UNIVERSIDADE ESTADUAL PAULISTA – UNESP

CENTRO DE AQUICULTURA DA UNESP

ESTRESSE, IMUNIDADE, SISTEMA ANTIOXIDANTE E METABOLISMO EM PEIXES EM CONDIÇÕES SIMULADAS COMUNS DA CRIAÇÃO

Mariana Maluli Marinho de Mello

Jaboticabal, São Paulo

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"Por vezes sentimos que aquilo que fazemos não é
senão uma gota de água no mar.
Mas o mar seria menor se lhe faltasse uma gota".

Madre Teresa de Calcutá

À nova vida que recebemos em nossa família, que enche nossos corações de amor, meu futuro sobrinho e afilhado, dedico.

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SUMÁRIO

RESUMO

Muitas situações inerentes da piscicultura intensiva desencadeiam resposta de estresse nos animais, podendo causar perdas na produtividade. Manejos como a perseguição e o transporte e alterações na qualidade de água como baixos níveis de oxigênio dissolvido (OD) e mudanças na temperatura são exemplos de condições estressantes para os peixes. O conhecimento do impacto dessas condições na fisiologia dos peixes é importante para antever respostas e melhorar a saúde e bem-estar dos peixes, evitando, em consequência, perdas na produção. O uso de imunoestimulantes, como o β-glucano, na aquicultura, é uma alternativa para minimizar os efeitos negativos do estresse, incluindo prejuízos no sistema imune. Além disso, tanto as respostas metabólicas do estresse quanto as respostas do sistema imune são energeticamente caras e podem ter efeito no sistema de produção de energia e balanço redox intracelular. Neste contexto, esta tese é composta por três experimentos que objetivaram avaliar o efeito de situações estressantes na resposta de estresse, metabólica, imune e antioxidante de peixes imunoestimulados ou não com β-glucano. No primeiro experimento, juvenis de pacu, alimentados com β-glucano, foram submetidos à estresse crônico (perseguição) ou/e agudo (transporte), e inoculação com *Aeromonas hydrophila* inativada pelo calor. No segundo experimento, que originou dois capítulos desta tese, salmões foram mantidos por seis semanas em hipóxia (40% OD) e posteriormente foram inoculados com *Aeromonas salmonicida* inativada com formalina. No terceiro experimento, pacus foram alimentados com β-glucano e passaram por um desafio térmico, com queda severa na temperatura da água antes da inoculação com lipopolissacarídeo (LPS). Todos os estressores utilizados neste estudo desencadearam uma resposta de estresse nos peixes, prejudicando a resposta imune, antioxidante e metabólica. Enquanto os estressores agudos (transporte e estresse térmico) provocaram aumento na concentração plasmática de cortisol e/ou glicose, os estressores crônicos (perseguição e hipóxia) fadigaram a responsividade do eixo HPI. A imunoestimulação, seja por LPS ou por bactéria potencializou os efeitos negativos dos estressores, especialmente na resposta antioxidante. O β-glucano apresentou efeito protetor, diminuindo picos na

concentração plasmática de cortisol e evitando a redução na atividade do sistema antioxidante.

PALAVRAS-CHAVE:

Estresse crônico, estresse agudo, pacu, salmão, inoculação.

ABSTRACT

Many inherent situations in intensive fish farming trigger stress response on animals, which can cause productivity losses. Chase, transport and changes in water quality, as low dissolved oxygen (DO) levels and alterations in water temperature are examples of stressful conditions for fish. Understanding the impact of stressful situations on fish physiology is important to predict responses and improving fish health and well-being, preventing production losses. The use of immunostimulants, as β-glucan, in aquaculture, is an alternative to minimize the negative impacts of stress, including those in immune system. In addition, both metabolic and immune system responses are of high energy cost, and can affect the cellular system of energy production and the intracellular redox balance. In this context, this thesis is composed of three experiments that aimed to evaluate the effects of stressful situations on the stress, immune, metabolic and antioxidant response of non-immunestimulated or glucan-immunestimulated fish. In the first experiment, pacu juveniles fed with β-glucan were subjected to chronic (chase) or/and acute (transport) stress and heat-killed *Aeromonas hydrophila*. In the second experiment, present in two chapters of this thesis, salmon were kept in hypoxia (40% DO) for six weeks before injection with formalin-killed *Aeromonas salmonicida*. In the third experiment, pacu fed with β-glucan were thermal challenged, with severe reduction in water temperature prior lipopolysaccharide (LPS) inoculation. All stressors applied in this study triggered a stress response in fish, impairing the immune, antioxidant and metabolic responses. While acute stressors (transport and thermal challenge) increased plasma cortisol and/or glucose concentration, chronic stressors (chase and hypoxia) reduced the responsiveness of HPI axis. Immunostimulation, either by LPS or bacteria, potentiated the negative effects of stressors, especially in the antioxidant response. The glucan showed a protective effect, reducing peaks in plasma cortisol concentrations and avoiding a decrease in antioxidant system activity.

KEY-WORDS:

Chronic stress, acute stress, pacu, salmon, inoculation.

CAPÍTULO 1

Introdução Geral

1. Panorama da Aquicultura

A aquicultura é uma prática de produção conhecida desde os tempos remotos e é considerada, ultimamente, a única alternativa para aumentar o suprimento de pescado para consumo (Jensen *et al*., 2014), atendendo à crescente demanda por alimentos de alto valor nutricional (Siqueira, 2017; FAO, 2018). Este sistema de criação utiliza-se da intervenção humana para o aumento da produção (Oliveira, 2009) e como consequência, tem sido o setor que mais cresce no mundo (Jensen *et al*., 2014).

A estimativa de crescimento mundial do setor até 2030 é de 46,3% (Siqueira, 2017; FAO, 2018) e a expansão deve ocorrer principalmente nos países em desenvolvimento (FAO, 2018). Dentre eles, o Brasil se destaca por possuir grande diversidade de peixes com potencial para aquicultura (Nakatani *et al*., 2001), clima favorável, uma extensa costa oceânica e cerca de 5,5 milhões de hectares de água doce, dos quais são explorados menos de 1% (Bueno *et al*., 2017). Desta forma, o Brasil é um país promissor, com grande capacidade para contribuir para a manutenção e aumento da produção aquícola mundial.

Esta pressão para a expansão da aquicultura leva à intensificação dos sistemas produtivos, caracterizados pelo adensamento populacional (Val *et al*., 2004), exposição a alterações na qualidade da água (Jorgensen *et al*., 2002; Costa *et al*., 2004) e alta frequência de manejos (Urbinati *et al*., 2004). Paradoxalmente, estas características do sistema intensivo geram uma situação estressante para o animal e podem resultar em redução na produção devido ao aumento da mortalidade (Urbinati e Carneiro, 2004).

Deste modo, é necessária a geração de conhecimento em prol de uma aquicultura sustentável, que preze pela saúde e bem-estar do animal e que seja competitiva no mercado.

2. Agentes estressores na aquicultura

No ambiente de criação intensiva, os animais são impostos à situações diferentes do ambiente natural (Urbinati e Carneiro, 2004), consideradas agentes estressores quando estimulam os animais a desenvolverem mecanismos

fisiológicos adaptativos e que têm sido as principais causas de perdas na produção (Oba *et al*., 2009)

Manejos, como perseguição e captura e transporte, são procedimentos inerentes à produção aquícola e, embora possam ser de curta duração, são capazes de causar alteração na condição fisiológica e, inclusive, injúrias físicas no peixe (Urbinati e Carneiro, 2004; Oba *et al*., 2009). Além disso, o ambiente aquático é extremamente dinâmico e as mudanças súbitas e severas na qualidade da água, como alterações na concentração de oxigênio dissolvido ou temperatura, podem gerar estresse e reduzir a capacidade de manutenção da homeostase (Oba *et al*., 2009).

2.1. Perseguição

A perseguição aos peixes é um procedimento comum no cultivo durante a captura. Quando são submetidos à perseguição, os peixes tendem à fuga, realizando intenso exercício físico, com possível abrasão de seu corpo contra a rede ou puçá, outros peixes e/ou limites do tanque (Urbinati e Carneiro, 2004; Inoue *et al*., 2008)

Durante o exercício físico acentuado, o animal pode exceder sua capacidade aeróbica de produção de energia, recorrendo à síntese anaeróbica, com consequente produção de lactato, que pode ser identificado na corrente sanguínea, juntamente com elevadas concentrações de cortisol (Barnett e Pankhurst, 1998). Bandeen e Leatherland (1997) observaram aumento na concentração de cortisol de *Catostomus commersonii* por até 3 horas após perseguição por 5 minutos, sendo os picos do hormônio evidenciados 15 minutos após o estresse. A abrasão mecânica também é considerada uma precursora de respostas de estresse (Ross e Ross, 1999; Chopin *et al*., 1996), causando escoriações e perda de escamas e muco, além de elevações plasmáticas de cortisol (Urbinati e Carneiro, 2004). O muco dos peixes é uma importante barreira física e química de proteção contra agentes invasores (Urbinati e Carneiro, 2004; Ellis, 1999), pois possui proteínas, como a lisozima, que age contra microrganismos (Palaksha *et al*., 2008; Urbinati *et al*., 2014). Portanto, o desequilíbrio na quantidade de muco deixa os peixes susceptíveis à doenças e favorece a disseminação de patógenos.

2.2. Transporte

O crescimento da aquicultura traz consigo o aumento no transporte de peixes vivos, considerada uma das práticas mais delicadas do sistema de cultivo. O tempo de transporte é muito variado, devido principalmente à distância do destino, condições do veículo e do trajeto (Diniz e Honorato, 2012; Urbinati *et al*., 2004; Fagundes e Urbinati, 2008), porém de maneira geral, envolve etapas como captura, manuseio, adensamento, alteração na qualidade da água, o transporte propriamente dito e a soltura, as quais podem induzir respostas de estresse nos animais (Wendelaar Bonga, 2011) e, eventualmente, causar mortalidade (Urbinati *et al*., 2004; Fagundes e Urbinati, 2008).

O transporte tem efeito agudo e desenvolve respostas primárias e secundárias características do estresse, incluindo alterações hormonais, metabólicas, hematológicas, de desequilíbrio hidroeletrolítico e de suscetibilidade a infestação de parasitas (Urbinati e Carneiro, 2004). O aumento da concentração de cortisol sanguíneo após o transporte foi observado em matrinxã (*Brycon cephalus*) (Carneiro e Urbinati, 2001; Urbinati *et al*., 2004), tambaqui (*Colossoma macropomum*) (Gomes *et al*., 2003) e pacu (*Piaractus mesopotamicus*) (Takahashi *et al*., 2006). Em juvenis de jundiá (*Rhamdia quelen*), as respostas causadas pelo transporte por quatro horas em diferentes densidades, incluíram aumento nos níveis plasmáticos de cortisol, sendo estes valores mais expressivos em maiores densidades (Carneiro *et al*., 2009). Como uma resposta secundária de estresse de transporte, concentrações de glicose plasmática aumentam, para suprir a maior demanda energética (Mommsen *et al*., 1999). Matrinxãs (*Brycon cephalus*) adultos, após transporte, apresentaram altas concentrações de cortisol, glicose e amônia plasmática, porém estes valores não foram tão altos quando houve adição de sal na água do manejo (Carneiro e Urbinati, 2001). Brandão *et al*. (2006) encontraram altas concentrações de cortisol e glicose plasmática em pirarucu (*Arapaima gigas*) após manejo de transporte. E Urbinati *et al*. (2004) relataram aumento nas concentrações de glicose e cortisol plasmáticos durante o manejo de transporte em matrinxã (*Brycon cephalus*) juvenil.

2.3. Hipóxia

Agentes estressores de natureza química, como variação na concentração de oxigênio dissolvido na água, por exemplo, podem ocorrer naturalmente ou por intervenção humana. Em cenários de alta densidade de estocagem, a demanda por oxigênio aumenta e o alto consumo deste gás favorece a instalação de uma situação de hipóxia. (Oba *et al*., 2009, Urbinati e Carneiro, 2004). Nos períodos mais quentes do ano, ou em locais com altas temperaturas, a hipóxia se instala com mais frequência no ambiente de criação, devido à baixa saturação do oxigênio em águas com temperaturas elevadas (Boyd, 1982). Além disso, durante a noite, quando não há produção de oxigênio pelas algas e macrófitas do tanque, e todos os seres estão realizando respiração, a quantidade de oxigênio dissolvido diminui (Oba *et al*., 2009).

Para a maioria dos peixes, a recomendação é que a concentração de oxigênio dissolvido esteja acima de 4 mg L^{-1} (Boyd, 1982; Oba *et al*., 2009) e a exposição à concentrações inferiores a esta podem alterar os sistemas fisiológicos dos animais. Matrinxã (*Brycon amazonicus*) exposto por 12 horas à hipóxia aumentou sua concentração plasmática de cortisol, glicose e lactato (Ferreira *et al*., 2010), o que também foi observado em bacalhau do Atlântico (*Gadus morhua*) exposto à hipóxia progressiva (Herbert e Steffensen, 2005). Após um dia de exposição à hipóxia, os níveis de cortisol e lactato plasmáticos aumentaram em salmão do Atlântico (*Salmo salar*) (Remen *et al*., 2012), enquanto em tilápia (*Oreochromis niloticus*) os níveis de cortisol aumentaram e houve diminuição na resistência à *Streptococcus agalactiae* (Evans *et al*., 2003).

Além dos efeitos prejudiciais diretos da hipóxia, o peixe, quando exposto a baixa quantidade de oxigênio dissolvido, aumenta a velocidade do batimento opercular (Oba *et al*., 2009), na tentativa de compensar a tomada de oxigênio. O aumento do batimento opercular eleva o nível tóxico de outros possíveis elementos presentes na água (Oba *et al*., 2009), devido ao aumento à exposição, já que os peixes captam o oxigênio dissolvido na água através de trocas gasosas ocorridas no epitélio branquial, onde há intensa aproximação entre o sangue e a água (Wedemeyer, 1996).

2.4. Temperatura da água

O sucesso da piscicultura está intimamente relacionado com as condições físicas da água onde ela é instalada (Urbinati e Carneiro, 2004). Dentre os principais parâmetros a serem monitorados na água de produção está a temperatura. Peixes são animais ectotérmicos e, portanto, variam a velocidade dos processos metabólicos e fisiológicos em função da temperatura da água (Urbinati e Carneiro, 2004), sendo mais ativos quando a temperatura se encontra dentro dos limites de tolerância da espécie (Lucas e Baras, 2011).

Além das mudanças sazonais na temperatura da água, devido ao aquecimento global, a temperatura da superfície terrestre tem aumentado no último século (Houghton *et al*., 2001) e as últimas três décadas registraram as temperaturas mais quentes já observada nos ambientes aquáticos desde os finais dos anos 1800 (Houghton *et al*., 2001, NOAA, 2020). Diminuições repentinas na temperatura da água podem ocorrer tanto devido a fatores naturais, como as tempestades, como antropogênicas, por exemplo pela liberação de efluentes térmicos em corpos de água (Donaldson *et al*., 2008). Alterações na temperatura da água pode ter consequências nos processos fisiológicos afetando, por exemplo, a imunidade (Prötner, 2002; Alborali, 2006), com impactos negativos na produção.

Neste contexto, pesquisas estão sendo realizadas com o intuito de elucidar os efeitos de mudanças na temperatura sobre a fisiologia dos peixes em diferentes habitats e seu impacto na piscicultura. Tilápia (*O. niloticus*) mantida no frio por 7 dias apresentou diminuição na quantidade de linfócitos e aumento na quantidade de neutrófilos (Junior *et al*., 2010) após 42 dias, o estímulo pelo frio determinou diminuição de leucócitos e linfócitos e aumento de monócitos e neutrófilos circulantes (Falcon *et al*., 2008). Em bagre do canal (*Ictalurus puncatus*), a diminuição da temperatura da água está associada à supressão de atividade dos linfócitos e aumento da mortalidade (Bly e Clem, 1991). Um estudo com kingfish do rabo amarelo (S*eriola lalandi*) mostrou que a temperatura ideal para o crescimento deste peixe é maior que a temperatura usada na criação de tanques-rede no sul da Austrália e Nova Zelândia, indicando a importância de estudos como este para a otimização da aquicultura (Abbink *et al*., 2012). Além disso, um interessante estudo com cinco espécies estuarinas (*Diplodus vulgaris, Diplodus sargus, Dicentrarchus labrax, Gobius niger* e *Liza ramada)* mostrou que biomarcadores de estresse

oxidativo, como peroxidação lipídica e atividade das enzimas antioxidantes catalase e glutationa-S-transferase, são extremamente sensíveis a mudanças na temperatura (Madeira *et al*., 2013).

3. Estresse em peixes

Existem diversas formas de se definir o estresse, porém, em essência, pode-se descrevê-lo como um conjunto de respostas de um organismo diante de estímulos extrínsecos ou intrínsecos que alterem seu equilíbrio fisiológico, chamado homeostase (Jobling, 1994; Urbinati e Carneiro, 2004; Urbinati *et al*., 2014; Urbinati *et al*., 2015). Assim, esse conjunto de respostas é uma reação adaptativa, que prepara o animal para se defender e sobreviver às adversidades desta nova condição (Jobling, 1994; Wendelaar Bonga, 1997).

Em 1936, Selye introduziu o conceito da Síndrome de Adaptação Geral (SAG), segundo o qual a resposta de estresse apresenta três diferentes estágios: a resposta primária ou reação de alarme, a resposta secundária ou estado de resistência e a resposta terciária ou exaustão (Urbinati *et al*., 2014). As etapas da resposta de estresse encontram-se sintetizadas na imagem baixo (**Fig.1**), compreendendo desde a percepção do estímulo estressor até as respostas secundárias e terciárias desencadeadas pela liberação das catecolaminas e do cortisol.

