters indicate differences between feeding strategies, lowercase letters (a, b) indicate differences between diets and lowercase letters (w, x, y) indicate differences among time points analyzed by Tukey test ($P<0.05$).

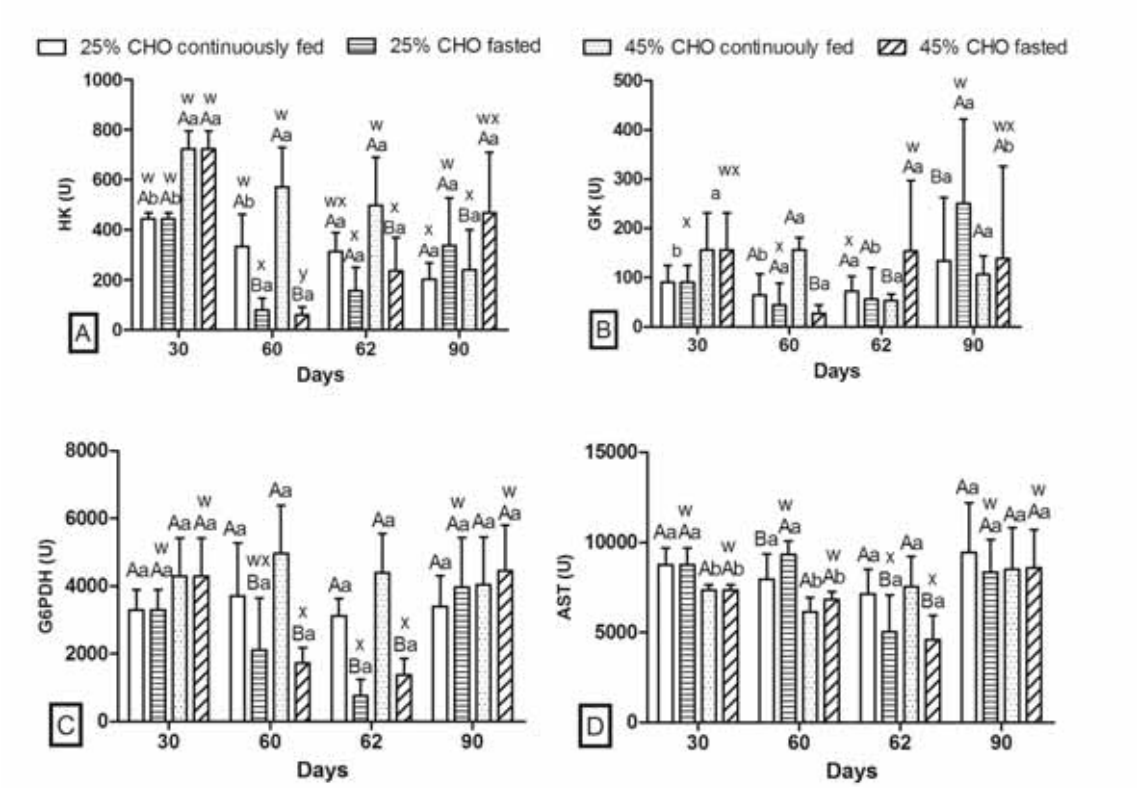

Figure 4. Effects of carbohydrates (CHO) and feeding strategy on the activities of hexokinase (HK), glucokinase (GK), glucose-6-phosphate dehydrogenase (G6PDH) and aspartate amino transferase (AST). Pacu fish fed diets containing 25% or 45% carbohydrates (CHO) were submitted to a sequence of feeding, fasting and re-feeding periods of 30 days each (Fasted), or were continuously fed. Fish were collected and analyzed after each period – days 30, 60 and 90 of the experimental protocol, and at day 62 for the evaluation of the acute response to re-feeding. Uppercase letters indicate differences between feeding strategies, lowercase letters (a, b) indicate differences between diets, lowercase letters (w, x, y) indicate differences among time points analyzed by Tukey test ($P<0.05$).

CAPÍTULO 3. Serum ammonia as indicator of unbalanced diet in pacu (*Piaractus mesopotamicus*)

Short communication

Introduction

Plasma ammonia is usually used as indicator of stress in fish. Stress results in increased levels of cortisol (Wendelaar Bonga, 1997), stimulating both glycogenesis and gluconeogenesis, by increased protein catabolism (Mommsen et al., 1999) and ammonia production (Randall and Tsui, 2002).

Ammonia and urea are the major nitrogenous end products in fishes, with ammonia comprising at least 80% of nitrogen excretion in most teleosts (Wright and Wood, 2012). The production of ammonia occurs mainly through the transamination of various amino acids (Forster and Goldstein 1969; Watts and Watts, 1974). The primary site for ammonia production is probably the liver (Randall and Tsui, 2002), but the necessary enzymes have also been located in the kidneys, gills, and skeletal muscle (Goldstein and Forster, 1961; Walton and Cowey, 1977; McBean et al., 1966).

Several studies evaluating the efficiency of different diets are based in results of growth performance, body composition and diet digestibility, however, they take long time to produce conclusive results. In this study, we propose to validate an alternative and punctual indicator of unbalanced diet in serum of pacu that is the measure of the circulating levels of ammonia.

Material and methods

The experimental procedures were approved by the Comissão de Ética no Uso de Animais (CEUA – Protocol 002112/12) and performed in accordance with the Guidelines of the Ethical Principles in Animal Experimentation, adopted by the Colégio Brasileiro de Experimentação (COBEA).