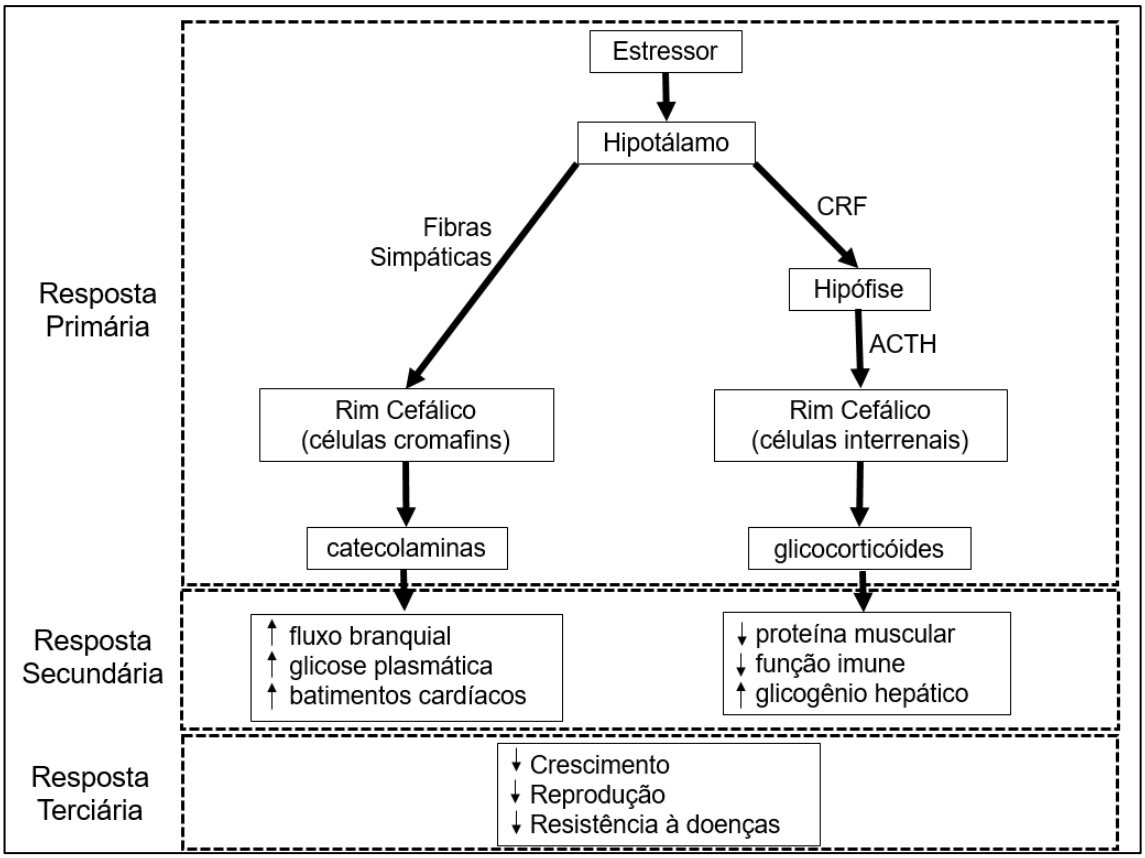

Fig. 1. Esquema das respostas primária, secundária e terciária do estresse em peixes. Adaptado de Barton e Iwama, 1991.

As respostas de estresse indicam o grau de alteração provocado pelo estímulo estressor e dependem da intensidade e duração deste estímulo (Wendelaar Bonga, 1997; Mommsen *et al*., 1999; Tort, 2011). A resposta primária de estresse nos peixes se inicia com o reconhecimento do agente estressor (Urbinati *et al*., 2014) e ocorre de forma a compensar o distúrbio (Oba *et al*., 2009). A resposta secundária envolve mecanismos de ajustes na tentativa de retorno à homeostase e, na resposta terciária, esses mecanismos perdem a capacidade adaptativa, e os sistemas biológicos se exaurem, podendo levar a uma condição patológica ou à morte. As respostas primárias e secundárias de estresse são decorrentes de estresse agudo, de curta duração, enquanto as terciarias se apresentam em decorrência de estresse crônico, de longa duração (Urbinati *et al*., 2014).

3.1. Resposta ao estresse agudo

Diante de um agente estressor de curta duração, o peixe possui a capacidade natural de responder adaptativamente, e a homeostase pode ser reestabelecida sem consequências graves para o seu organismo (Mommsen *et al*., 1999; Barton *et al*., 2002; Wendelaar Bonga, 1997).

A resposta primária de estresse em peixes tem início segundos após a detecção de um fator estressor e é caracterizada por um complexo mecanismo neuro-endócrino de alarme, o qual envolve dois eixos distintos: o eixo sistema nervoso simpático-células cromafins e o eixo hipotálamo-hipófise-interrenal (Wendelaar Bonga, 1997). No primeiro, a ativação de nervos do sistema nervoso autônomo simpático estimula as células cromafins, presentes na porção cefálica do rim, a produzir as catecolaminas (adrenalina, noradrenalina e dopamina – em menor quantidade) (Barton e Iwama, 1991; Sumpter, 1997; Wendelaar Bonga, 2011; Urbinati *et al*., 2014). O eixo hipotálamo-hipófise-interrenal é ativado em uma cascata de reações. Seguindo a ativação desta via, o sistema nervoso central estimula o hipotálamo a liberar o hormônio liberador de corticotrofina (CRH), o qual age na hipófise promovendo a produção e liberação de corticotropina (ACTH) por células localizadas na porção anterior da glândula. Por fim, o ACTH liberado na corrente sanguínea age nas células interrenais presentes na porção cefálica do rim, estimulando a produção e liberação de glicocorticoides, especialmente o hormônio cortisol (Barton e Iwama, 1991; Sumpter, 1997; Wendelaar Bonga, 2011; Urbinati *et al*., 2014). Durante a ativação da resposta de estresse, o hipotálamo age como um centro integrador do sistema nervoso central, comunicando-se com vários outros centros nervosos para iniciar a cascata de efeitos que culmina no acionamento das respostas fisiológicas de estresse (Urbinati e Carneiro, 2004).

A secreção de catecolaminas e cortisol leva a alterações fisiológicas, metabólicas, osmorregulatórias e imunológicas, que caracterizam a resposta secundária de estresse. Estas alterações visam mobilizar e garantir a produção e distribuição de energia aos tecidos e órgãos responsáveis por funções consideradas prioritárias, de acordo com a demanda da nova condição biológica do animal (Urbinati e Carneiro, 2004; Urbinati *et al*., 2014).

As catecolaminas são responsáveis pela regulação das funções cardiorrespiratórias, como aumento do fluxo sanguíneo e permeabilidade branquial, e aumento do número de eritrócitos, devido à contração esplênica (McDonald e

Milligan, 1992, Urbinati *et al*., 2014). Estas ações facilitam a oxigenação dos tecidos, assegurando a produção de energia pelas células.

Além do oxigênio, as células também são abastecidas com glicose durante a condição de estresse. A glicose, que é o principal carboidrato de reserva e representa a fonte primária de energia para o peixe, é mobilizada dos locais de armazenamento, como fígado, e é liberada na corrente sanguínea. O aumento da glicose circulante após um estímulo estressor ocorre, inicialmente, devido à ação das catecolaminas na quebra do glicogênio hepático (glicogenólise) (Wendelaar Bonga, 2011; Urbinati *et al*., 2014) e é mantido pela ação do cortisol, que atua principalmente no aumento da capacidade gliconeogênica (Urbinati *et al*., 2014).

O fígado, intestino e brânquias são órgãos-alvo do cortisol em peixes. Estes órgãos refletem as principais ações do cortisol: balanço hidromineral e metabolismo energético (Oba *et al*., 2009). O cortisol estimula a gliconeogênese, mobiliza aminoácidos e lipídeos provenientes da quebra de proteínas corporais (Urbinati *et al*., 2014), e de reservas (Vijayan *et al*., 1994) respectivamente e, desta forma, mantem a disponibilidade energética a longo prazo (Wendelaar Bonga, 2011). A concentração basal de cortisol plasmático varia entre as diferentes espécies de peixes. Salmonídeos em condições de repouso apresentam níveis em torno de 10 ng ml^{-1}, por exemplo, porém, em situações agudas de estresse, um rápido aumento nessa concentração é observado, chegando a valores próximos de 200 ng ml^{-1} (Oba *et al*., 2009). Assim, a concentração plasmática de cortisol é utilizada como um dos principais indicadores de estresse (Barton e Iwama, 1991), enquanto que a alteração na concentração de glicose plasmática é uma das respostas secundárias mais utilizadas para a quantificação de estresse em peixes (Barton e Iwama, 1991; McDonald e Milligan, 1992; Wendelaar Bonga, 2011).

A resposta secundária de estresse também é caracterizada por alterações nos parâmetros hematológicos. Frente a estressor agudo, peixes podem aumentar a quantidade de eritrócitos circulantes (Wedemeyer, 1997; Oba *et al*., 2009), a concentração de hemoglobina (Wedemeyer, 1997) e a afinidade dessas células pelo oxigênio (Aota *et al*., 1990) para otimizar o suprimento de oxigênio aos tecidos. Em relação às células brancas, o efeito é dependente da intensidade e duração do estímulo. Uma reação rápida do cortisol, apesar de ser uma resposta menos

proeminente, tende a elevar a atividade imune, o que pode ser essencial para a recuperação do animal, garantindo a sobrevivência (Martin, 2009).

Diferentes estudos analisaram a resposta de estresse em peixes expostos à estressores de curta duração. Zebrafish (*Danio rerio*) adensados por 3 horas apresentaram concentrações de cortisol 4 vezes maior que os níveis observados antes da exposição ao estressor (Ramsay *et al,* 2009). Red porgy (*Pagrus pagrus)* mostraram aumento das concentrações de cortisol e glicose plasmática após serem perseguidos por 8 minutos com puçá (Rotllant eTort, 1997). Expostos a estresse por confinamento, truta-arco-íris (*Oncorhynchus mykiss*) diploides e triploides mostraram aumento no cortisol e glicose plasmáticos e no hematócrito (Benfey e Biron, 2000).

3.2. Resposta ao estresse crônico

A resposta de estresse desenvolve um mecanismo adaptativo frente a ameaças agudas e de curto período, porém se a condição desfavorável for crônica ou contínua, a resposta terciária de estresse se instala, podendo resultar em danos ao sistema imune, ao crescimento e à reprodução dos peixes (Wendelaar Bonga, 1997).

Em relação ao crescimento, Davis *et al*. (1985) relataram uma redução de aproximadamente dois terços no peso final de bagre do canal (*Ictalurus punctatus*) quando comparados com o grupo controle, após a administração de cortisol na dieta. Já Barton *et al*. (1987) observaram diminuição de 45% no ganho de peso de truta arco-íris (*Oncorhynchus mykiss*) após dez semanas de alimentação de dieta com inclusão de cortisol. A cessação da alimentar durante a exposição a um estresse crônico contribui para o prejuízo no crescimento, porém a ação do cortisol na proteólise muscular é a principal causa da perda de peso de animais cronicamente estressados (Barton e Iwama, 1991). Estes autores sugerem que o catabolismo da proteína em glicose, estimulado pelo cortisol em uma situação de estresse, fornece energia à custa de crescimento. Porém, catecolaminas e cortisol também inibem o crescimento somático ao estimularem o consumo energético, a lipólise, além de atuarem negativamente sobre hormônios que promovem o crescimento (Wendelaar Bonga, 2011).

Quanto ao efeito do cortisol na reprodução, Carragher *et al*. (1989) observaram que o aumento do cortisol plasmático obtido por implante provocou a diminuição do peso das gônadas e das concentrações dos hormônios sexuais em machos e fêmeas de truta marrom (*Salmo trutta*). E, em fêmeas de tilápia (*Oreochromis mossambicus*), que também receberam implante de cortisol, ocorreu a diminuição do índice gonadossomático, tamanho do ovócito, testosterona e estradiol plasmáticos, após sete dias ou mais de tratamento (Foo e Lam, 1993).

Os efeitos prolongados do cortisol no sistema imune são muito estudados. O cortisol, por meio da circulação sanguínea, atinge importantes órgãos linfopoieticos como o fígado, o baço e o rim, diminuindo a produção de células brancas (Ellis, 1981; Anderson e Siwick, 1994), o que favorece o aparecimento de enfermidades oportunistas. Trutas arco-íris implantadas com cortisol foram mais suscetíveis ao parasita *Cryptobia salmositica* e liberaram menos anticorpos em relação ao tratamento controle (Woo *et al*., 1987), enquanto bagres do canal com elevação crônica de cortisol apresentaram diminuição na quantidade de linfócitos (Ellsaesser e Clem, 1987). Além disso, por ser um esteroide lipossolúvel, o cortisol é capaz de entrar nas células e ligar-se a agentes promotores nucleares, regulando a expressão de genes importantes relacionados à imunidade, como proteínas do sistema complemento e lisozima (Aluru e Vijayan, 2009). Como evidência disso, dourada (*Sparus aurata*), após estresse contínuo, apresentou diminuição na atividade do complemento e concentração de lisozima (Sunyer e Tort, 1995).

4. Sistema imune de peixes

Os peixes possuem um sistema de defesa complexo, formado por componentes celulares e humorais (substâncias solúveis), capaz de distinguir os elementos do próprio organismo de substâncias estranhas (Tizard, 2002; Secombes e Wang, 2012), como microrganismos, toxinas ou células malignas, que estimulam este sistema (Biller-Takahashi e Urbinati, 2014)

A ação do sistema imune é parecida na maioria das espécies de peixes teleósteos (Zelikoff *et al*., 2000) e assemelha-se à imunidade de outros vertebrados (Beaman *et al*., 1999; Saurabh e Sahoo, 2008). O sistema imune de peixes apresenta respostas imunes inatas ou não específicas, que funcionam como uma

primeira barreira contra infecções, indiferente do tipo de patógeno, e respostas imunes adaptativas ou específicas, que atuam mais lentamente, pois dependem do reconhecimento de antígenos, produção de anticorpos específicos e memória imunológica (Bernstein *et al*., 1998). Tanto o sistema imune inato quanto o adaptativo são divididos em defesa mediada por células e fatores humorais, que agem em conjunto para destruir invasores ou desencadear processos de defesa (Urbinati *et al*., 2014). Quando um patógeno penetra no peixe pela primeira vez, normalmente os mecanismos do sistema imune inato são suficientes para prevenir a infecção, porém, ao mesmo tempo, os mecanismos de defesa adaptativos são acionados, os quais produzirão a memória imunológica, bloqueando o desenvolvimento de uma nova infecção causada pelo mesmo patógeno (Wedemeyer, 1997).

As células de defesa dos peixes teleósteos são produzidas por tecidos e órgãos linfoides como o rim, timo, baço, e tecidos linfoides associados à mucosa (Urbinati *et al*., 2014). O rim, atua como órgão hematopoiético, responsável pela produção de linfócitos, monócitos, granulócitos (Torroba e Zapata, 2003; Urbinati *et al*., 2014), enquanto o timo constitui o local de desenvolvimento e maturação de linfócitos (Bowden *et al*., 2005). O baço produz linfócitos e macrófagos (Urbinati *et al*., 2014), é uma fonte de memória imunológica (Arkoosh e Kaattari, 1991) e estudos mostram que seu tamanho relativo tem uma associação positiva com resistência à doenças (Hadidi *et al*., 2008). Os tecidos linfoides associados à mucosa, que incluem os tecidos da mucosa do trato gastrointestinal, brânquias e pele, além de serem responsáveis pela produção de macrófagos, linfócitos, mastócitos e granulócitos (Bowden *et al*., 2005; Urbinati *et al*., 2014), produzem muco que contém proteínas importantes na barreira contra invasores, como a lisozima e proteínas do sistema complemento (Dalmo *et al*., 1997).

4.1. Sistema imune inato

Embora ambas as respostas, inatas e adaptativas, exerçam papéis fundamentais na defesa contra patógenos, a resposta imune inata em peixes é mais importante em relação à adquirida quando comparado aos mamíferos (Urbinati e Carneiro, 2004; Saurabh e Sahoo, 2008), pois fornece uma proteção rápida e

inespecífica, sendo a primeira linha de defesa do organismo (Bols *et al*., 2001; Secombes e Wang, 2012; Biller-Takahashi e Urbinati, 2014).

Entre os componentes do sistema imune inato estão o tegumento (pele e muco), os componentes celulares (granulócitos, monócitos/macrófagos e células *natural killer*) e os componentes humorais (sistema complemento, sistema de enzimas antimicrobianas e mediadores como o intérferon e as interleucinas) (Ellis, 1999; Urbinati *et al*., 2014).

O tegumento, formado por pele e muco, constitui-se de uma barreira de células especializadas que atuam para barrar a entrada de microrganismos nocivos. A superfície do corpo do peixe se apresenta como uma barreira física para microrganismos, porém o muco presente na pele, contém compostos antimicrobianos como proteínas do sistema complemento, lisozima e peptídeos bactericidas (Urbinati *et al*., 2014) que auxiliam no combate a microrganismos invasores.

Quando as barreiras do tegumento não são suficientes para evitar a entrada de patógenos, componentes do sistema imune irão reconhecê-lo e atuar na tentativa de degradá-los (Ellis, 1999). Tanto os componentes celulares quanto os componentes humorais do sistema imune inato de peixes reconhecem regiões específicas denominadas PAMPs (Pathogen Associated Molecular Patterns) de moléculas de agentes infecciosos, como lipopolissacarídeos, peptídeoglicanos, DNA bacteriano, RNA viral ou outras moléculas encontradas nas membranas de microrganismos multicelulares (Elward e Gasque, 2003).

Os componentes celulares do sistema imune inato incluem os trombócitos, leucócitos (monócitos/macrófagos e granulócitos - neutrófilos, eosinófilos e basófilos) e células citotóxicas (*natural killers*) (Secombes, 1996), as quais atuam no reconhecimento e eliminação de patógenos. Em peixes, os trombócitos são considerados células de defesa, pois são capazes de realizar fagocitose e normalmente são encontrados em focos inflamatórios (Tavares-Dias *et al*., 1999). Os monócitos são células em trânsito na corrente sanguínea periférica e durante o processo inflamatório migram para o tecido conjuntivo onde se transformam em macrófagos (Cuesta *et al*., 1999). O macrófago além de ser uma célula fagocítica, produz e libera citocinas (grupo de moléculas envolvidas na emissão de sinais entre

as células durante o desencadeamento das respostas imunes, como os intérferons e as interleucinas) e estão envolvidas na apresentação de antígenos aos linfócitos, (Secombes e Fletcher, 1992; Cuesta *et al*., 1999). Os neutrófilos são as células envolvidas nos estágios iniciais da inflamação em peixes (Manning, 1994) com grande capacidade fagocítica. Impreterivelmente, são os macrófagos que possuem a maior capacidade fagocítica, ingerindo grande quantidade de partículas, entretanto, em infecções bacterianas, os neutrófilos podem ser encontrados em maior quantidade no sangue (Suzuki, 1984), permitindo que o seu perfil seja utilizado como indicativo de infecção (Kindt *et al*., 2006). Estas células são os primeiros leucócitos a deixarem os vasos sanguíneos e chegarem ao sítio da inflamação (Griffin, 1984; Biller-Takahashi e Urbinati, 2014).

Os neutrófilos e macrófagos possuem grande importância na defesa do organismo contra patógenos, pois possuem a capacidade de fagocitar e produzir ânions superóxidos ($O_2^{\bullet -}$) (Biller e Takahashi, 2018), que atuam como bactericida extracelular (Plyzycz *et al*., 1989; Secombes, 1996), através do processo denominado “explosão oxidativa” (do inglês, *oxidative burst*). O processo de fagocitose se inicia com o englobamento do agente patogênico por meio de pseudópodos, seguido da internalização e formação de fagossoma, o qual se une ao lisossoma formando o fagolisossoma, com início de eventos microbicidas e de degradação (Roitt *et al*., 1998). Durante o processo da fagocitose, ocorre aumento no consumo de oxigênio molecular, dependente da respiração mitocondrial (Secombes, 1996). Durante a respiração celular, o oxigênio é reduzido em ânion superóxido ($O_2^{\bullet -}$), um agente bactericida fraco, que age em proteínas bacterianas (Biller e Takahashi, 2018). Porém, o ânion superóxido ($O_2^{\bullet -}$) forma compostos com uma potente ação bactericida, como o peróxido de hidrogênio (H_2O_2) e hipoclorito (HClO) (Verlhac *et al*., 1998; Biller e Takahashi, 2018). Tanto o hipoclorito (HClO) quanto o peróxido de hidrogênio (H_2O_2) oxidam a parede celular e membranas de microrganismos, contribuindo ativamente para a sua destruição (Verlhac *et al*., 1998). A produção de EROs durante a explosão oxidativa pode ser detectada por ensaio simples e tem sido usada como indicador da imunidade inata (Biller e Takahashi, 2018). O ensaio é baseado na redução do corante *nitroblue tetrazolium* (NBT), o qual é capaz de captar elétrons do superóxido, convertendo-se à forma reduzida. Como consequência dessa redução, são formados, no interior do

fagócito, precipitados de material insolúvel, denominados grânulos de *formazan* (Klein, 1990), os quais podem ser detectados em esfregaços sanguíneos (Abreu *et al*., 2009) ou pela densidade óptica, após a lise dos leucócitos (Biller-Takahashi *et al*., 2013).

Os fagócitos também podem regular a resposta imune através da produção e liberação de citocinas. Todos os componentes do sistema imune são regulados por uma complexa via neuroimunoendócrina bidirecional (Fast *et al*., 2008), que envolve as citocinas. Citocinas são glicoproteínas ou polipeptídeos de baixo peso molecular que controlam a comunicação do sistema imune com o sistema endócrino e nervoso, regulando a rede de defesa do hospedeiro (Savan e Sakai, 2006). A interleucina-1β (IL-1β) foi a primeira citocina clonada em peixes e é classificada como uma citocina pró-inflamatória, pois aumenta a fagocitose, a proliferação de linfócitos e a produção de ânions superóxidos ($O_2^{\bullet -}$) (Engelsma *et al*., 2003; Savan e Sakai, 2006). A interleucina-8 (IL-8), também secretada pelos fagócitos, é um potente mediador inflamatório, pois promove o recrutamento e ativação dos neutrófilos e monócitos para o foco inflamatório (Gerszten *et al*., 1999), além de estimular a atividade respiratória dessas células (Urbinati *et al*., 2014).

Portanto, os macrófagos e neutrófilos são os principais agentes em defesa do organismo, se apresentando como células essenciais para a fagocitose e morte do patógeno, além de necessárias para construir a rede de resposta imune inata e adaptativa (Kantari *et al*., 2008).

Os eosinófilos podem ser encontrados na corrente sanguínea quando há infestação de parasitos e, apesar de sua função não estar totalmente esclarecida, estas células atuam nos processos de inflamação (Urbinati *et al*., 2014), enquanto os basófilos são raros na maioria dos peixes (Hine, 1992). As células denominadas "*natural killer*" (NK) são células citotóxicas não específicas, cuja função principal é lisar células estranhas ou infectadas por vírus, sem que estas expressem algum antígeno ativador da resposta imune, e, desta forma, não estimula a resposta imune específica e, consequentemente não há memória imunológica (Raulet, 2004).

Além de células, várias moléculas estão envolvidas nos mecanismos de defesa inata, conhecidas como mediadores químicos solúveis ou componentes humorais (Urbinati *et al*., 2014). Estas moléculas estão presentes no soro, na forma inativa

ou de precursores, e suas concentrações elevam-se rapidamente durante uma infecção (Verlhac e Gabaudan, 1997). Os componentes humorais inatos incluem fatores inibidores do crescimento de bactérias, como a transferrina, antiproteases peptídeos antibacterianos, proteína C reativa, lisozima e as proteínas do sistema complemento (Dalmo *et al*., 1997; Urbinati *et al*.; 2014).

Os fatores inibidores do crescimento bacteriano são importantes para evitar a disseminação da colônia e consequentes danos teciduais (Stafford *et al*., 2001). Entre estes fatores, a transferrina se caracteriza por ser uma proteína solúvel, ativadora de macrófagos com alta afinidade pelo íon ferro (Stafford *et al*., 2001). Esta proteína liga-se ao íon ferro e transporta-o na corrente sanguínea, tornando este metal indisponível para as bactérias e, consequentemente, dificultando a instalação da infecção (Bullen e Griffiths, 1987). As antiproteases atuam sobre proteínas proteolíticas de bactérias, evitando a obtenção de energia pelas bactérias, e, desta forma, evitam sua multiplicação (Urbinati *et al*., 2014). Peptídeos bacterianos, também são proteases, porém atacam as membranas dos patógenos (Ellis, 2001) e estão presentes principalmente no muco (Braun *et al*., 1990). A proteína C reativa é encontrada em grandes concentrações no sangue e muco de peixes e liga-se à parede de diversos microrganismos, favorecendo a ingestão do patógeno pela célula fagocítica (opsonização) e promovendo a ligação das proteínas do sistema complemento (Goldsby *et al*., 2000).

A lisozima é uma enzima não específica importante do sistema imune inato, que apresenta atividade lítica contra bactérias, parasitas e vírus (Magnadotir, 2006). Esta enzima catalisa a hidrólise da parede celular de bactérias gram-positivas, digere carboidratos de alto peso molecular presentes na parede bacteriana e destrói o esqueleto glicano presente em muitas bactérias (Paulsen *et al*., 2003). Estas propriedades tornam a lisozima capaz de lisar a maioria das bactérias gram-positivas e, agindo em conjunto com as proteínas do sistema complemento, atuam também sobre bactérias gram-negativas (Paulsen *et al*., 2003). Por ser produzida por leucócitos, é encontrada nos tecidos que possuem grande quantidade destas células, como tecidos linfoides, soro e plasma, além de locais onde a probabilidade de ocorrer invasão bacteriana é alta, como pele, boca, brânquias e trato gastrointestinal (Yousif *et al*., 1991; Palaksha *et al*., 2008). Segundo Saurabh e

Sahoo (2008), a mensuração da concentração sérica de lisozima pode ter valor diagnóstico na determinação da condição imune do animal.

O sistema complemento representa o principal efetor da resposta imune humoral, composto por um conjunto de cerca de 35 proteínas envolvidas em uma cascata de reações enzimáticas que, quando ativadas, participam de respostas inatas (via alternativa) e/ou adquiridas (via clássica) (Urbinati *et al*., 2014). O sistema complemento destrói patógenos pela ruptura da membrana celular, fixa opsoninas no agente patogênico permitindo a fagocitose (opsonização), atrai células fagocíticas para o local da lesão (quimiotaxia) nos processos inflamatórios, (Holland e Lambris, 2002) e é responsável pela inativação de toxinas liberadas por bactérias (Secombes, 1996, Nakao *et al*., 2011). As proteínas do sistema complemento podem ser encontradas na circulação na sua forma inativa (Biller-Takahashi *et al*., 2012; Urbinati *et al*., 2014), e sua ativação pode ocorrer por três diferentes vias: a) via clássica, ativada por complexo antígeno-anticorpo, dependente de anticorpo; b) via alternativa, ativada por moléculas de superfície de microrganismos, inespecífica; c) via lectina, ativada por ligação de carboidratos de superfície bacteriana. Apesar de as três vias já terem sido identificadas em peixes (Holland e Lambris, 2002), a via alternativa é a mais comum e considerada a mais importante, indicando a importância da resposta inata nesses animais (Yano, 1996). Independente da via de ativação, as reações enzimáticas subsequentes culminam no aparecimento de peptídeos que podem romper a membrana do patógeno pela formação de poros que permitem o influxo descontrolado de água e íons e consequente lise celular, no recrutamento de macrófagos e neutrófilos para o local da infecção (quimiotaxia), na inativação de toxinas liberadas pelos agentes patogênicos ou revestimento dos microrganismos patogênicos (opsonização), facilitando sua aderência à membrana do fagócito para sua subsequente destruição (Holland e Lambris, 2002). O sistema complemento é muito utilizado como indicador da condição imune e sua atividade pela via alternativa pode ser determinada pela atividade hemolítica do soro quando em contato com eritrócitos estranhos (Yano, 1992; Biller-Takahashi *et al*., 2012).

4.2. Sistema imune adaptativo

Ao contrário do sistema imune inato, o sistema de defesa específico age contra um antígeno específico. A presença do antígeno desencadeia uma cascata de reações que irá culminar no aumento de anticorpos próprios circulantes e na memória imune (Bernstein *et al*., 1998). A imunidade específica refere-se à proteção que existe num organismo quando este já sofreu exposição prévia ao patógeno e em peixes, assim como nos demais vertebrados, o sistema imune adaptativo requer tempo para que seja ativado (Carey *et al*., 1999). O sistema imune adaptativo também possui componentes celulares e humorais.

As células do sistema imune adaptativo são chamadas de linfócitos e são responsáveis pela resposta imune adaptativa humoral e celular. Estas células, que circulam por todo o corpo através do sangue, são altamente diferenciadas e possuem capacidade de resposta frente aos estímulos imunológicos (Ellis, 1999). Os linfócitos se distinguem em dois grupos de populações, com ações diferentes, chamados linfócitos T e B.

Em uma resposta imune adaptativa, as células apresentadoras de antígenos (macrófagos e células dendríticas) apresentam o antígeno ao linfócito T auxiliar, o qual reconhece este antígeno na presença de moléculas de histocompatibilidade (MHC) (Urbinati *et al*., 2014). Após a fagocitose de um patógeno por um macrófago, fragmentos celulares ligam-se a marcadores na superfície da célula e é utilizado na apresentação deste patógeno pelo macrófago ao linfócito T auxiliar. Em seguida ao reconhecimento, os linfócitos T auxiliares liberam mensageiros químicos solúveis (citocinas) que estimulam a atividade de outras células como os fagócitos, os linfócitos B, responsáveis pela produção de anticorpos (imunoglobulinas), e linfócitos T citotóxicos, que destroem as células infectadas por indução à apoptose (Tizard, 2002; Secombes e Wang, 2012), caracterizando a resposta imune adaptativa celular.

A resposta imune adaptativa humoral envolve componentes denominados imunoglobulinas (expressa na membrana) ou anticorpos (imunoglobulina secretada), moléculas com alta especificidade que se ligam e marcam agentes invasores para que sejam fagocitados (Ellis, 2001). Como resposta ao estímulo das citocinas liberadas pelos linfócitos T, os linfócitos B se multiplicam e formam células que sofrem diferenciação, originando plasmócitos (linfócitos B ativos) e células de memória (Goldsby *et al*., 2000). As células de memória são linfócitos capazes de

memorizar a estrutura do patógeno e elaboram respostas mais rápidas e eficientes no caso de uma reinfecção (Roitt *et al*., 1998; Abbas e Lichtman, 2005), enquanto que os plasmócitos são as células responsáveis pela produção e liberação de anticorpos na corrente sanguínea (Goldsby *et al*., 2000). As imunoglobulinas neutralizam toxinas bacterianas e partículas virais, ativam o sistema complemento pela via clássica através do complexo antígeno-anticorpo, ligam-se aos patógenos e ativam a fagocitose e promovem a opsonização (Ellis, 2001) na tentativa de exterminar o agente patogênico.

5. Estresse oxidativo

As reações de estresse oxidativo celular são resultado do maior uso de oxigênio pela célula na tentativa de aumentar a produção de energia, seja por demanda do organismo ou por aumento da atividade leucocitária (Aschbacher *et al,* 2013, Biller e Takahashi, 2018).

Durante a respiração celular, em seu metabolismo basal, a célula sintetiza ATP por meio da redução completa do O_2 na crista mitocondrial, formando água (Sies e Murphy, 1991; Storey, 1996). Porém, o oxigênio pode ser reduzido de forma incompleta, produzindo espécies reativas de oxigênio (EROs). As EROs, como o ânion superóxido ($O_2^{\bullet -}$) e o radical hidroxil (OH●) também podem ser classificadas como radicais livres, pois possuem elétrons não pareados (Barreiros e David, 2006; Halliwell e Gutteridge, 2000) e, por isso, interagem com elétrons de outros átomos para se tornarem estáveis (Del Maestro, 1980), atuando em diferentes alvos biológicos (Biller e Takahashi, 2018). O peróxido de hidrogênio (H_2O_2) é uma espécie intermediária, que apesar de não possuir elétrons desemparelhados, é capaz de produzir facilmente o radical hidroxil (OH●) pela reação de Fenton (Del Maestro, 1980). O radical hidroxil (OH●) é considerado a ERO mais prejudicial, pois não há antioxidante capaz de prevenir a ação desta molécula, devido à sua meia-vida curta (Biller e Takahashi, 2018).

A interação das EROs com moléculas que integram estruturas celulares, como lipídeo (lipoperoxidação) e DNA, causa o dano oxidativo, podendo levar à morte da célula (Halliwell e Gutteridge, 2000; Nordberg e Arner, 2001). Porém, a formação destas moléculas é fundamental na defesa contra microorganismos invasores e, por

isso, fagócitos em plena atividade, produzem quantidades significativas de EROs pela ação da enzima NADPH oxidase presente em suas membranas (Diaz *et al*., 1998; Biller e Takahashi, 2018).

Para limitar as concentrações intracelulares de EROs e impedir a indução de danos, o organismo é dotado de mecanismos de defesa, conhecido como sistema antioxidante (Sies, 1993). Desta forma, o dano oxidativo ocorre quando há um desequilíbrio celular entre a produção de EROs e os mecanismos antioxidantes que as neutralizam (Biesalski, 2000, Aschbacher *et al,* 2013; Biller e Takahashi, 2018).

6. Sistema antioxidante em peixes

A neutralização da ação das EROs nos sistemas biológicos ocorre por componentes do sistema antioxidante. Antioxidantes são substâncias que atrasam ou evitam a oxidação (Halliwell e Gutteridge, 1989; Biller e Takahashi, 2018), e são produzidos naturalmente pelas células (Storey, 1996; Van der Oost *et al*., 2003).

Os antioxidantes podem ser classificados, de acordo com sua função biológica em duas categorias: a) Sistema enzimático - de maneira preventiva, inibem ou retarda a geração de EROs ou ainda impedem a proximidade das EROs com os alvos celulares; b) Sistema não enzimático – reparam as lesões causadas pelas EROs (Abdalla, 1993). Os componentes do sistema antioxidante enzimático, são enzimas, encontradas abundantemente nos tecidos de peixes (Van Der Oost *et al*., 2003), que agem de maneira profilática na produção de EROs. A catalase (CAT), superóxido dismutase (SOD), glutationa peroxidase (GSH-Px) e glutationa-S-transferase (GST) são enzimas primárias do sistema antioxidante (Halliwell e Gutteridge, 1989; Bonorden e Pariza, 1994). Os componentes do sistema antioxidante não-enzimáticos são representados pela glutationa reduzida (GSH), os carotenoides, as vitaminas A, C e E, os bioflavonóides, o ácido úrico, indóis e catecóis (Halliwell e Gutteridge, 1989; Sies e Murphy, 1991, Bonorden e Pariza, 1994).