Fish and experimental conditions

We used 150 (15.8± 1.2 g) fish, obtained from a fish farm and initially held in 10 100 L plastic tanks (15 fish $tank^{-1}$) for one week acclimatization, being fed with a commercial diet. During this period, water temperature (27 ± 1°C) and oxygen levels (> 5.0 mg L^{-1}) were monitored. Photoperiod was 12 h light: 12 h dark.

Experimental design and sampling

Fish were distributed in two groups and fed with two diets (25 and 45% CHO) during 30 days, offered twice a day (9:00 AM and 5:00 PM) at 4 % of body weight. At the end of this period, 10 fish of each treatment (two fish per tank) were anaesthetized with benzocaine (0.1 g L^{-1}) for blood sampling and weight and length measurement. The blood, drawn from the caudal vessels, was dispensed in microtubes without anticoagulant. Blood was kept at room temperature to clot and then centrifuged to separate serum. After blood sampling, fish were euthanized by brain destruction for liver removal, and a fragment was stored at -80 ºC for further determination of enzyme activity.

Specific procedures

Experimental diets

Two isonitrogenous and isolipidic experimental diets were formulated varying to contain different (Table 1). Dry ingredients were weighed and mixed until to obtain a homogenous mixture. Water (40 %) was added to the mixture and then pelletized. After dried, the diets were stored at -20ºC until needed.

Table 1. Formulation and nutrient composition of experimental diets.

Ingredients	Experimental diets	
	25 % CHO	45 % CHO
Fish meal	18.75	13.00
Soy bran	9.03	14.00
Viscera meal	13.00	15.98
Corn	11.00	14.00
Rice bran	16.60	3.00
Wheat bran	9.00	9.00
Corn starch	2.00	26.00
Soy oil	0.40	3.00
Bicalcic phosphate	0.50	0.50
Premix[1]	0.50	0.50
BHT	0.020	0.02
Caulim	19.20	1.00
Proximate composition (% dry matter)		
Digestive protein (%)	21.70	21.80
Digestive energy (kcal kg^{-1})	2,438.20	3,339.40
Fat (%)	7.50	7.50
Carbohydrate (%)	25.00	44,50

[1]Premix: vitamin A 860,000 UI; vitamin D3 240,000 UI; vitamin E 10,500 UI; vitamin K3 1,400 mg; vitamin B1 2,100 mg; vitamin B2 2,150 mg; vitamin B6 2,100 mg; vitamin B12 2,200 mcg;

Niacin 10,000mg; calcium pantotenate 5,600 mg; folic acid 580 mg; biotin 17mg; vitamin C 18,000 mg; metionin 100,000 mg; colin 60,000 mg; cooper 1,800 mg; manganese 5,000 mg; zinc 8,000 mg; iodine 90 mg; cobalt 55 mg; selenium 30 mg

Serum ammonia concentration

Serum ammonia determination followed protocol proposed by Verdouw et al. (1978) with modifications. We used 60 µL serum treated with 20 µL trichlorine acetic acid (15%) and then centrifuged (10000 rpm during 3 minutes), and the supernatant was separated to prevent the N-protein interference in final results. Reaction was carried out adding 30 µL of the supernatant to a mixture containing 0.01 mM sodium nitroprusside, 0.32% sodium hipochloride, 64.5 mM sodium hydroxide, 87.1 mM sodium citrate and 161.16 mM sodium salicylate. After well shaked, the reaction occurred for 2 hours in dark for blue-coloured indophenol formation. Solution was then read at room temperature, using a spectrophotometer (Thermo Scientific; Genesys 10S), set to 540nm. Ammonia chlorite was linearly diluted to calculate ammonia samples concentration.

Aspartate aminotransferase (AST, EC 2.6.11) activity assay

Samples were homogenized in 1:4 ice-cold Tris-HCl buffer containing 0.1 mM EDTA and 0.1% (v/v) Triton X-100, pH 7.8. Homogenates were centrifuged at 14,000 ×g for 30 min, at 4 °C, and the supernatants were separated in aliquots and stored at −80 °C for further enzymes assays. The assay was carried out in kinetic assay (5 minutes, 340 nm, at 25 °C). Enzyme activity was measured by production or degradation of NADPH using a Multskan microplate reader (Thermo Scientific, USA). The substrate dilution was determined using a pool of all samples verifying the linearity and the ideal curve inclination. The enzymatic reactions were initiated by the addition of the tissue extract and followed adaptations of Pérez-Jiménez et al. (2009).

Aspartate aminotransferase (AST) activity was determined as previously described by Singer et al. (1990). Reaction mixture contained 50 mM imidazole–HCl buffer (pH 7.4), 10 mM α-ketoglutarate, 0.3 mM NADH, 0.05 mM pyridoxal phosphate, 3 units mL^{-1} MDH and 25 mM L-aspartate. AST activity is express as milliunits per milligram of soluble protein (specific activity). One unit of enzyme activity was defined as the amount of enzyme required to transform 1 µmol of substrate per mg of protein

under the above assay conditions. Soluble protein concentration was determined using the method of Bradford (1976), with bovine serum albumin used as a standard.

Statistical analysis

The experiment was conducted in an entirely randomized design and results from both fish groups were compared by T-test. $P<0.05$ was used as the level of statistical significance. Values in figures are means ± standard deviation (S.D.) of the mean.