A catalase (CAT) é uma enzima, encontrada principalmente nos peroxissomos de eritrócitos, tecido hepático e renal, que catalisa a redução do peróxido de hidrogênio (H_2O_2) em água (H_2O) e oxigênio (O_2) (Gaetani *et al*., 1989, Biller e Takahashi, 2018), evitando a formação do radical hidroxil (OH●). Em diversos

peixes, entretanto, não foi encontrada atividade desta enzima em eritrócitos (Wilhelm-Filho e Marcon, 1996), e o fígado tem sido o órgão utilizado na avaliação da atividade da CAT. A ação da superóxido dismutase (SOD) contribui na defesa contra as EROs catalisando a dismutação do ânion superóxido ($O_2^{\bullet -}$), formado nos peroxissomos e mitocôndrias, em peróxido de hidrogênio (H_2O_2), que é menos reativo e pode ser degradado pela CAT (McCord, 1987). Segundo McCord e Fridovich (1969), a SOD é a enzima mais abundante nas células aeróbicas, encontrada no citosol ou mitocêndria (Biller e Takahashi, 2018), e apresenta um metal em seu sítio catalítico, podendo este ser o manganês (Mn), ferro (Fe), cobre (CU) e Zinco (Zn) (McCord,1987; Halliwell e Gutteridge, 1989). A glutationa peroxidase (GSH-Px) é a enzima responsável pela redução de diversos tipos de peróxidos, inclusive o peróxido de hidrogênio (H_2O_2), formando seus correspondentes álcoois (Hayes *et al*., 1997; Halliwell e Gutteridge, 2007). Nesta reação, a GSH-Px, utiliza a glutationa reduzida (GSH) como co-fator, gerando a glutationa oxidada (GSSG) como produto (Hayes *et al*., 1997; Halliwell e Gutteridge, 2007). A GSH-Px tem alta atividade no fígado (Halliwell e Gutteridge, 1989) e é considerada um importante indicador de estresse oxidativo, uma vez que atua na redução de uma gama maior de peróxidos, e não somente o de hidrogênio como ocorre com a CAT, ampliando a sua avaliação (Mourente, 2002; Van Der Oost *et al*., 2003).

No esquema abaixo (**Fig. 2**) encontram-se sintetizados os mecanismos de ação do sistema antioxidante sobre as EROs.

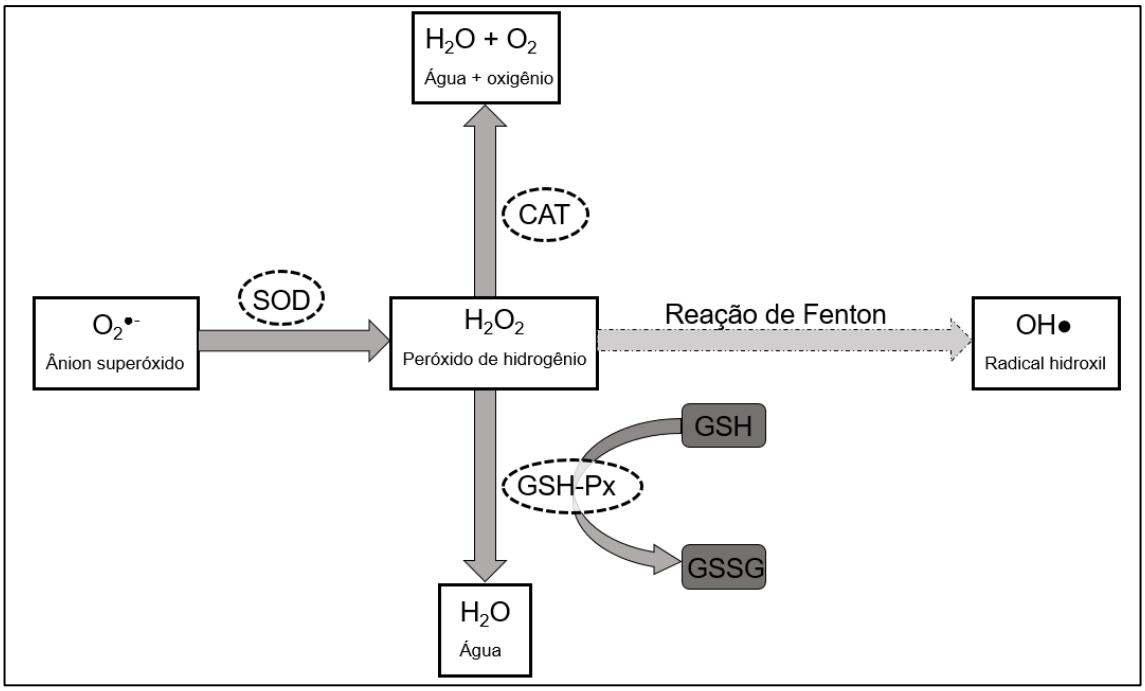

Fig. 2. Esquema das respostas antioxidante sobre as espécies reativas de oxigênio (EROs). Adaptado de Barbosa *et al*., 2010.

7. Imunoestimulantes

Imunoestimulante é uma substância que promove a ativação dos mecanismos de defesa inatos ou adaptativos (Saurabh e Sahoo, 2008), por interagir diretamente com as células do sistema imune, melhorando a imunocompetência dos animais. Imunoestimulantes podem ter origem química, sintética ou biológica e, de maneira geral, ligam-se a receptores específicos nas membranas dos leucócitos para ativá-los (Sakai, 1999). Desta forma, estas substâncias aumentam a atividade de monócitos/macrófagos e neutrófilos e amplificam o processo de fagocitose e a produção de linfócitos, anticorpos e lisozima (Sakai, 1999, Lim e Webster, 2001).

Na prática, os imunoestimulantes são suplementos com potencial de aumentar a resistência a infecções por parasitos, fungos, vírus e bactérias oportunistas (Sakai, 1999; Raa, 2000) e, por isso, representam uma alternativa viável para a prevenção de doenças (Raa, 2000; Abasali e Mohamad, 2010) e para reduzir os efeitos negativos do estresse na piscicultura (Siwicki *et al*., 1998). O uso de diferentes imunoestimulantes na dieta mostrou melhora na resposta imune em carpa indiana (*Labeo rohita*) (Sahoo e Mukherjee, 2001), truta arco-íris (*Oncorhynchus mykiss*) (Siwicki *et al*., 1994); salmão do Atlântico (*Salmo salar*)

(Engstad *et al*., 1992), bagre-do-canal (*Ictalurus puncatus*) (Chen e Ainsworth, 1992), dourada (*Sparus aurata)* (Ortuño *et al*., 2002), tambaqui (*Colossoma macropomum*) (Chagas *et al*., 2013), peixe-lápis (*Nannostomus trifasciatus*) (Abreu *et al*., 2014), pacu (*Piaractus mesopotamicus*) (Biller-Takahashi *et al*., 2014) e matrinxã (*Brycon cephalus*) (Zanuzzo *et al.,* 2012).

7.1. β-glucano

Entre os diferentes imunoestimulantes utilizados na aquicultura, o β-glucano é um dos mais promissores (Ringo *et al*., 2012; Meena *et al*., 2013). β-glucanos são compostos bioativos, encontrado em cereais (Genc *et al*., 2001), bactérias e fungos, muito utilizados na aquicultura, seja como aditivo alimentar, adjuvante em vacinas ou em banhos profláticos (Dalmo e Bøgwald, 2008; Meena *et al*., 2013; Yamamoto *et al*., 2018).

A conformação estrutural dos β-glucanos em forma de hélice é reconhecida pelos receptores das células imunes do hospedeiro iniciando uma cascata de reações inflamatórias que levam ao aumento de respostas imunes (Dalmo e Bøgwald, 2008), como a melhora na atividade fagocítica e citotóxica nos macrófagos (Tsiapali *et al*., 2001) e amplificação da produção de proteínas líticas como a lisozima e as proteínas do sistema complemento (Paulsen *et al*., 2001; Nakao *et al*., 2011). .

Vários estudos reportam o uso de β-glucano na melhora da resposta imune e resistência a doenças. Bagni *et al*. (2005) observaram que a atividade das proteínas do sistema complemento e da lisozima melhorou após 15 dias de administração de β-glucano incluso na dieta para robalo (*Dicentrarchus labrax*). Em carpas (*Cyprinus carpio*) infectadas por *Aeromonas hydrophila*, a administração intraperitoneal de concentrações acima de 500 µg de β-glucano por grama de peixe melhorou a sobrevivência. Neste mesmo estudo, os autores observaram que após a administração do β-glucano, a contagem total de leucócitos e a produção de espécies reativas de oxigênio pelos macrófagos aumentaram (Selvaraj *et al*., 2005). A alimentação de carpa indiana (*Labeo rohita)* com diferentes níveis de β-glucano mostrou que os animais que receberam 250 mg de β-glucano kg^{-1} de ração, por 42 dias, apresentaram maior número de leucócitos, e maior atividade de lisozima e do

sistema complemento, sendo o grupo com maior índice de sobrevivência (80%) após desafio bacteriano com *Aeromonas hydrophila (*Misra *et al*., 2006). Pacus alimentados por sete dias com dietas com inclusão de 0.1% e 1% de β-glucano e infectados experimentalmente com *Aeromonas hydrophila* apresentaram melhores taxas de sobrevivência que o grupo controle (Biller-Takahashi *et al*., 2014).

A imunoestimulação com β-glucano em peixes não promove somente uma resposta imune mais efetiva como também minimiza os efeitos negativos do estresse (Anderson e Jeney, 1992), pois confere ao animal uma condição fisiológica adequada para suportar estressores. Jeney *et al*. (1997) suplementaram a dieta de truta arco-íris (*Oncorhynchus mykiss*) com β-glucano e avaliaram os efeitos nas respostas imunes inatas dos peixes após transporte de 2 horas. Estes autores observaram que baixas doses de β-glucano na dieta diminuíram a concentração de cortisol plasmático e atenuaram as alterações secundárias do estresse. Em pacu (*Piaractus mesopotamicus*), a alimentação por 10 dias com ração suplementada com 0.5% de β-glucano aumentou a atividade respiratória de leucócitos suprimida pelo estresse de manejo (Sabioni *et al*., 2019) e em matrinxã (*Brycon amazonicus*), a alimentação por 15 dias com 0.1% de β-glucano aumentou a mobilização e a atividade de leucócitos após inoculação com *Aeromonas hydrophila* (Montoya *et al*., 2018). Um interessante estudo com pacus (*Piaractus mesopotamicus*), alimentados com dieta suplementada com 0.1% de β-glucano e injetados com *Aeromonas hydrophila* após 4 horas de transporte, mostrou que este imunoestimulante, além de aumentar a atividade do sistema complemento, possui um efeito protetor, pois impediu o aumento exagerado dos níveis de cortisol após inoculação em peixes estressados (Mello *et al*., 2019).

Alguns estudos também mostraram a ação do β-glucano no sistema antioxidante de peixes. A suplementação da dieta de *Lutjanus peru* com β-glucano aumentou a atividade de SOD e CAT após injeção com LPS (Guzmán-Villanueva *et al*., 2013) e em carpa capim (*Ctenopharyngodon idella*) a atividade destas mesmas enzimas foram maiores quando os peixes receberam β-glucano por injeção intraperitoneal (Kim *et al*., 2009). Estes autores ainda observaram que o β-glucano impediu a redução da atividade dessas enzimas provocadas pela inoculação com o vírus da hemorragia da carpa capim (GCHV) (Kim *et al*., 2009).

8. Gênero *Aeromonas*

Bactérias do gênero *Aeromonas* estão presentes nos ambientes aquáticos do mundo todo e, dentro da aquicultura, é o patógeno responsável por grande parte das doenças dos animais de cultivo, gerando notáveis perdas econômicas (Carraschi, *et al*., 2012). Diante deste quadro, o uso de bactérias do gênero *Aeromonas* é uma interessante estratégia experimental para estimular a resposta do sistema imune em peixes (Ellis, 1999), simulando infecções que ocorrem naturalmente no ambiente aquático.

A *Aeromonas hydrophila* é uma bactéria gram-negativa com forma de bastonete móvel (Stoskopf, 1993), considerada um dos mais importantes patógenos oportunistas de peixes de água doce, pois apresentam ampla distribuição geográfica, crescendo em temperaturas que variam de 5°C a 37°C (Figueiredo e Leal, 2008). No Brasil, esta bactéria é causadora de surtos de doença em diversas espécies de animais aquáticos nativos utilizados em cultivo (Godoy *et al*., 2008).

O aparecimento de infecção por *A. hydrophila* normalmente está relacionado com o excesso de matéria orgânica na água ou situações de estresse (Post, 1987) e representa um sério risco à saúde dos peixes. Segundo Vieira (2004), a infecção por *A. hydrophila* pode ser considerada um grave problema econômico, pois os sinais característicos da doença e a pronunciada mortalidade impossibilitam o comércio do pescado.

A *Aeromonas salmonicida* é uma bactéria gram-negativa, que causa a furunculose, uma doença responsável por grandes perdas econômicas na piscicultura (Møyner *et al*., 1993). Esta doença afeta principalmente salmonídeos, mas fatores associados às células (lipopoissacarídeos – LPS) e fatores extracelulares (exotoxinas) têm sido relacionados ao desenvolvimento dos sinais ulcerativos ou generalizados da furunculose em diferentes espécies de peixes marinhos e de água-doce (Magnadottir *et al*., 2002). Experimentos da década de 30 mostraram que peixes que sobreviveram à furunculose podem se tornar portadores assintomáticos da *A. salmonicida* (Bullock e Stuckey, 1975), favorecendo o contágio de peixes sadios.

Ulcerações, focos hemorrágicos, síndrome ulcerativa epizoótica e erosão das nadadeiras em peixes cultivados e selvagens são lesões normalmente

relacionadas com patógenos do gênero *Aeromonas.* A aquicultura intensiva tem aumentado tanto a ocorrência quanto a gravidade de infecções por *Aeromonas* ssp., emergindo como uma das principais causas de mortalidade na criação de peixes (Silva *et al*., 2012; Song *et al*., 2014), principalmente devido ao aumento do estresse ambiental (Plumb *et al*., 2011).

9. Modelos biológicos

9.1. Pacu (*Piaractus mesopotamicus*)

O pacu (*Piaractus mesopotamicus,* Holberg 1887) (**Fig. 3**) é um peixe da família Characidae de ampla distribuição na América do Sul, encontrado desde a bacia do rio Orinoco, na Venezuela até o rio da Prata, no Uruguai e, no Brasil, sua maior distribuição ocorre na região Centro-Oeste, no Pantanal do Mato Grosso (Petrere Jr, 1989). O pacu alimenta-se principalmente de folhas, frutos e resíduos vegetais, portanto, trata-se de uma espécie herbívora com preferência frugívora (Urbinati *et al*., 2020) com fácil adaptação à alimentação artificial. Esta característica, somada ao fato deste animal possuir um rápido crescimento, ganho de peso elevado, rusticidade e carne branca de excelente qualidade (Abimorad e Carneiro, 2007) o torna um dos peixes de maior importância na aquicultura brasileira (Oliveira, 2009), além de ser uma das espécies de peixe mais estudada nas regiões sul, sudeste e centro-oeste do Brasil (Urbinati *et al*., 2020). Segundo a FAO (2020), o pacu, juntamente com o cachara (*Pseudoplatystoma coruscans*), o tambaqui (*Colossoma macropomum*) e seu híbrido tambacu, é a terceira espécie de peixe de água doce mais produzida no país. Porém, seu cultivo intensivo o coloca em uma condição em que diversos fatores estressantes estão presentes, predispondo-o à imunossupressão e consequentemente, doenças bacterianas e parasitárias (Urbinati e Carneiro, 2004).

Fig. 3. Pacu (*Piaractus mesopotamicus*, Holberg, 1987)

9.2. Salmão do Atlântico (*Salmo salar*)

O salmão do Atlântico (*Salmo salar*, Linnaeus 1758) (**Fig. 4**) é o peixe da família Salmonidae mais produzido no mundo, representando, junto com as outras espécies de salmão, 13% do valor de produção da aquicultura mundial (Ashe e Bjørdal, 1990). Seu cultivo é realizado em águas frias e sua produção ocorre principalmente em pisciculturas da Noruega, Chile e Canadá (Torrissen *et al*., 2011).

O salmão é uma espécie carnívora, se alimentando principalmente de crustáceos, e migradora anádroma, ou seja, migra do mar para os rios para se reproduzir (Ashe e Bjørdal, 1990). O ciclo de vida dos salmonídeos é complexo e dotado de variadas estratégias de vida (Thorpe,1994; Fleming, 1996). O salmão do Atlântico se reproduz em água doce e os ovos fertilizados desenvolvem-se e eclodem no rio. Os alevinos podem permanecer por um ou vários anos na água doce até se tornarem fisiologicamente adaptados ao ambiente salino e migrar para o mar. Vivendo no mar, esta espécie torna-se sexualmente madura e retorna para seu local de reprodução no rio (Ashe e Bjørdal, 1990; Fleming, 1996). Grande parte dos adultos morre de exaustão quando termina a época reprodutiva e apenas uma minoria volta ao mar, podendo reproduzir-se novamente (Fleming, 1996).

Fig. 4. Salmão do Atlântico (*Salmo salar*, Holberg, 1987)

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CAPÍTULO 2

Effects of β-glucan on stress, innate immune and antioxidant response of pacu injected with heat-killed *Aeromonas hydrophila* under different stress conditions

ABSTRACT

Glucans are polysaccharides with immunostimulatory properties. These compounds have shown effect on several innate immune parameters and antioxidant indicators. Recently, 1-3-β glucan was shown to modulate cortisol circulating levels differently after acute stress and bacterin inoculation. The present study evaluated stress response, innate immune system and antioxidant enzymes indicators in pacu under different stress conditions. Fish were fed with β-glucan and injected with *A. hydrophila* after 15 days of daily chasing, after transport and after the exposure to the combined stressors. Plasma cortisol and glucose concentration, serum lysozyme concentration, respiratory activity of leucocytes, hemolytic activity of complement system, lipid peroxidation and antioxidant enzymes activity were measured and spleen and hepatosomatic index calculated. We found that both acute and chronic stress had an immunosuppressive effect; the respiratory activity of leucocytes being reduced mainly by chronic chasing and the lysozyme concentration by transport. Additionally, the acute stressor combined with the chronic stressor or with bacterin injection increased the oxidative damage, evaluated by lipid peroxidation. Otherwise, β-glucan showed a protective effect in stress response and oxidative damage, delaying the increase in plasma cortisol concentration after bacterin inoculation and reducing the increase of the hormone after transport followed by inoculation, besides to reducing lipid peroxidation.