Results

We used diets with distinct CHO levels (25 and 45 %) and with intentional unbalance in protein/energy ratio (25% CHO diet) to induce metabolism to shift to gluconeogenesis in pacu. We observed higher AST activity and higher serum ammonia levels in fish fed with 25% CHO diet (Fig. 1 A and B).

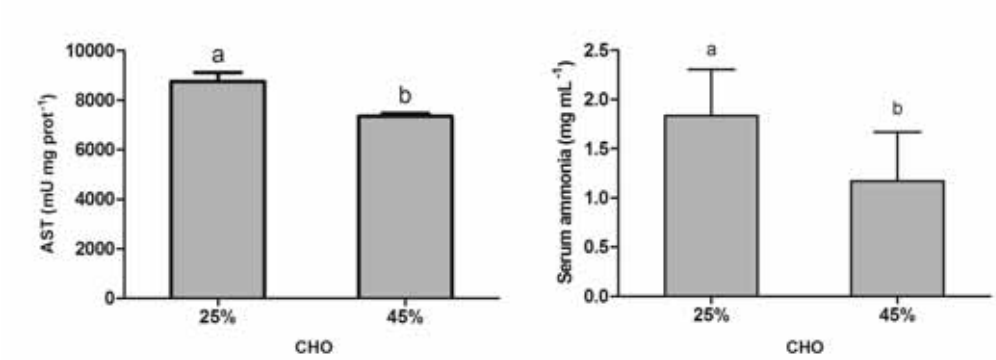

Figure 1. AST (*aspartate aminotransferase)* activity and serum ammonia of pacu fed with 25 and 45% CHO during 30 days. Different letters indicate difference by T test ($P<0.05$).

Discussion

In this study, we modified the protocol of Verdouw et al. (1978) to determine serum ammonia for the first time as an alternative assay to verify the occurrence of unbalance diets in fish. Based in our results, 25% CHO diet induced protein catabolism in pacu, due to the unbalance in protein and energy levels. Some benefits of appropriate protein/energy ratio have been reported, such as better growth, better food

utilization, higher protein retention and reduced nutrient excretion (Einen and Roem, 1997).

High protein/energy ratio observed in 25% CHO diet (8.9 g CP $kcal^{-1}$) compared to 45% (6.5 g CP $kcal^{-1}$) resulted in excess of amino acids that fish can use for energy propose, which were converted to ammonia in the liver. Transaminases in the liver convert amino acids to glutamate, and the accompanying alpha-keto acid (Forster and Goldstein, 1969; Watts and Watts, 1974). As dietary protein is the most expensive component, it contributes significantly to the cost of the diet. Thus, it is desirable that this protein is used for growth and not to supply the energy needs of the fish (Kumar et al., 2010).

Higher AST activity in 25% CHO diets observed in this study has two main roles. The first is the deamination of amino acids to use carbonic skeletal as energy source, and a second function has a protective function against ammonia. Ammonia toxicity can be ameliorated by the formation of less toxic compounds, as glutamine and urea (Randall, et al., 1989). Thus, serum ammonia determination by colorimetric assay showed to be easy, relatively fast without to kill the fish unnecessarily.

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CAPÍTULO 4. Energy deficit does not affect immune responses of experimentally infected pacu (*Piaractus mesopotamicus*)*

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http://www.sciencedirect.com/science/article/pii/S1050464815000066

Abstract

We investigated if the energy deficit following a 30-day starvation period could affect the ability of fish to mount immune responses after experimental exposure to *Aeromonas hydrophila*. Fish were submitted to two feeding strategies during 30 days: starvation and continuously feeding. Fish were then sampled to allow for the assessment of baseline metabolic and immune system indicators, were next intraperitonially inoculated with *Aeromonas hydrophila*, and finally were sampled at 3 and 24 h after the challenge. The respiratory activity of leukocytes was lower in starved fish at baseline, increasing after bacterial inoculation to levels similar to those seen among fed fish. Levels of serum lysozyme were higher in starved fish at baseline. The same response profile was observed 3 hours after inoculation, but among fed fish, these levels increased to values similar to those of starved fish 24 hours after infection. Among starved fish, lysozyme concentration did not change over the course of the experiment. The serum ACH activity was lower in starved fish at baseline and increased after bacterial inoculation in both fish groups. Baseline levels of blood glucose of starved fish were lower than those of fed fish and increased 3 hours after bacterial inoculation in both fish groups, decreasing in both groups at 24 hours after inoculation. Baseline liver glycogen levels were similar in both fish groups and higher than at 3 and 24 hours after inoculation. Three hours after bacterial inoculation, liver glycogen was less reduced in fed fish. Baseline levels of blood triglycerides were lower in starved fish and the profile remained unchanged 3 hours after inoculation. There was a gradual decrease in fed fish, and the levels of starved fish remained unchanged throughout the observation period. Blood glycerol levels at baseline were higher in starved fish than in fed fish and remained unaltered at 3 hours after inoculation. However those levels increased at 24 hours. In fed fish there was a gradual increase of glycerol levels up to 24 hours after bacterial inoculation. Baseline liver lipid levels of starved fish were lower and this difference in the response profile remained

unchanged 3 and 24 hours after inoculation. The liver lipid levels of fasted fish decreased after inoculation, and remained unchanged in fed fish. As observed in liver lipid, muscle lipid levels of starved fish were lower than in fed fish, throughout the experiment. Fasted fish levels remained unchanged; however fed fish levels decreased 24 hours after bacterial inoculation. Levels of cortisol were higher in starved fish at baseline and increased in both fish groups 3 hours after bacterial inoculation, reaching intermediary levels 24 hours after inoculation. Our results show that in pacu, although mounting an immune response triggered after bacterial exposure is an energy-expensive process, fish under energetic deficit status were able to display protection against infection.

Keywords: innate immune responses, energy deficit, pacu.

Introduction

Immune defense is an energetically costly physiological process. The production and maturation of immune cells in response to a pathogen, as well as the increased synthesis and activity of humoral components, such as proteins of the innate and acquired immune systems, are energetically costly [1, 2]. Both starvation and infection promote important alterations in animal physiology, which are sustained by mobilization of reserves to meet this energy demand [3-6]. The adaptive physiological response of fish to starvation is aimed at conserving the health and function of key organs and redistributing essential resources for biological systems [7].

Previous research on the effects of starvation in fish has focused mainly on metabolic responses and endocrine pathways [5, 7-9]. Immune responses have also been evaluated in starved fish [5, 8, 10, 11], however, little is known about immune responses after infection in starved fish [5]. Thus, it is still unclear how energy deficits affect the different metabolic pathways involved in the immune responses triggered after bacterial exposure.

Understanding the modulation of immune responses under conditions of energetic deficit is particularly important considering that food restriction is a feeding strategy widely used in the fish farming industry to promote compensatory growth [12] while improving growth efficiency [13] and preventing water quality deterioration [14].

However, it is possible that while growth is more efficient at restricted levels of energy, this reduction of the energy budget might affect biological processes such as mounting of immune responses. This possibility would be in line with the notion that host defense against parasites and pathogens is a costly life-history trait that can generate trade-offs with other fitness components [15]. In this study, we investigated the effects of energetic deficit following starvation on innate immune and metabolic responses of pacu, an important farmed fish in South America, after experimental pathogen exposure. Published studies have concerned pacu response to starvation [17,18], stress [19], metabolism [20] and immune responses [21] but none associated metabolic mobilization to an experimental bacterial infection.

Material and methods

The experimental procedures were approved by the Comissão de Ética no Uso de Animais (CEUA – Protocol 002112/12) and performed in accordance with the guidelines of the ethical principles in animal experimentation, adopted by the Colégio Brasileiro de Experimentação (COBEA).

Fish and experimental conditions

We used a total of 96 (345.6 ± 16.4 g) fish, obtained from the CAUNESP (Centro de Aquicultura da UNESP) and initially held in 8 fiber tanks of 460 L (12 fish $tank^{-1}$) for one week acclimatization, being fed a commercial diet. During this period, water temperature (28.5 ± 1.0 °C) and oxygen levels (> 5.0 mg L^{-1}) were monitored. Photoperiod was 13 h light: 11 h dark.

Experimental design

Fish were submitted to two different strategies during 30 days: starvation (S) and continuous feeding (F) groups (4 tanks per treatment). Fed fish were fed until apparent satiation with experimental diet twice a day (9:00 AM and 5:00 PM). At the end of the 30-day trial, 12 fish per treatment were quickly sampled to allow for the assessment of baseline metabolic and immune system indicators. The remaining fish of each tank were intraperitoneally injected with Aeromonas hydrophila and then sampled at 3 and 24 h after the challenge (n = 12). The suspension of $1x10^2$ CFU cells

mL1 of bacteria was previously determined to provoke only infection and not severe mortality (data not shown), to allow the stimulation of the fish immune system.

Sampling and experimental infection

Fish (12 from each treatment) were anaesthetized with benzocaine (0.1 g L^{-1}) and blood samples, drawn from the caudal vessel, were dispensed in heparinized microtubes (plasma), microtubes containing anticoagulant, and microtubes without anticoagulant (serum). Whole blood was immediately used to measure the leukocyte respiratory burst. For plasma separation, blood samples were quickly centrifuged (10 min at 3.000 xg), and glucose and triglyceride concentrations were determined immediately. Blood was allowed to clot at room temperature for 3 hours, thereafter centrifuged and serum was stored at –80 ºC until analysis of cortisol, glycerol, complement system activity, and serum lysozyme concentration. After blood sampling, fish were euthanized (benzocaine, 0.4 g L^{-1}) for mesenteric fat removal, whose weight was used to calculate the mesenteric fat index (MFI) [(mesenteric fat weight / body weight) x 100] and liver and dorsal portions of white muscle were removed and stored at –20 ºC for further determination of lipids and glycogen.

Specific procedures

Experimental diets

The practical diets were formulated and prepared aiming to supply pacu requirements (22% crude protein and 4,200 kcal kg^{-1}) [16] and stored at –20 ºC until needed.

Metabolic assays

The blood glucose and triglyceride concentrations were measured by enzymatic method (Labtest kit, Sao Paulo, Brazil, codes 84 and 87, respectively). Serum was used to determine glycerol by the enzymatic method (Bioclon, Belo Horizonte-MG, Brazil, code 76) and cortisol concentration by ELISA (Enzyme-Linked ImmunoSorbent Assay) with a commercial kit (DRG International, Inc., USA; Cortisol ELISA – EIA –1887) following manufacturer's instructions. Liver glycogen level was measured by lysis in amyloglycosidase after extraction with perchloric acid (7%) [17]

and liver and muscle lipid levels were determined after extraction in chloroform and methanol solution [18].