KEY-WORDS:

Acute stress, chronic stress, transport, chase, immunostimulant.

1. Introduction

β-(1-3)(1-6)-D-glucans, hereafter referred to as “β-glucans”, are highly conserved polysaccharides found naturally in cell wall of different organisms including plants, algae, fungi, yeast and bacteria (Douglas, 2001; Synytsya and Novak, 2014; Douxfils *et al*., 2017). Glucans have been widely used in aquaculture and numerous studies have highlighted the immunostimulatory and beneficial properties of glucans in a several fish species (Robertsen *et al*., 1990; Cain *et al*., 2003; Biller-Takahashi *et al*., 2014; Douxfils *et al*., 2017; Montoya *et al*., 2018; Amphan *et al*., 2019; Mello *et al*., 2019; Sabioni *et al*., 2020). The immunostimulatory effect of β-glucan is attributed to its binding to multiple toll-like receptors on leukocytes membrane, resulting in the stimulation of immune responses (Dalmo and Bøgwald, 2008) including increase of lysozyme and the complement system concentration, components that enhance the phagocytic activity of macrophages and consequently protection against infections (Nikl *et al*., 1991; Selvaraj *et al*., 2005; Siwicki *et al*., 2010). Antioxidant enzymes may also be influenced by β-glucan. Grass carp injected with β-glucan showed increase in superoxide dismutase (SOD) and catalase (CAT) activities (Kim *et al*., 2009). Higher liver SOD and CAT activities in relation to control were also observed in Pacific red snapper fed with β-glucan and injected with LPS (Guzmán-Villanueva *et al*., 2013). Interestingly, a recent study showed modulatory effect of glucan on cortisol circulating levels in pacu injected with heat-killed *Aeromonas hydrophila* following acute stress (Mello *et al*., 2019). The authors showed that glucan modulated differently the plasma cortisol concentration, sustaining higher levels of cortisol after transport and preventing the additional increase after bacterial injection, highlighting a protector effect of the immunostimulant. Based on this observation, to elucidate the effect of glucan as cortisol modulator in different stress conditions the present study was designed. To achieve our aim, fish previously fed with β-glucans were inoculated with heat-killed *A. hydrophila* after exposure to acute, chronic and acute plus chronic stressors and then we measured stress response, innate immunity, and antioxidant system biomarkers.

2. Material and Methods

The experimental procedures were approved by the Comitê de Ética no Uso de Animais (CEUA) of the Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal campus (Protocol 014928/18).

2.1. Animals and experimental design

We distributed 320 juvenile fish (87.6 ± 15.7 g and 16.3 ± 0.9 cm) into 32 100-L tanks (10 fish per tank) with constant water renewal and aeration, and were fed with an extruded commercial diet twice a day (3% of body mass) for 7 days until acclimatization to experimental conditions. After acclimatization, fish were randomly sorted into two treatments: fed with a control diet (commercial diet – glucan free) or fed with a glucan supplemented diet (commercial diet with addition of 0.1% β-glucan) for 15 days (16 tanks/160 fish per treatment) twice a day with 3% of their body mass. The β-glucan concentration was chosen according to Mello *et al*. (2019). In each treatment, fish were distributed in two groups with 16 replicates each (16 tanks/160 fish per group) (see Fig.1). During the experimental feeding, one group was kept undisturbed (WS – without stress) and the other group was chased with a net for 5 minutes twice a day (CS – chronic stress). At the end of the 15-day period, two fish from each replicate (n=8) were sampled (basal sampling) and a half of the remainders fish were transported and half remained in the tanks. To transport, fish were packed in plastic bags (16 bags/9 fish per bag; density of 166 g L^{-1}) inflated with oxygen and transported for 4 hours. All fish were inoculated with heat-killed *Aeromonas hydrophila*: not transported fish were inoculated after basal sampling and transported fish were inoculated at the return to the lab. For that, fish were anesthetized (benzocaine, 0.05 g L^{-1}) and injected in the mesenteric cavity with 0.5 µL g^{-1} of heat-killed *A. hydrophila* and then returned to the tanks. The fish were then sampled 3, 6 and 24 hours post inoculation (n=8).

During the experimental period, the water oxygen levels, temperature and un-ionized ammonia were 5.86 ± 0.65 mg L^{-1}, 29.9 ± 0.5°C and 0.03 mg L^{-1}, respectively. The photoperiod was 12 hours light: 12 hours dark. Immediately before transport, the oxygen levels were 7.06 ± 0.51 mg L^{-1}, temperature were 29.9 ± 0.6 °C and un-ionized ammonia was 0.18 ± 0.02 mg L^{-1} in the plastic bags water. After

transport, the plastic bags water oxygen levels were 9.06 ± 1.43 mg L^{-1}, temperature were 33.0 ± 0.6 °C and un-ionized ammonia was 0.64 ± 0.17 mg L^{-1}.

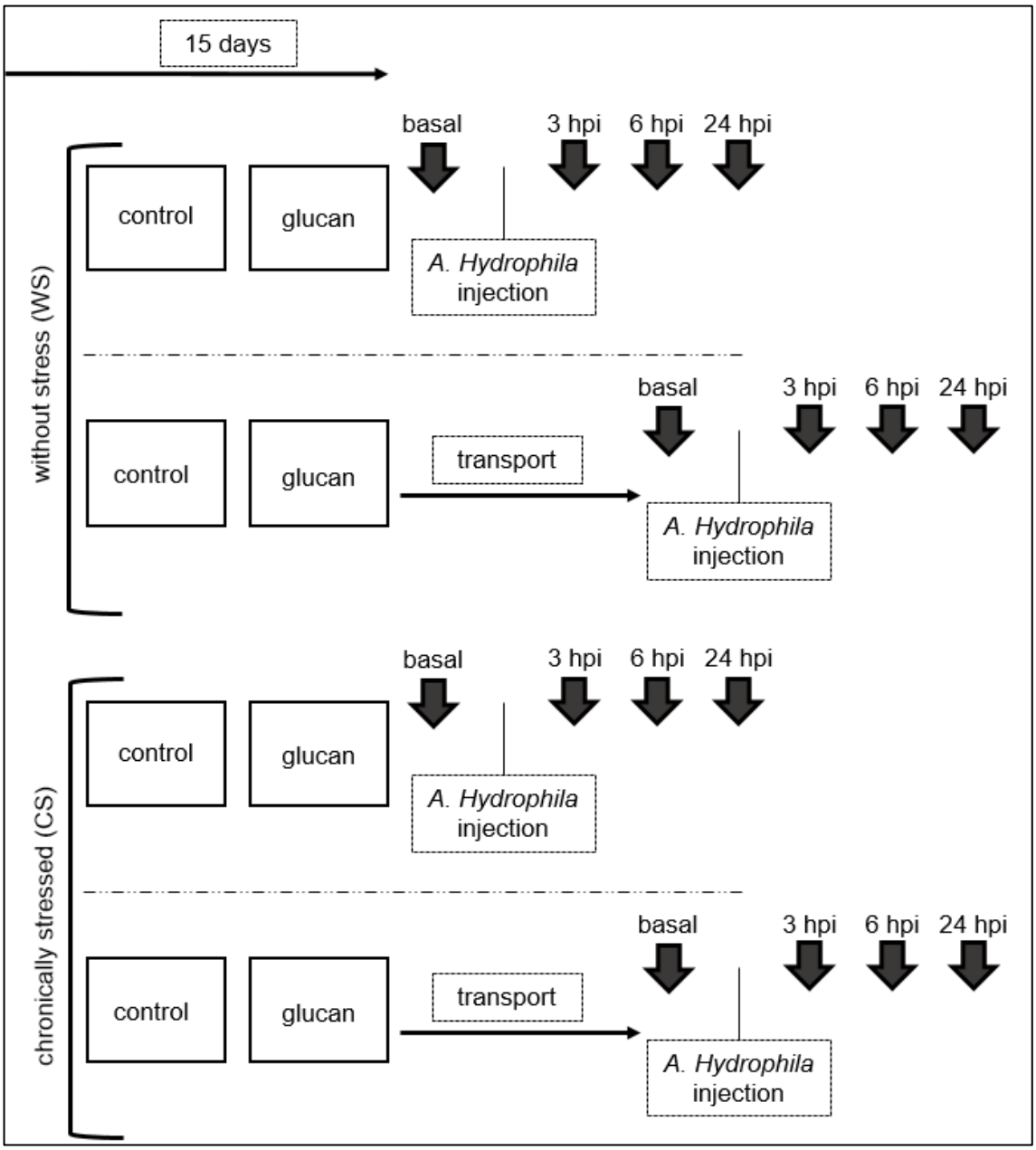

Fig. 1. Flowchart of experimental design.

2.2. Experimental diet

To prepare the experimental diets, an extruded commercial feed (Fri-Aqua® Onivoros Engorda: 8–10 mm, 12% moisture, 28% crude protein) was ground and mixed with 0.1% β-glucan (Macrogard®, batch Q513187, Biorigin, Brazil). The diet was moistened with 40% of water, granulated in a food processor and dried at 40

°C for 24 hours. The control diet was processed following the same procedure without the inclusion of β-glucan.

2.3. Bacteria preparation and inoculation

The *Aeromonas hydrophila* strain supplied by the Laboratório de Microbiologia e Parasitologia de Organismos Aquáticos (LAPOA/CAUNESP) was identified by sequencing of the 16S rDNA (Genbank identification as KJ561014) (Sebastião *et al*., 2015). Bacteria were stored in TSB culture medium (Tryptic Soy Broth, Hi-Media) at -80°C until inoculation. For use, an aliquot of 20 µl of strain was resuspended into 5 ml of TSB solution, autoclaved and incubated at 28 °C for 24 hours. After this period, 700 ml of TSB medium was added to the solution, autoclaved, and incubated again for 24 hours. The bacterial suspension was centrifuged at 8000 g for 30 min, and the pellets were washed twice in PBS (Phosphate buffer saline - 0.01 M), before be centrifuged again. This operation was repeated twice. The bacteria were diluted at 10^8 CFU ml^{-1} for spectrophotometer reading (DO 625=0.08, corresponds to $1.5x10^8$) and inactivated at 50 °C for 40 min. For inoculation, fish were anesthetized (benzocaine, 0.05 g L^{-1}) and injected in the mesenteric cavity with 0.5 µL g^{-1} of heat-killed *A. hydrophila.*

2.4. Samplings

In each sampling, fish were anesthetized with benzocaine (0.05 g L^{-1}), weighed, bled by puncture of the caudal vessels and quickly euthanized by medullar section. An aliquot of blood was dispensed in microtube with Glistab® (EDTA 6 g dL^{-1} and KF 12 g dL^{-1}, Labtest, São Paulo, Brazil, code 29) to separate plasma for glucose and cortisol determination. Another aliquot of blood was dispensed in microtube with heparin (5.000 UI ml^{-1}) to measure the respiratory activity of leukocytes and the remainder blood without anticoagulant was centrifuged (10 min at 20000 g) to extract serum to determination of lysozyme concentration and the hemolytic activity of the alternative pathway of serum complement system. Liver and spleen were weighed to hepatosomatic index and spleen somatic index calculation, respectively [(tissue weight / body weight) x 100] and liver was stored at -80 °C for further determination of antioxidant defenses.

2.5. Stress indicators

Plasma cortisol and glucose concentrations were determined using commercial kits (DRG® Cortisol ELISA - EIA-1887 and Labtest - code 133, respectively).

2.6. Immunological indicators

To determine the respiratory activity of leucocytes we followed the methodology of Anderson and Siwicki (1995) with modifications for pacu (Biller-Takahashi *et al*., 2013). This method is based on the reduction of NBT (Nitroblue tetrazolium chloride - Sigma Aldrich®, N6876) during the oxidative burst of leukocytes, which produces precipitates called formazan granules measures. The optical density of the solution was determined in a spectrophotometer at 540 nm.

The hemolytic activity of the alternative pathway of serum complement system was determined according to Zanuzzo *et al*. (2017). A kinetic assay determined the time (in seconds) necessary for the serum sample to lyse 50% of a rabbit erythrocyte suspension, with readings every 20 sec for 20 min, at 700 nm.

The serum lysozyme concentration was determined according to Demers and Bayne (1997) with modifications for pacu by Zanuzzo *et al*. (2015) based on lysis of the *Micrococcus lysodeikticus* suspension (Sigma Aldrich®, M3770), measuring the reduction of the absorbance of this solution at 450 nm.

2.7. Antioxidant system

Liver samples will be homogenized (1:10 w/v) in a PBS buffer 0.1 M, pH 7.0 (lipid peroxidation - LPO, superoxide dismutase - SOD, catalase - CAT, reduced glutathione - GSH) and 7.6 (glutathione peroxidase - GPx), centrifuged, and the supernatants stored at -80ºC for further analysis. The protein content was determined according to Bradford (1976) for the biochemical biomarkers. Lipid peroxidation (LPO) was determined by thiobarbituric acid reactive substances (TBARS) assay, performed according to Camejo *et al*. (1998). The SOD activity was determined according to McCord *et al*. (1969), based on the inhibition of the reduction rate of cytochrome c by the superoxide radical, at 550 nm, 25ºC. The

activity of CAT was determined according to Aebi (1984), based on the consumption of exogenous hydrogen peroxide (H_2O_2) by CAT, generating water and oxygen, which absorbance read at 240 nm, every 15 sec for 5 min. The GPx activity was measured according to Hafeman *et al*. (1974), based on the reduction of GSSG (oxidized glutathione) in reduced glutathione (GSH) in the presence of NADPH promoted by glutathione reductase. The reduction of the absorbance is read every 15 sec for 5 min, at 340 nm. The glutathione-S-transferase (GST) concentration was determined according to Keen *et al*. (1976). This method is based on glutathione reduction by 1-chloro-2,4-dinitrobenzene substrate (CDNB) creating glutathione-2,4-nitrobezene. The reduction was read at 340 nm and the results were expressed in nmol mg protein^{-1}. The concentration of reduced glutathione (GSH) was determined following Beutler *et al*. (1963), using 5,50-dithiobis-2-nitrobenzoic acid (DTNB), at 412 nm, against a GSH standard curve. TBARS concentrations is expressed in nmol MDA mg protein^{-1}, SOD activity is expressed in units of mg protein^{-1}, CAT activity is expressed as nmol mg protein^{-1} min^{-1}, GPx activity is expressed mmol mg protein^{-1} min^{-1}, GST concentration is expressed in nmol mg protein^{-1} and GSH concentration is expressed in mmol mg protein^{-1} min^{-1}.

2.8. Statistical analysis

The data were submitted to normality (Shapiro-Wilk) and homoscedasticity (Brown-Forsythe) tests. To adjust cortisol, glucose and lysozyme LPO and GST concentrations, complement system, leucocytes respiratory, GSH, GPx activity and spleen somatic index data for normality, they were transformed using log10. Hepatosomatic index, catalase and SOD activity was not transformed. We used a completely randomized design with a factorial arrangement of two treatments (control and glucan 0.1%) vs. three stress conditions (acute stress, chronic stress and chronic plus acute stress) vs. four time point (basal, 3 hpi, 6 hpi and 24 hpi). Means were compared by Duncan's post-hoc tests using SAS software (version 9.0). P value < 0.05 was used to estimate the level of significance for statistical differences. N=8.

3. Results

We evaluated the effect of 15-day dietary supplementation with β-glucan on the stress, innate immune and antioxidant responses of pacu, either chronically or acutely stressed. The chronic stressor was daily chasing of fish during the experimental feeding and the acute stressor was 4-h transport of fish. Additionally, fish were inoculated with heat-killed *A. hydrophila* after being stressed. There was no mortality during the experiment.

3.1 Stress response

3.1.1. Plasma cortisol concentrations

Plasma cortisol concentration increased in fish WS (not stressed) and not transported (Fig.1A) fed control diet at 3, 6 and 24 hpi (p=0.0390, a), while in fish fed glucan diet, cortisol concentration increased at 24 hpi exceeding basal levels (p=0.0118, a). In CS (chronically stressed) fish, not transported and fed with control diet, the plasma cortisol concentrations increased (p=0.0260, a) 3 hpi and returned at basal levels (p=0.0168, b) at 6 hpi. In CS fish, not transported and fed with glucan, cortisol levels also increased (p=0.0063, a) at 3 hpi, but they did not return at basal levels until 24 hpi. In WS fish, transported and fed with both diets, cortisol concentration peaked 3 hpi, although the cortisol levels in fish of control diet exceeded that of glucan diet (p=0.0161, A). Comparing WS and CS fish not transported in each sampling time, at 6 and 24 hpi, cortisol levels were higher in WS compared to CS in fish fed with control diet (6 hpi – p=0.0353; 24 hpi – p=0.0419, *).

Additionally, comparing not transported and transported fish groups, cortisol concentration increased independent of diet and stress condition in transported fish (Fig. 1B) (WS-control diet – p=0.0009; WS-glucan diet – p=0.0049; CS-control diet – p=0.0046, CS-glucan diet – p=0.0083, ●). In WS fish, not transported, cortisol levels were higher at 24 hpi independent of diet (control – p=0.0103; glucan – p=0.0154, ●).