Immunological assays

Leukocyte respiratory burst

The production of reactive oxygen species (ROS) was measured using NBT (Nitrotetrazolium Blue chloride – Sigma-Aldrich – N6876), following the protocol of [19] modified by [20]. Immediately after fish bleeding, 100 µL of heparinized blood was incubated with an equal volume of NBT buffer (0.2%) at room temperature for 30 min. Thereafter, 1 mL of dimethylformamide (DMF, Sigma, St Louis, MO, USA – 227056) was added to the samples, and they were read using a spectrophotometer (Thermo Scientific; Genesys 10S), at room temperature, and set to 540nm.

Serum lysozyme concentration

Serum lysozyme concentration was determined according to [21] modified for use with pacu blood. The assay is based on the lysis *Micrococcus lysodeikticus* (Sigma-Aldrich – M3770) suspension using hen egg white lysozyme (Sigma-Aldrich – L6876) as standard. The assay was performed into a 96-well plate in triplicate. The rate of decrease in absorbance for each sample (∆OD) was then compared to that obtained with the standard curve so that lysozyme concentration could be expressed in ng μL^{-1}.

Complement system activity: Alternative pathway (ACH activity)

Serum complement hemolytic activity was measured according to [22] and [23] modified for use with pacu blood. Rabbit blood was collected and processed to isolate red blood cells (RaRBC) whose suspension was added to serum, then complement hemolytic activity was measured as time (in seconds) necessary to lyse 50% of RaRBC in kinetic assay at 700 nm, using a Genesys 10S spectrophotometer (Thermo Scientific®).

Statistical analysis

The experiment was conducted in an entirely randomized design and results were analyzed by a two-way ANOVA. Two feeding strategies (starved and fed) x 3 samplings (baseline, 3, and 24 h after bacteria inoculation) factorial, followed by Tukey's post-hoc tests, were used to examine the effect of the energetic status on the response to the pathogen after being tested for normality (Cramer Von Mises) and homoscedasticity tests (Brown-Forsythe). $P<0.05$ was used as the level of statistical significance. Values in the text and figures are means ± standard deviation (S.D.) of the mean.

Results

To evaluate how the energetic status of pacu affected the metabolic response profile according to the immune strategy adopted by infected fish, we used starvation as a tool to achieve different energetic status, and bacterial inoculation with intraperitonial injection to induce the immune responses. Differences in energetic status between the two groups of fish were confirmed at the end of the feeding trial, as starved fish lost weight (-7.7 g ± 1.1) whereas fed fish gained weight (105.2 g ± 22.7). Similarly, MFI values were lower in starved (2.8% ± 0.7) compared to fed fish (3.7% ± 0.8).

Metabolic responses

Baseline levels of blood glucose of starved fish were significantly lower ($P < 0.05$) than those of fed fish. Blood glucose levels increased significantly in both groups following challenge when compared to the baseline values (Fig. 1A). Highest levels occurred at 3 h post-inoculation (hpi). There was no significant difference in blood glucose levels between the groups at 3 and 24 hpi. Both groups showed similar liver glycogen levels at baseline, higher than at 3 and 24 h after inoculation. Three hours after bacterial inoculation liver glycogen from both groups was reduced, but less in fed fish. Both fed and starved fish continued consuming liver glycogen 24 hpi with the same levels in both groups (Fig. 1B).

Baseline levels of blood triglycerides were significantly lower in starved than in fed fish. 3 hpi this difference in the response profile remained unchanged; however, there were no differences in triglycerides levels between the groups 24 hpi. This effect

was prompted by a gradual decrease in triglyceride levels in fed fish, as such levels remained unchanged among starved fish throughout the observation period (Fig. 1C).

Blood glycerol levels at baseline were significantly higher in starved fish than in fed fish and remained unaltered at 3 hpi. However those levels increased at 24 h. In fed fish there was a gradual increase of glycerol levels up to 24 hpi (Fig. 1D).

At baseline liver lipid levels of starved fish were significantly lower than in fed fish and this difference in the response profile remained unchanged 3 and 24 hpi. The liver lipid levels of starved fish decreased after inoculation and remained unchanged in fed fish (Fig. 1E). As observed in liver lipid, muscle lipid levels of starved fish were significantly lower than in fed fish throughout the experiment. Starved fish levels remained unchanged; however fed fish levels decreased 24 h after bacterial inoculation (Fig. 1F).

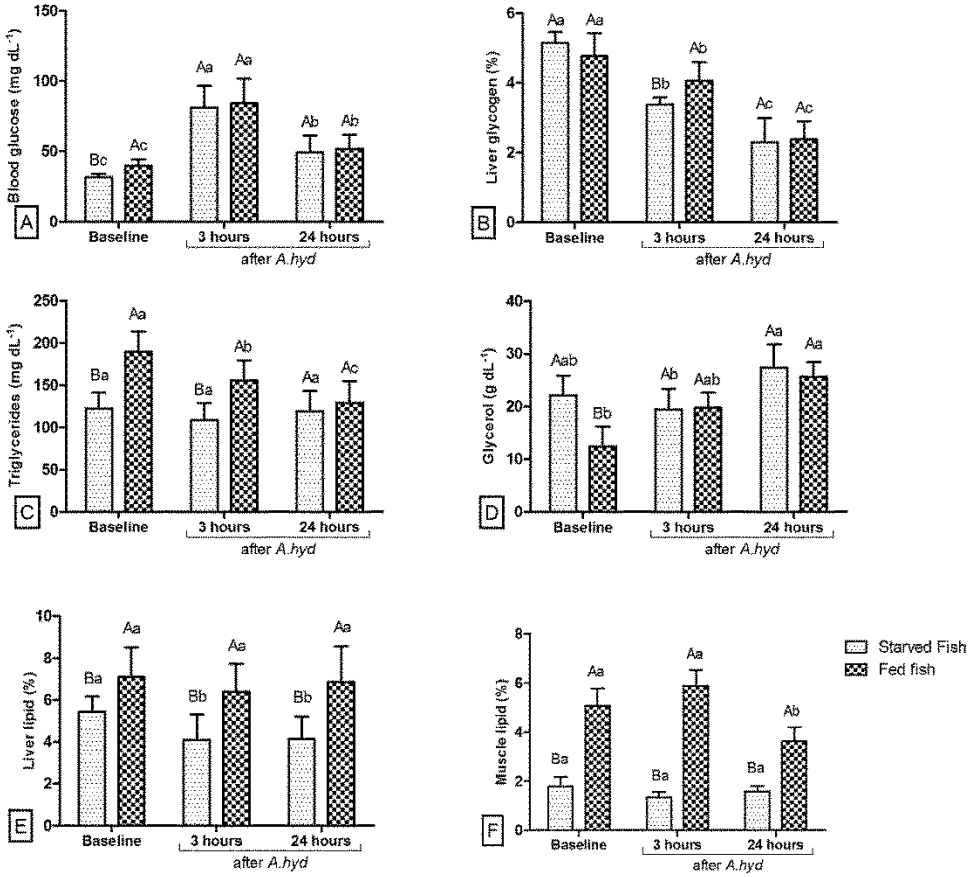

Figure 1. Metabolic parameters of pacu starved for 30 days or fed continuously. Different capital letters indicate difference ($P < 0.05$) between treatments at each

sampling, whereas dissimilar lower case letters indicate difference of treatments among samplings. Values are means ± standard deviation (SD).

Immune responses

The respiratory activity of leukocytes was significantly lower in starved fish only at baseline, increasing after bacterial inoculation to levels similar to those seen among fed fish (Fig. 2A).

Levels of serum lysozyme at baseline were significantly higher in starved fish. The same response profile was observed 3 hpi, but among fed fish these levels increased to values similar to those of starved fish 24 h later. Among starved fish, lysozyme concentration did not change over the course of the experiment (Fig. 2B).

Serum ACH activity was 102.8% significantly lower in starved fish at baseline. However, bacterial inoculation promoted the activity of the complement system in both fish groups. Although a difference in activity was observed 3 and 24 hpi, relative differences between the groups dropped to 19.5% and 25.1%, respectively (Fig. 2C).

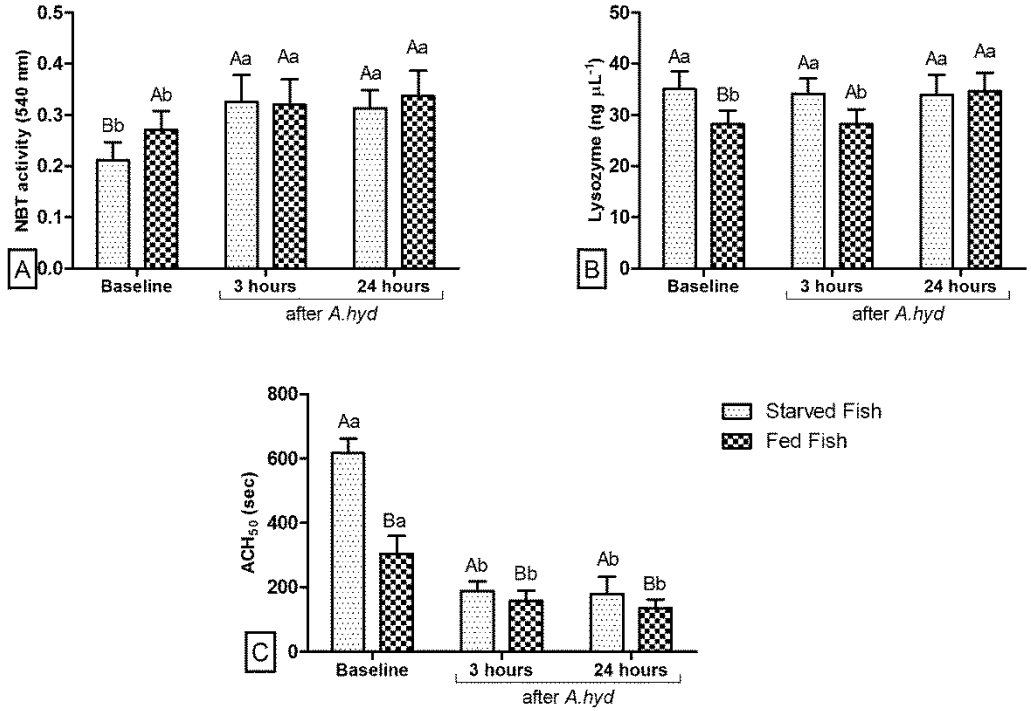

Figure 2. Innate immune indicators of pacu starved for 30 days or fed continuously. Different capital letters indicate difference ($P < 0.05$) between treatments at each

sampling, whereas dissimilar lower case letters indicate difference of treatments among samplings. Values are means ± standard deviation (SD).