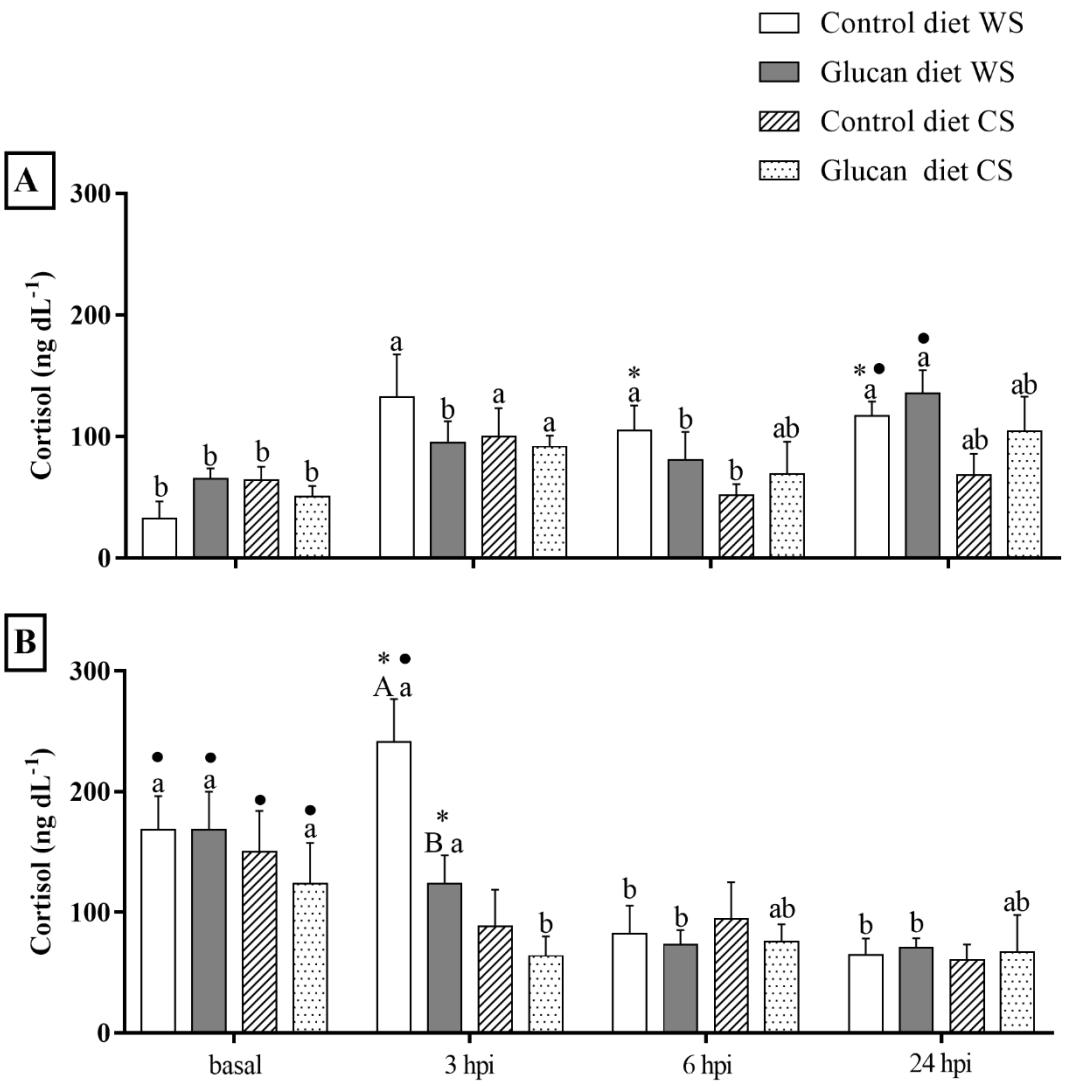

Fig. 2. Plasma cortisol concentration in not transported (A) or transported (B) pacu fed with control diet and diet containing 0.1% β-glucan, without stress and after chronic stress (basal) and 3, 6 and 24 h post inoculation with heat-killed *Aeromonas hydrophila*. Different capital letters indicate difference between treatments at particular group in same sampling time. Different lower letters indicate differences between sampling times within the same group. Asterisk indicates difference between stress condition (without stress or chronic stress). Balls indicates difference between not transported and transported groups. Symbols were omitted when there was no statistical difference. Values are means ± standard error (n=6).

3.1.2 Plasma glucose concentrations

The plasma glucose concentration increased 3 hpi in control diet (p=0.0198, a) and 24 hpi in glucan diet (p=0.0038, a) in WS not transported fish (Fig. 3A). At 6 hpi, glucose was higher (p=0.0278, A) in CS-glucan diet fish compared with those fed with CS-control diet. At 24 hpi, WS-control diet not transported fish showed higher

levels of plasma glucose compared with CS (p=0.0128, *) and transported (p=0.0091, ●) fish.

Immediately after transport, fish from both diets and stress conditions (WS and CS) showed increased levels of glucose (WS-control diet – p<0.0001; WS-glucan diet – p<0.0001; CS-control diet – p<0.0001; CS-glucan diet – p<0.0001, ●) remaining elevate until 3 hpi in WS fish from both diets (control – p=0.0204; glucan – p=0.0192, ●).

In transported fish, the glucose concentration decreased 3 hpi and remained lower until 24 hpi without influence of diet, transport and stress condition (CS-control diet not transported– p=0.0128; CS-glucan diet not transported – p=0.0305; WS-control diet not transported – p=0.0011; WS-glucan diet not transported – p=0.0213; CS-control diet transported – p=0.0278; CS-glucan diet transported – p=0.0316; WS-control diet transported – p=0.0009; WS-glucan diet transported – p=0.0305, b) (Fig. 2A and B).

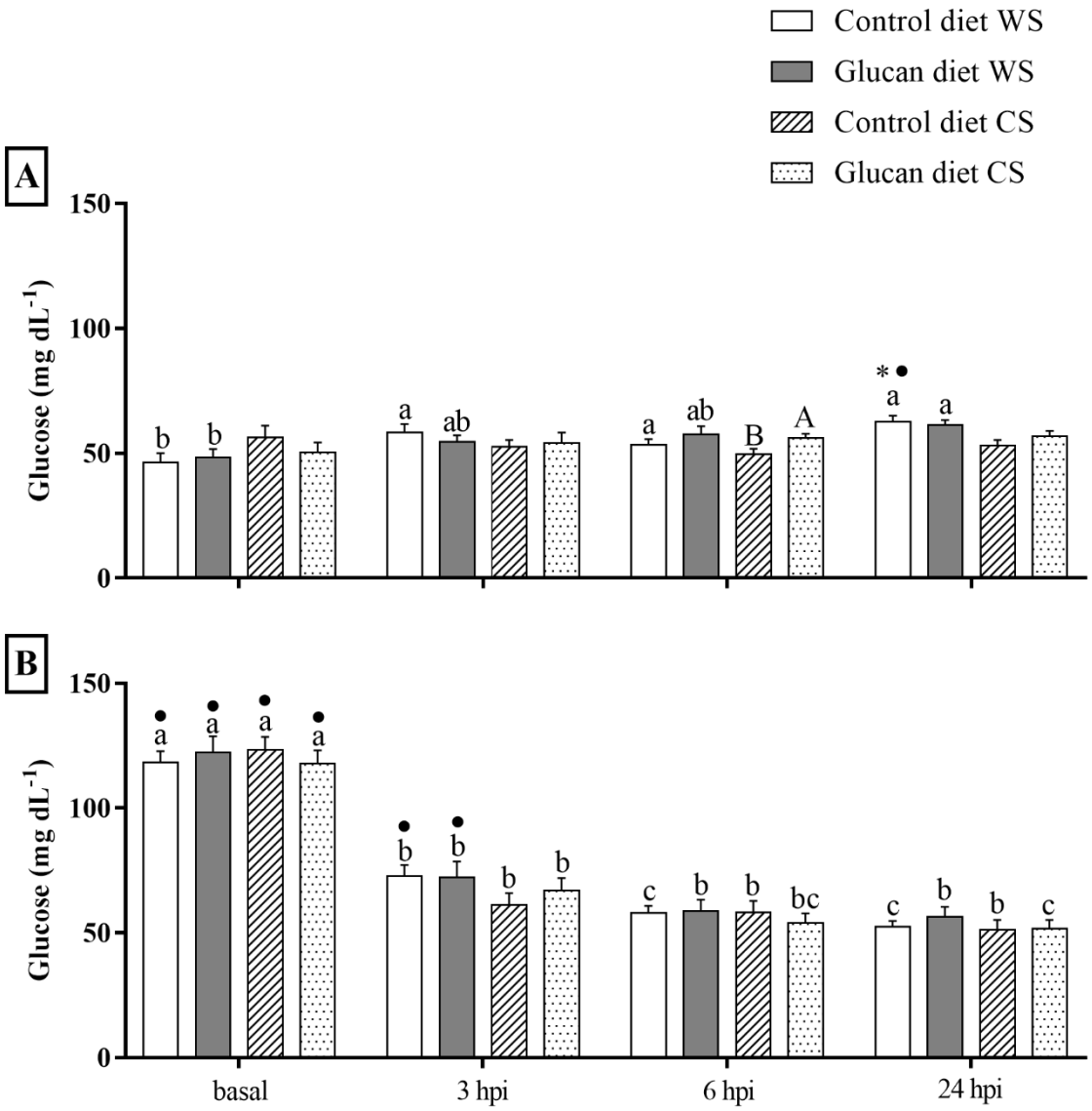

Fig. 3. Plasma glucose concentration in not transported (A) or transported (B) pacu fed with control diet and diet containing 0.1% β-glucan, without stress and after chronic stress (basal) and 3, 6 and 24 h post inoculation with heat-killed *Aeromonas hydrophila*. Different capital letters indicate difference between treatments at particular group in same sampling time. Different lower letters indicate differences between sampling times within the same group. Asterisk indicates difference between stress condition (without stress or chronic stress). Balls indicates difference between not transported and transported groups. Symbols were omitted when there was no statistical difference. Values are means ± standard error (n=8).

3.2 Innate immune response

3.2.1 Respiratory activity of leucocytes (RAL)

At basal sampling (Fig.3A), in not transported fish, the respiratory activity of leucocytes (RAL) was higher (p=0.0381, A) in CS-control diet fish compared with

those of fed with CS-glucan diet. At 6 hpi, RAL increased in WS fish without influence of diets (control – $p=0.0238$; glucan – $p<0.0001$, a). In CS fish, the RAL decreased 3 hpi (control – $p=0.0070$; glucan – $p=0.0222$, b) and returned to basal levels 6 hpi.

In transported fish (Fig. 3B), RAL increased in WS control-diet fish at 3 hpi ($p=0.0148$, a) and at 24 hpi in glucan-diet group ($p=0.0088$, a), while in CS group, RAL decreased at 6 hpi (control – $p=0.0009$; glucan – $p=0.0503$, b) and returned to basal levels at 24h. The RAL decreased at 6 hpi without influence of diets in WS (control – $p=0.0148$; glucan – $p=0.0088$, c) and CS (control – $p=0.0009$; glucan – $p=0.0503$, b) groups.

At basal and 3 hpi samplings fish from all transported groups showed higher RAL than those not transported (Basal: WS-control diet – $p<0.001$; WS-glucan diet – $p<0.0001$; CS-glucan diet – $p=0.00368$; 3 hpi: WS-control diet – $p=0.0003$; CS-control diet – $p=0.0021$; WS-glucan diet – $p=0.0158$; CS-glucan diet – $p=0.0022$, ●). At 6 hpi, fish from all transported groups showed less activity than not transported fish (WS-control diet – $p=0.0022$; CS-control diet – $p<0.0001$; WS-glucan diet – $p=0.0081$; CS-glucan diet – $p=0.0021$, ●).

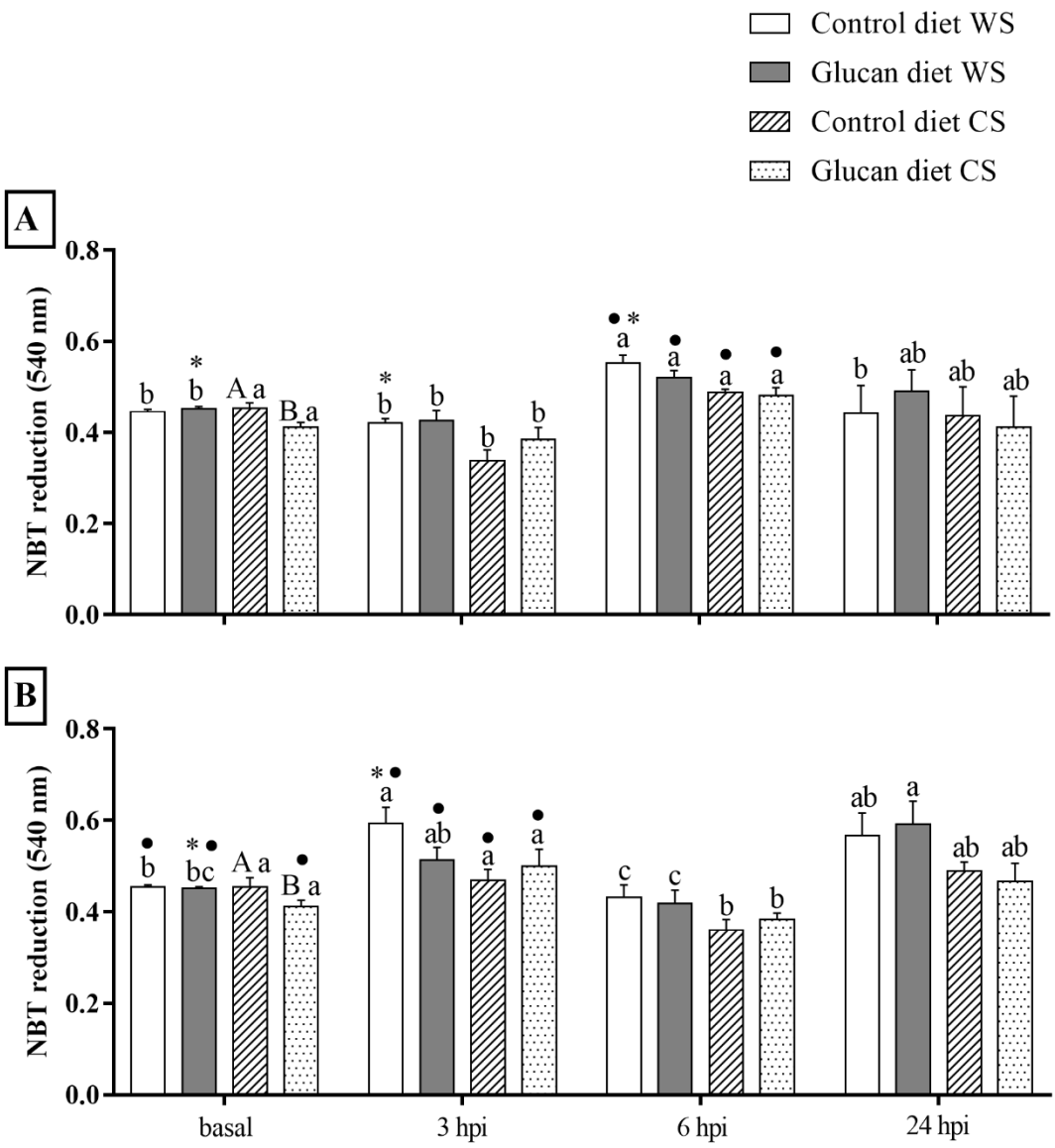

Fig. 4. Respiratory activity of leucocytes in not transported (A) or transported (B) pacu fed with control diet and diet containing 0.1% β-glucan, without stress and after chronic stress (basal) and 3, 6 and 24 h post inoculation with heat-killed *Aeromonas hydrophila*. Different capital letters indicate difference between treatments at particular group in same sampling time. Different lower letters indicate differences between sampling times within the same group. Asterisk indicates difference between stress condition (without stress or chronic stress). Balls indicates difference between not transported and transported groups. Symbols were omitted when there was no statistical difference. Values are means ± standard error (n=8).

3.2.2 Serum hemolytic activity of the alternative pathway of the complement system (HA-AP)

The lowest serum hemolytic activity of complement system was observed at 3 hpi independent of the diet, transport and stress (WS-control diet not transported –

p=0.0158; WS-control transported – p=0.0352; CS-control diet not transported – p=0.0074; CS-control diet transported – p=0.0155; WS-glucan diet not transported – p<0.0001; WS-glucan diet transported – p=0.0163; CS-glucan diet not transported – p=0.0229; CS-glucan diet transported – p=0.0002, a) with return to basal levels at 6 hpi (Fig. 4A and B). The HA-AP was higher in WS fish fed with control diet at 3 hpi independent of transport (not transported – p=0.0108; transported – p=0.0154, A), however in CS fish, this situation was the opposite, with higher activity in not transported fish fed with glucan diet (p=0.0231, A).