Hormonal response

Baseline levels of cortisol were significantly higher in starved fish. 3 hpi those levels increased in both fed and starved fish reaching similar values. Twenty-four hours after inoculation, the levels of cortisol reduced in both fish groups, to values that did not differ from those observed at baseline and 3 hpi (Fig. 3).

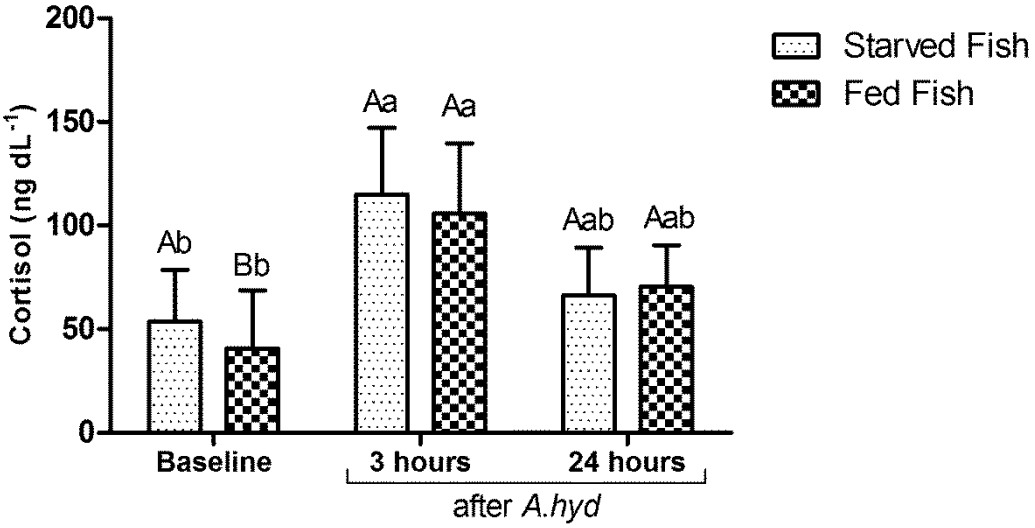

Figure 3. Serum cortisol levels of pacu starved for 30 days or fed continuously. Different capital letters indicate difference ($P < 0.05$) between treatments at each sampling, whereas dissimilar lower case letters indicate difference of treatments among samplings. Values are means ± standard deviation (SD).

Discussion

Our study describes several immune indicators of pacu under energetic deficit status to determine the ability of these fish to sustain an immune response following experimental bacterial infection, which included the respiratory activity of leukocytes, serum lysozyme concentration, and ACH activity. We also evaluated which metabolic reserves are allocated by starved pacu after experimental pathogen exposure. To infect fish we inoculated with A. hydrophila, a ubiquitous gram-negative bacterium that can be commonly isolated from freshwater ponds and is also a normal inhabitant of

the gastrointestinal tract of fish [29, 30]. In Brazil, A. hydrophila is one of the most important pathogens described on aquaculture farms (Godoy et al., 2008).

The respiratory burst is an antimicrobial response in phagocytic cells. Previous study demonstrated that LPS from Gram-negative bacteria has the ability to increase the respiratory burst activity of Atlantic salmon macrophages [31]. Additionally, different types of macrophages, derived from goldfish (Carassius auratus) kidney leukocyte culture and primed for respiratory burst activity, displayed enhanced respiratory burst responses 6e24 h after stimulation [32].

Regarding the lack of the lysozyme concentration response and the prompt activation of the complement, the explanation may be related to the bacteria used. In general, the fish complement system displays bactericidal activity against non-virulent Gram-negative bacteria, but not against Gram-positive bacteria or virulent Gram-negative bacteria [33]. Lipopolysaccharide in the cell walls of Gram-negative bacteria directly activates the alternative complement pathway and can result in lysis of the bacterial cell. Alternatively, lysozyme (which is also bactericidal), hydrolyzes the b-(1,4) linkages between N-acetylmuramic acid and N-acetylglucosamine in the cell walls of Gram-positive bacteria, thus preventing them from invading. Gram-negative bacteria are not directly damaged by lysozyme. When the outer cell wall of Gram-negative bacteria is disrupted by the complement and other enzymes, exposing the inner peptidoglycan layer of bacteria, then lysozyme becomes effective [29].

Our work shows that the activation of immune response by the infection was an expensive energetic process. At baseline, the energetic status of starved and fed fish was different. The mobilization of energy sources was different depending on the energetic status of fish. Starved fish lost body weight, presenting less visceral lipid, unaltered liver glycogen, less circulating glucose and triglycerides, less liver and muscle lipid, and more circulating glycerol, indicating the use of lipids to meet energy needs during starvation. After bacterial inoculation, liver glycogen was gradually depleted by both fish groups; however lipid utilization differed between the two groups. Starved fish mobilized liver lipid, keeping unaltered the blood triglycerides and the muscle lipid reserve. Otherwise, in fed fish blood triglycerides were gradually reduced in association with elevating blood glycerol, while liver lipid was unchanged and muscle dropped only at 24 h. It seems that, in starved fish, the blood triglycerides and

muscle lipid, lower at baseline, were preserved at the expense of liver lipid. Maintenance of high metabolic rates following the depletion of energy reserves during starvation would compromise animals' ability to survive [34], however the energetic deficit of starved pacu did not impair their ability to respond to the bacterial inoculation. The responses of the respiratory burst and complement system were associated with the mobilization of distinct energy sources depending on the energetic status of fish at baseline. Changes in the respiratory activity of leukocytes and blood glucose levels at baseline and 3 hpi followed the same pattern, suggesting that glucose was supplied by the glycogen depletion. During phagocytosis, phagocytes increase their consumption of molecular oxygen through the NADPH oxidase and generate various reactive oxygen species (ROS) such as the superoxide anion radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), singlet oxygen ($O_2^{\cdot}$), and the hydroxyl radical ($^{\cdot}OH$) in a process called the respiratory burst. Superoxide and hydrogen peroxide are highly toxic ROS and form the basis of a potent antibacterial system [35]. However, 24 hpi, blood glucose levels were not as high, and lipids were the main donor of substrate to this pathway by b-oxidation.

Although starved pacu showed a lower activation of the complement system at baseline, these fish were able to respond promptly after bacterial exposure, with a significant increase in ACH activity that was maintained up to 24 hpi. This response was associated with increased glucose and liver lipid mobilization to provide the required energy to starved fish. Gene expression of several factors of the component system has been shown to increase in response to acute bacterial infection [36], as similarly observed for our control group. Our results are, however, the first to confirm this observation among starved fish. Previous results on the ACH activity under conditions of energetic deficit had shown no alteration [37], decreased gene expression of some complement system factors [5,9], or down-regulation of gene C3, which controls the complement system cascade [5].

We measured the blood cortisol levels because cortisol strongly interacts with the immune system and is known to modulate several immune mechanisms [38], besides regulating several physiological processes including energetic metabolism [39]. Regarding the increased levels of cortisol in starved pacu at baseline, the literature is controversial. Previous studies reported increased cortisol levels in starved

Gillichthys mirabilis [40] and Rhamdia quelen [41], decreased cortisol levels in starved Ictalurus puntactus [42], or no influence of starvation on cortisol release in Salvelinus alpinus [43]. Despite these controversial results, the higher baseline levels of cortisol in starved fish indicates possible effect of the hormone in catabolic processes triggered to produce energy during starvation [39]. This higher baseline level of cortisol in starved fish may also be modulating the immune response in those fish, resulting in higher lysozyme levels and reduced activity of the complement system. Cortisol can modulate immune system differently according to the species and/or the nutritional status. In normally fed Perca fluviatilis, cortisol modulates the abundance of the C3 component of the complement system [38], otherwise in rainbow trout, the cortisol implant resulted in higher levels of lysozyme and complement system activity [44]. However this effect is not clear after the bacterial infection. The inoculation promoted elevation of circulating cortisol after 3 h and return to baseline levels at 24 h, in both fish groups. In these samplings, lysozyme levels were unchanged (except in fed fish, at 24 h, which increased) and the complement system decreased after 3 h, remaining unchanged at 24 h. In both samplings, complement activation was higher in fed fish. Higher lysozyme levels of starved fish at baseline and 3 hpi, compared to the fed fish, reinforces that this enzyme does not damage directly gram-negative bacteria [45].

The demonstration that mounting an immune response in pacu is metabolically costly is in line with a previous study [3] showing prolonged elevation in oxygen consumption after vaccination, concomitantly with higher plasma antibody titer and lysozyme activity in this period. Studies have shown the effects of starvation on metabolic and endocrine responses [5,7,9] and on immune response of fish [5,8,10,11]; however, to our knowledge, only one study has focused on the effect of starvation on the immune responses triggered after bacterial exposure [5], as our study did. Concluding, we demonstrate in pacu that although mounting immune response triggered after bacterial exposure is an expensive energetic process, fish under energetic deficit status were able to display protection against infection. This is particularly important considering that food restriction is a feeding strategy widely used in the fish farming industry to promote compensatory growth [13], and the benefits of food restriction as promoter of compensatory growth of pacu and consequent reduction of costs in its production have been reported [17,18].

Acknowledgments

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CAPÍTULO 5 – Considerações finais

Baseado nos trabalhos apresentados nesta tese, podemos concluir que juvenis de pacu utilizam o carboidrato da dieta de forma eficiente, melhorando o ganho em peso, a taxa de crescimento específica e a taxa de eficiência proteica. Além disso, estes peixes são capazes de suportar longos períodos de jejum, através de ajustes na atividade de enzimas metabólicas, fazendo com que os pacus poupem energia em situações nutricionais desfavoráveis ou restaurem as reservas energéticas com o retorna do fornecimento da dieta.

Em função do desbalanço entre proteína e energia da dieta, há aumento da produção de amônia devido ao uso da proteína como fonte de energia. Assim, sugerimos a determinação da amônia circulante como indicador prático do melhor aproveitamento da proteína da dieta.

E por último verificamos a capacidade de resposta imune do pacu em condição de déficit energético, uma vez que o jejum muitas vezes é utilizado como forma de manipular o crescimento e a qualidade de água da criação. Nossos resultados mostram que o pacu mesmo em jejum é capaz de responder a injeção experimental de *A. Hydrophila*, além da importância do cortisol como modulador das respostas imunes (principalmente lisozima e sistema complemento).