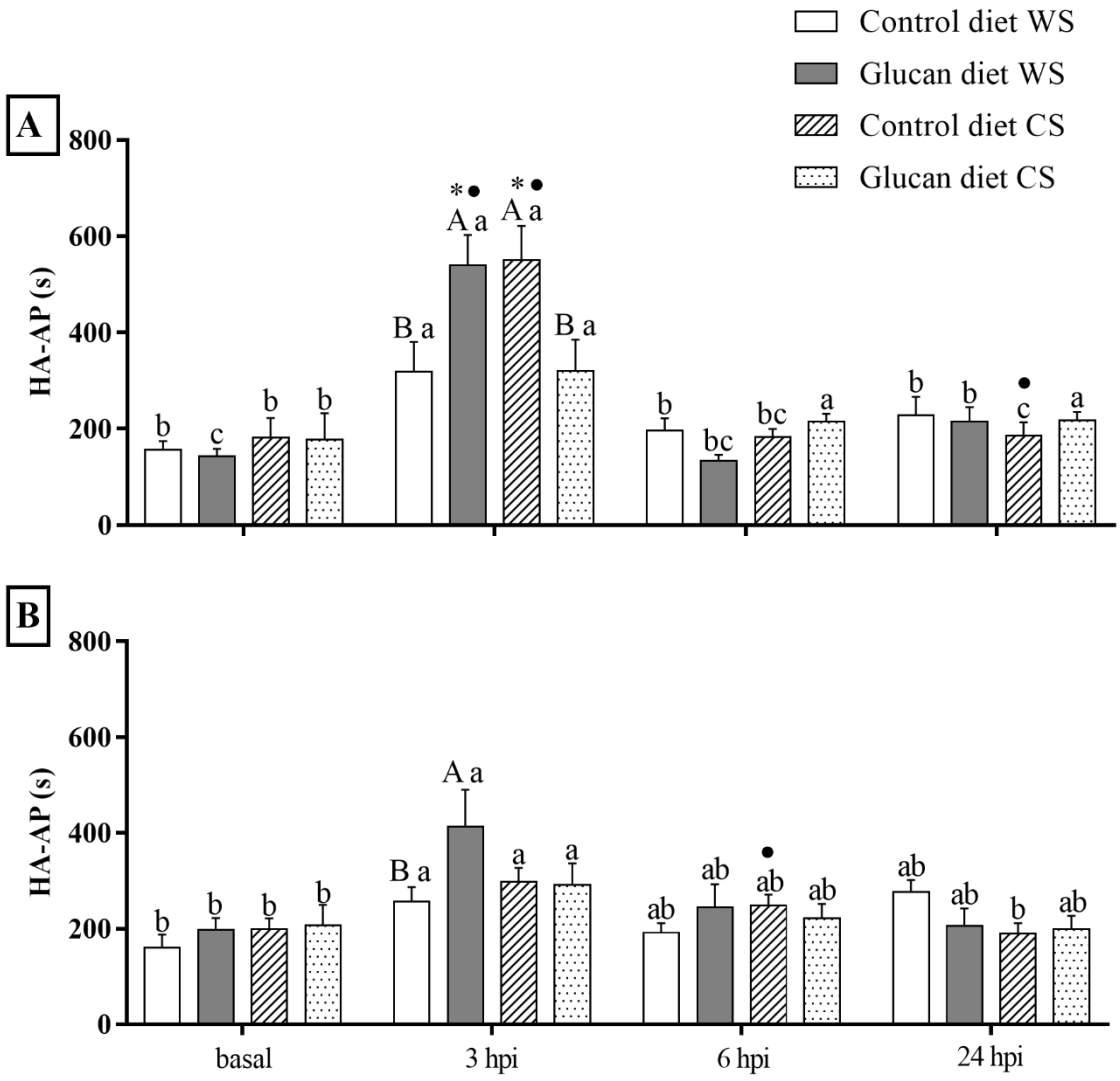

Fig. 5. Serum hemolytic activity of the complement system in not transported (A) or transported (B) pacu fed with control diet and diet containing 0.1% β-glucan, without stress and after chronic stress (basal) and 3, 6 and 24 h post inoculation with heat-killed *Aeromonas hydrophila*. Different capital letters indicate difference between treatments at particular group in same sampling time. Different lower letters indicate differences between sampling times within the same group. Asterisk indicates difference between stress condition (without stress or chronic stress). Balls indicates difference

between not transported and transported groups. Symbols were omitted when there was no statistical difference. Values are means ± standard error (n=8).

3.2.3 Serum lysozyme concentration

At basal sampling, both WS control fish (not transported and transported) had higher serum lysozyme concentration compared with those fed with glucan diet (not transported - p=0.0056; transported – p=0.0251A) (Fig. 5A and B). At 3 hpi the same was observed in WS transported fish (p=0.0005, A). Lysozyme concentration increased 24 hpi in fish from both diets, transport and type of stressor (WS-control diet transported – p=0.0006; CS-control diet not transported – p=0.0374; CS-control diet transported – p<0.0001; WS-glucan diet not transported – p=0.0018; WS-glucan diet transported – p<0.001; CS-glucan diet not transported – p=0.0014; CS-glucan diet transported – p=0.0008, a).

Comparing not transported and transported groups, lysozyme concentration increase in not transported fish 3 hpi in both diets CS (control – p=0.0420; glucan – p=0.0442, ●) and WS-glucan diet (p=0.0002, ●) and 6 hpi in glucan-diet independent of stress condition (WS – p=0.0301; CS – p=0.0114, ●). At 24 hpi, WS and CS transported fish fed with control diet showed increase (WS – p=0.0153; CS – p=0.0048, ●) in lysozyme concentration compared those not transported.

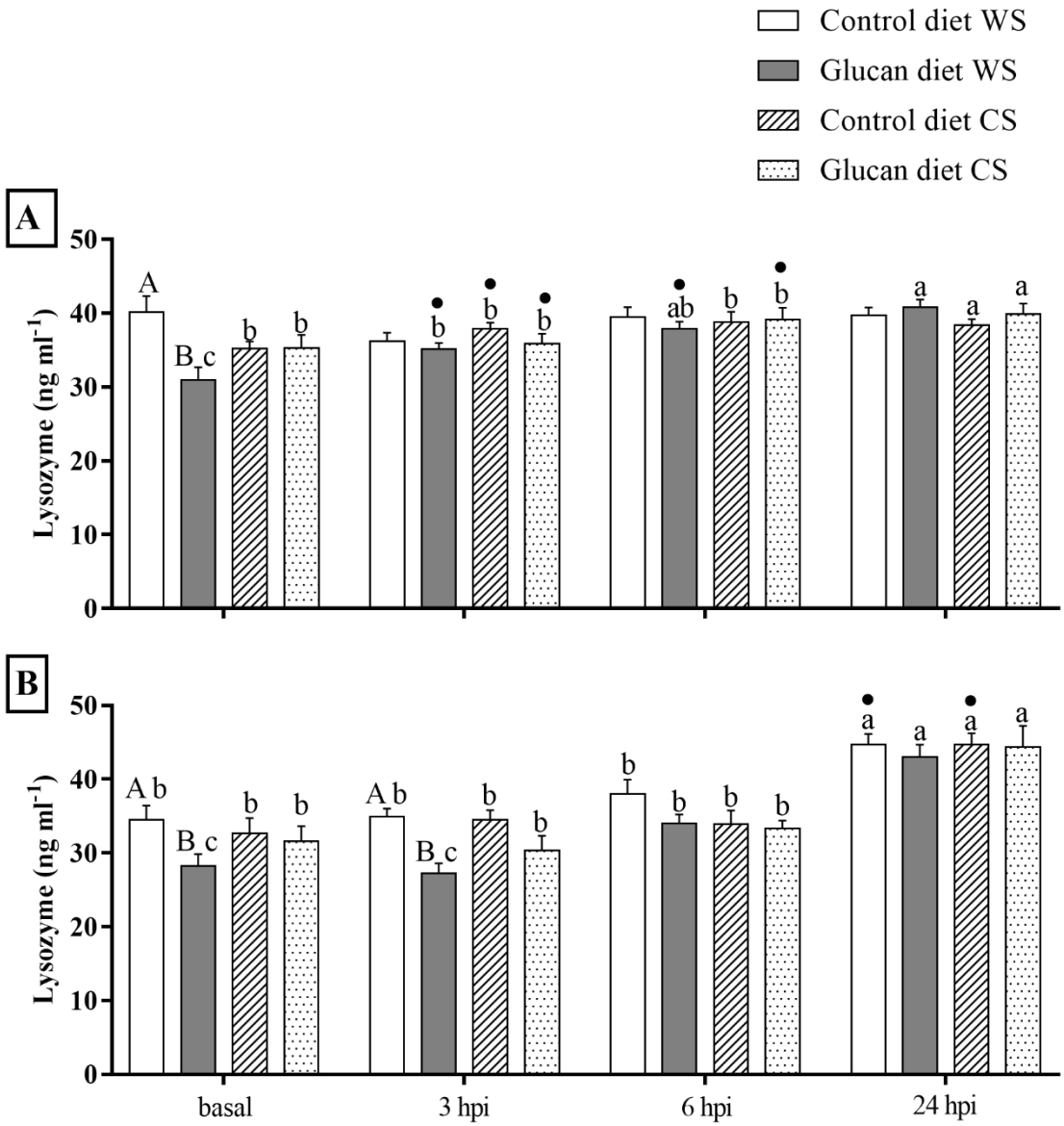

Fig. 6. Lysozyme concentration in not transported (A) or transported (B) pacu fed with control diet and diet containing 0.1% β-glucan, without stress and after chronic stress (basal) and 3, 6 and 24 h post inoculation with heat-killed *Aeromonas hydrophila*. Different capital letters indicate difference between treatments at particular group in same sampling time. Different lower letters indicate differences between sampling times within the same group. Asterisk indicates difference between stress condition (without stress or chronic stress). Balls indicates difference between not transported and transported groups. Symbols were omitted when there was no statistical difference. Values are means ± standard error (n=8).

3.3 Antioxidant system

3.3.1 Lipid peroxidation (LPO)

At basal sampling, both WS control fish (not transported and transported) had higher lipid peroxidation compared with those fed glucan diet (not transported -

p=0.0015; transported - p=0.0049, A) and, at 24 hpi, the same was observed in CS not transported fish (p=0.0149, A). Among fish transported and fed with glucan diet, CS group showed higher (p=0.0015, *) LPO than WS group at basal sampling. At 24 hpi, fish WS fed with glucan diet showed higher (p=0.0167, *) LPO than those from CS group. LPO increased after heat-killed *A. hydrophila* injection in glucan diet 24 hpi and 3hpi (p=0.0477, a) in not transported and transported (p=0.0007, a) fish, respectively. Transported fish showed increase in LPO in WS group at basal sampling in glucan diet (p=0.0392, ●) 3 hpi (control diet – p=0.0204; glucan diet – p=0.0040, ●) independent of diet.

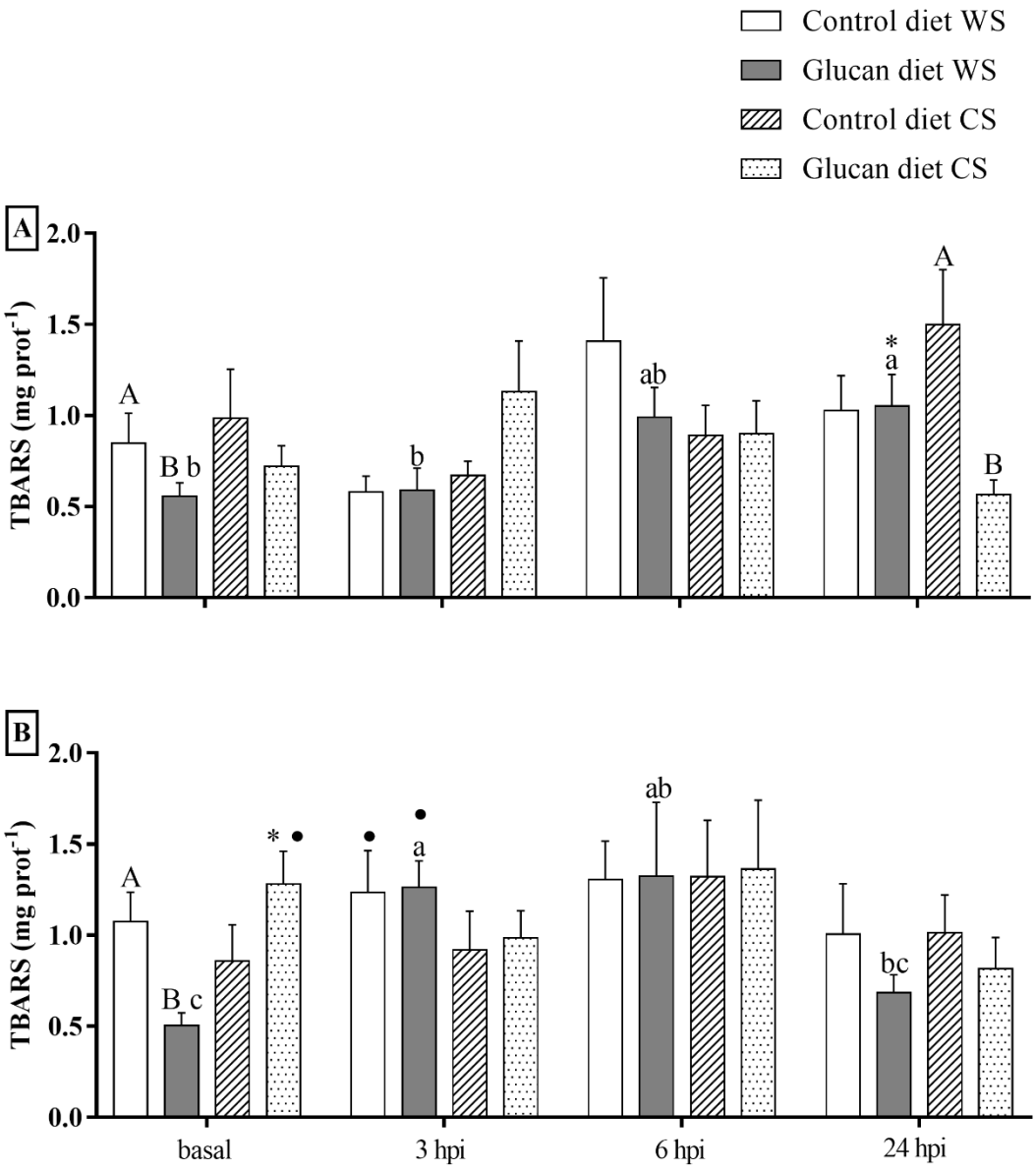

Fig. 7. Liver lipid peroxidation in not transported (A) or transported (B) pacu fed with control diet and diet containing 0.1% β-glucan, without stress and after chronic stress (basal) and 3, 6 and 24 h post inoculation with heat-killed *Aeromonas hydrophila*. Different capital letters indicate difference between treatments at particular group in same sampling time. Different lower letters indicate differences between sampling times within the same group. Asterisk indicates difference between stress condition (without stress or chronic stress). Balls indicates difference between not transported and transported groups. Symbols were omitted when there was no statistical difference. Values are means ± standard error (n=8).

3.3.2 Superoxide dismutase activity (SOD)

The SOD activity decreased (p=0.0290, b) in fish CS not transported fed with control diet at 6 hpi (Fig. 7A).

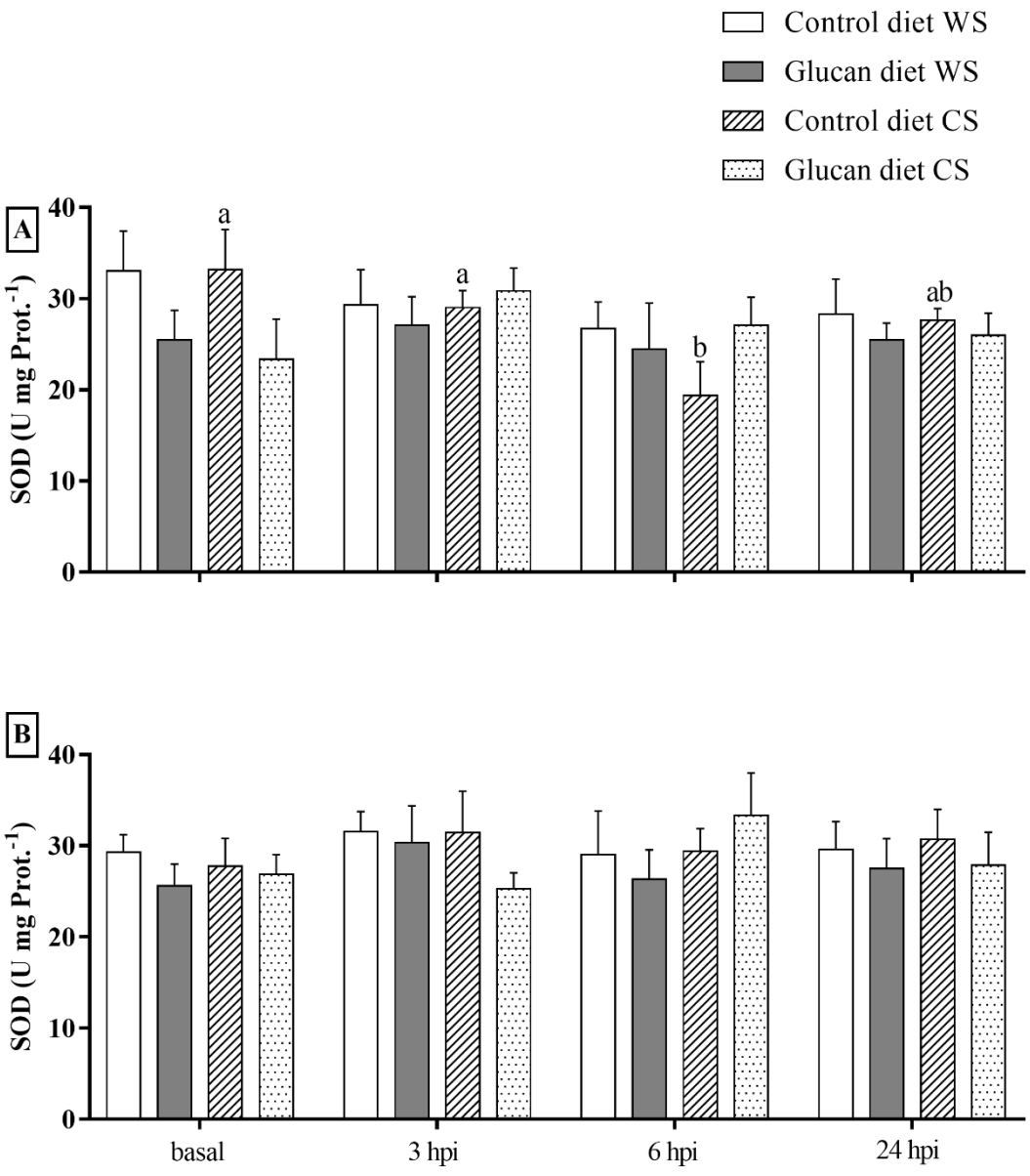

Fig. 8. Superoxide dismutase (SOD) activity in not transported (A) or transported (B) pacu fed with control diet and diet containing 0.1% β-glucan, without stress and after chronic stress (basal) and 3, 6 and 24 h post inoculation with heat-killed *Aeromonas hydrophila*. Different capital letters indicate difference between treatments at particular group in same sampling time. Different lower letters indicate differences between sampling times within the same group. Asterisk indicates difference between stress condition (without stress or chronic stress). Balls indicates difference between not transported and transported groups. Symbols were omitted when there was no statistical difference. Values are means ± standard error (n=8).

3.3.3. Catalase activity (CAT)

At basal sampling, not transported WS fish fed with glucan diet showed higher CAT activity compared with those transported (p=0.0316, ●) (Fig. 8A and B). At 3hpi, CAT decreased in WS group fed with glucan diet, not transported, and control diet transported fish (not transported glucan diet – p=0.0342; transported control diet – p=0.1098, b). At this time, in transported fish, CS group fed with control diet showed higher CAT activity than the WS group fed with control diet (p=0.0316, *).

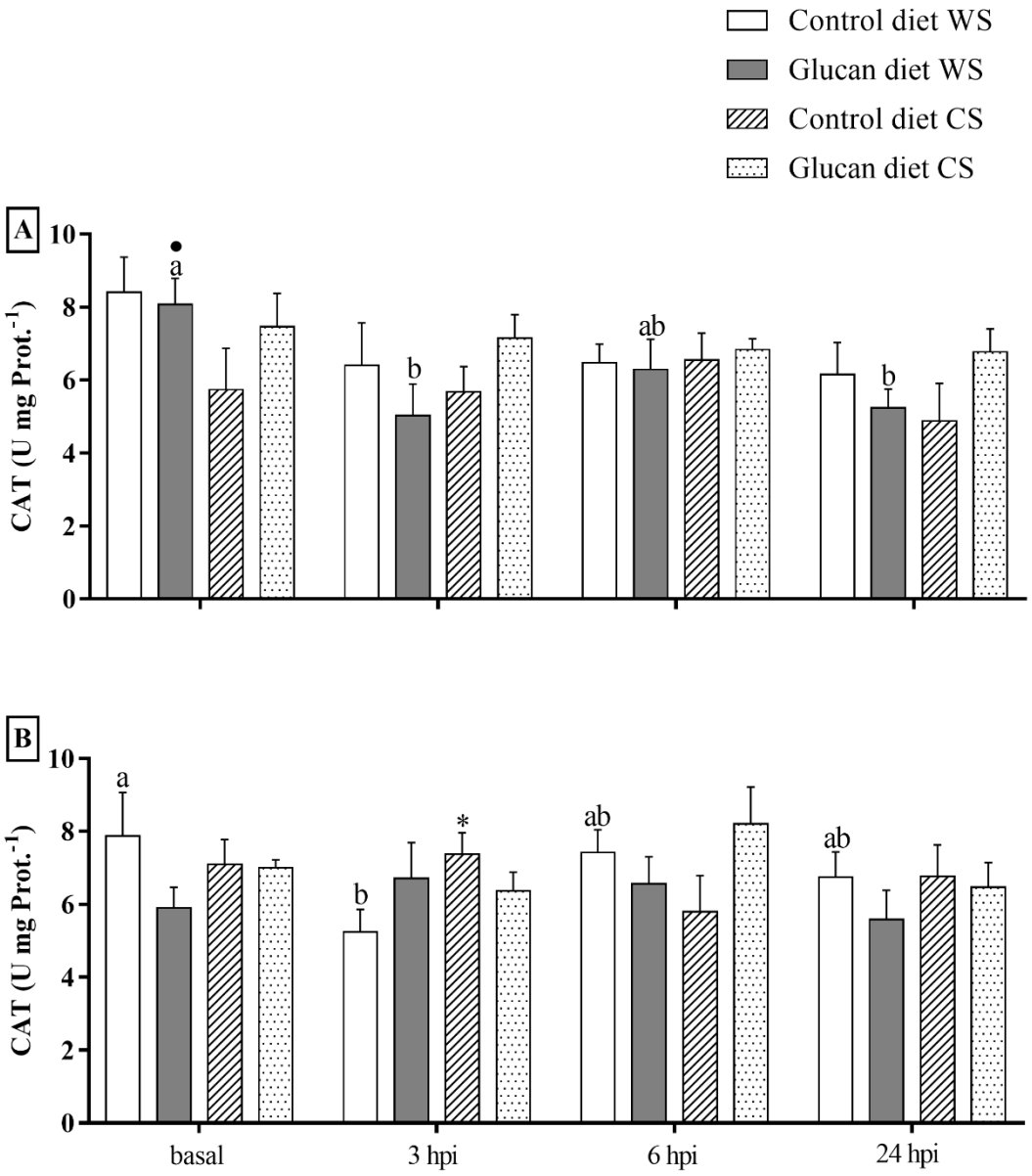

Fig. 9. Catalase (CAT) activity in not transported (A) or transported (B) pacu fed with control diet and diet containing 0.1% β-glucan, without stress and after chronic stress (basal) and 3, 6 and 24 h post inoculation with heat-killed *Aeromonas hydrophila*. Different capital letters indicate difference between treatments at particular group in same sampling time. Different lower letters indicate differences between sampling times within the same group. Asterisk indicates difference between stress condition (without stress or chronic stress). Balls indicates difference between not transported and transported groups. Symbols were omitted when there was no statistical difference. Values are means ± standard error (n=8).

3.3.4. Glutathione peroxidase activity (GPx)

At 6 hpi and 24 hpi, the GPx activity increased in WS fish fed with control diet in both not transported and transported groups, respectively (not transported –

p=0.0023; transported – p=0.0026, a) (Fig 9A and B). In WS fish fed with glucan diet, the increase was observed at 24 hpi and 3 hpi in both not transported and transported groups, respectively (not transported – p=0.1085; transported – p=0.0858, a). At 6 hpi, in not transported groups, WS fish fed with control diet showed higher (p=0.0431, A) GPx activity when compared to WS fish fed with glucan diet. The opposite was observed in transported fish at 3 hpi, when WS fish fed with glucan diet showed higher (p=0.0149, A) GPx activity when compared to WS fish fed with control diet.

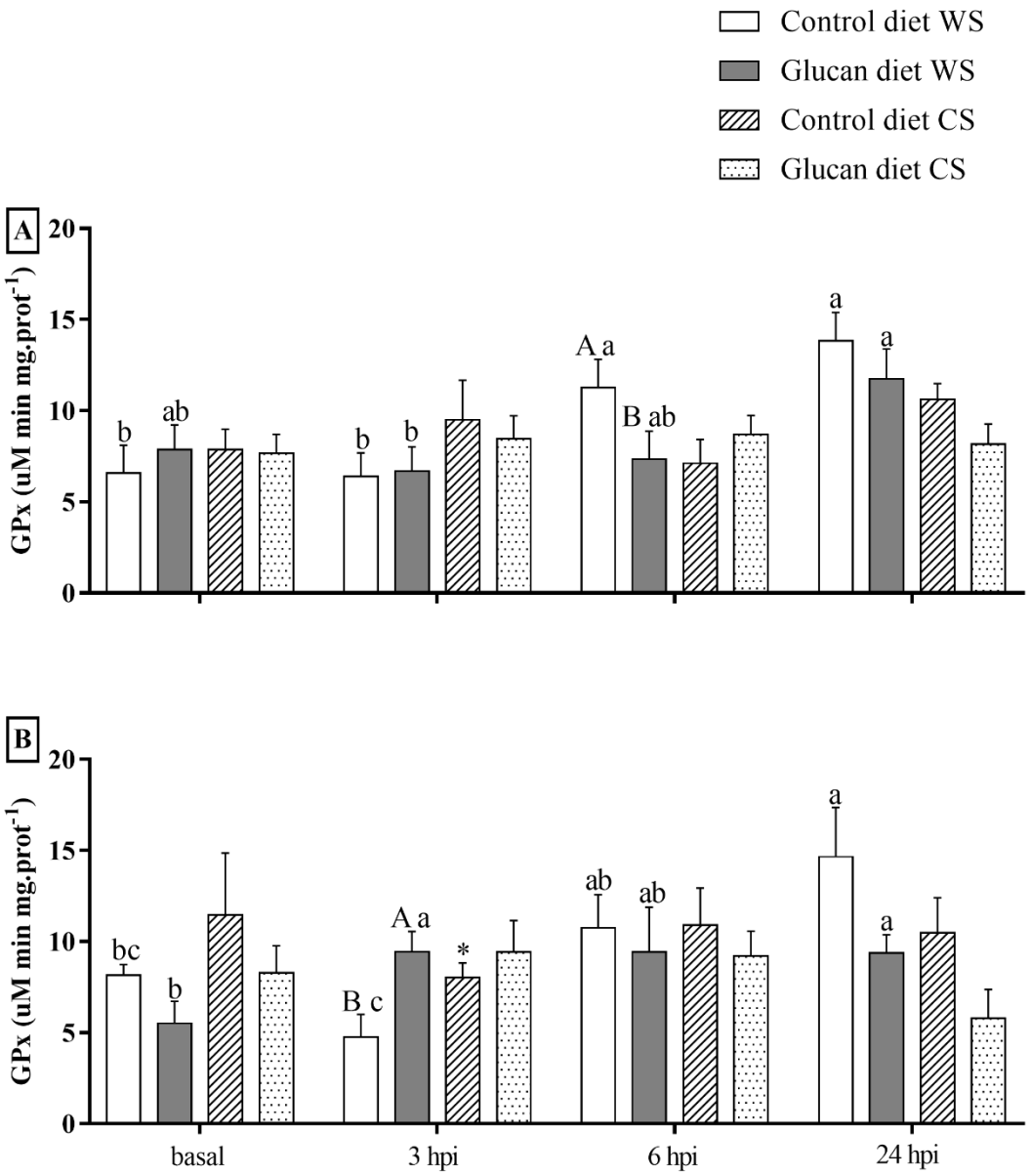

Fig. 10. Glutathione peroxidase (GPx) activity in not transported (A) or transported (B) pacu fed with control diet and diet containing 0.1% β-glucan, without stress and after chronic stress (basal) and 3,

6 and 24 h post inoculation with heat-killed *Aeromonas hydrophila*. Different capital letters indicate difference between treatments at particular group in same sampling time. Different lower letters indicate differences between sampling times within the same group. Asterisk indicates difference between stress condition (without stress or chronic stress). Balls indicates difference between not transported and transported groups. Symbols were omitted when there was no statistical difference. Values are means ± standard error (n=8).

3.3.5. Glutathione-S-transferase concentration (GST)

The GST activity decreased (p=0.1098, b), at 3 hpi, in WS fish fed with control diet and not transported. At basal sampling, WS fish fed with control diet and not transported showed higher GST activity compared to transported fish (p=0.0013, ●) (Fig. 10A)

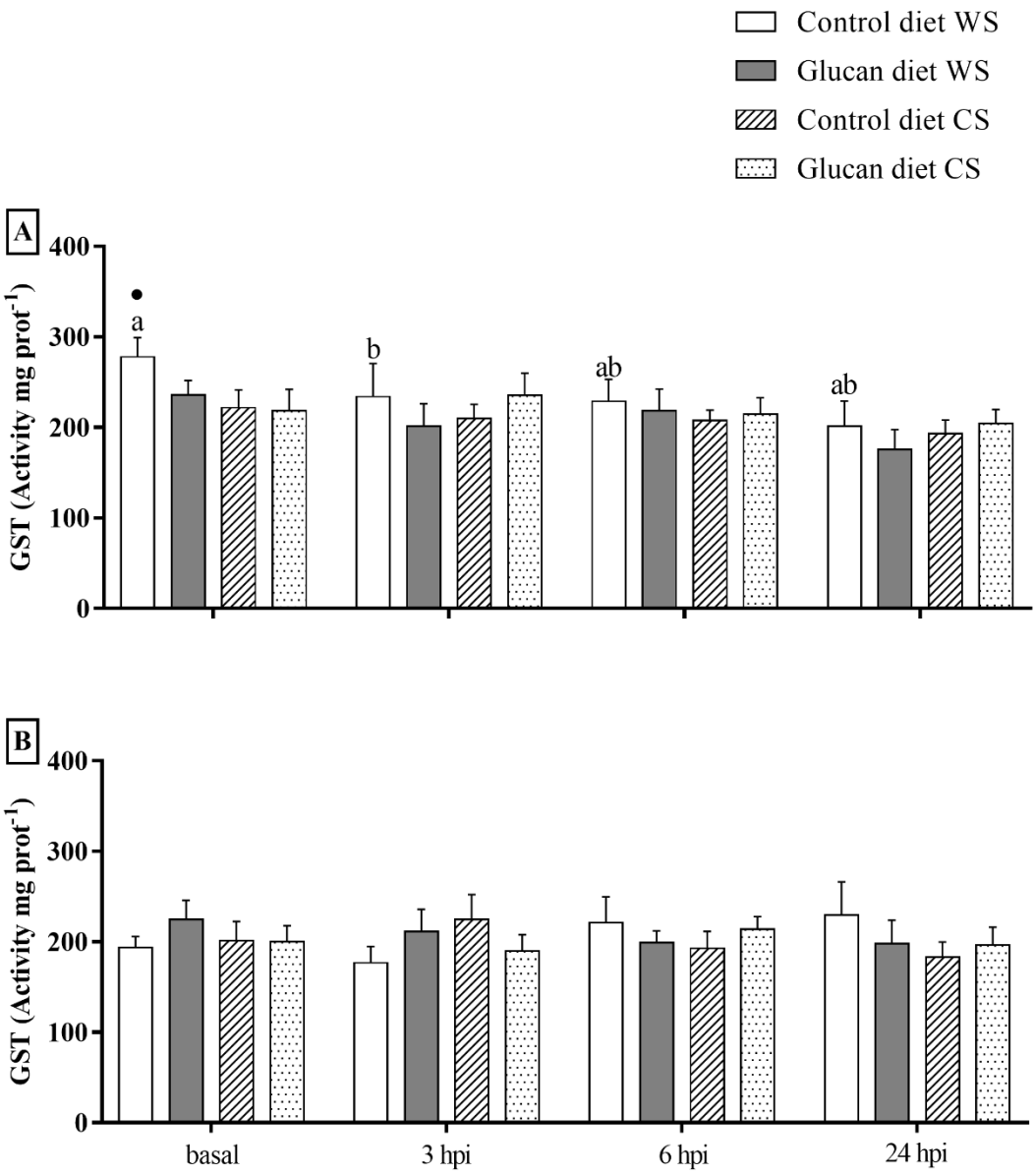

Fig. 11. Glutathione-S-transferase (GST) concentration in not transported (A) or transported (B) pacu fed with control diet and diet containing 0.1% β-glucan, without stress and after chronic stress (basal) and 3, 6 and 24 h post inoculation with heat-killed *Aeromonas hydrophila*. Different capital letters indicate difference between treatments at particular group in same sampling time. Different lower letters indicate differences between sampling times within the same group. Asterisk indicates difference between stress condition (without stress or chronic stress). Balls indicates difference between not transported and transported groups. Symbols were omitted when there was no statistical difference. Values are means ± standard error (n=8).

3.3.6. Reduced glutathione (GSH) concentrations

At basal sampling and 24 hpi, in not transported fish (Fig. 11A), WS fish fed with control diet showed higher (basal – p=0.0248; 24 hpi – p=0.0464, A) GSH concentration than WS fish fed with glucan diet. At 3 hpi, CS fish fed with glucan diet, and not transported, showed higher (p=0.0081, A) GSH concentration than CS fish fed with control diet, and not transported, while CS fish fed with glucan diet, and transported, showed lower (p=0.0145, B) GSH concentration than CS fish fed with control diet, and transported (Fig. 11B). In not transported WS fish fed with control feed fish, the GSH concentration decreased 6 hpi (p=0.0746, b). In CS fish the decrease was observed 3 hpi in fish fed with control diet, and not transported (p=0.0183, b), and fish fed with glucan diet, and transported (p=0.0334, b). The not transported fish showed higher GSH concentration compared with transported ones at basal and 3 hpi samplings in WS group for both diets (basal-control diet – p=0.0003; basal-glucan diet – p=0.0103; 3 hpi-control diet – p=0.0018; 3 hpi-glucan diet – p=0.0223, ●), at 6 hpi independent of treatment or stress condition (WS-control diet – p=0.0026, WS-glucan diet – p=0.0006; CS-control diet – p=0.0165; CS-glucan diet – p=0.0256, ●) and at 24 hpi in WS fish fed with control diet (p=0.0021, ●) and CS fish fed with glucan diet (p=0.0034, ●). At basal sampling and 3 hpi, in transported fish, WS fish fed with glucan diet had higher (basal - p=0.0494; 3 hpi - p=0.0033,*) GSH concentration than CS fish fed with glucan diet. At 3 hpi and 24 hpi, in not transported fish, WS fish fed with control diet had higher (3 hpi – p=0.0030; 24 hpi – p=0.0024, *) GSH concentration than CS fish fed with control diet.

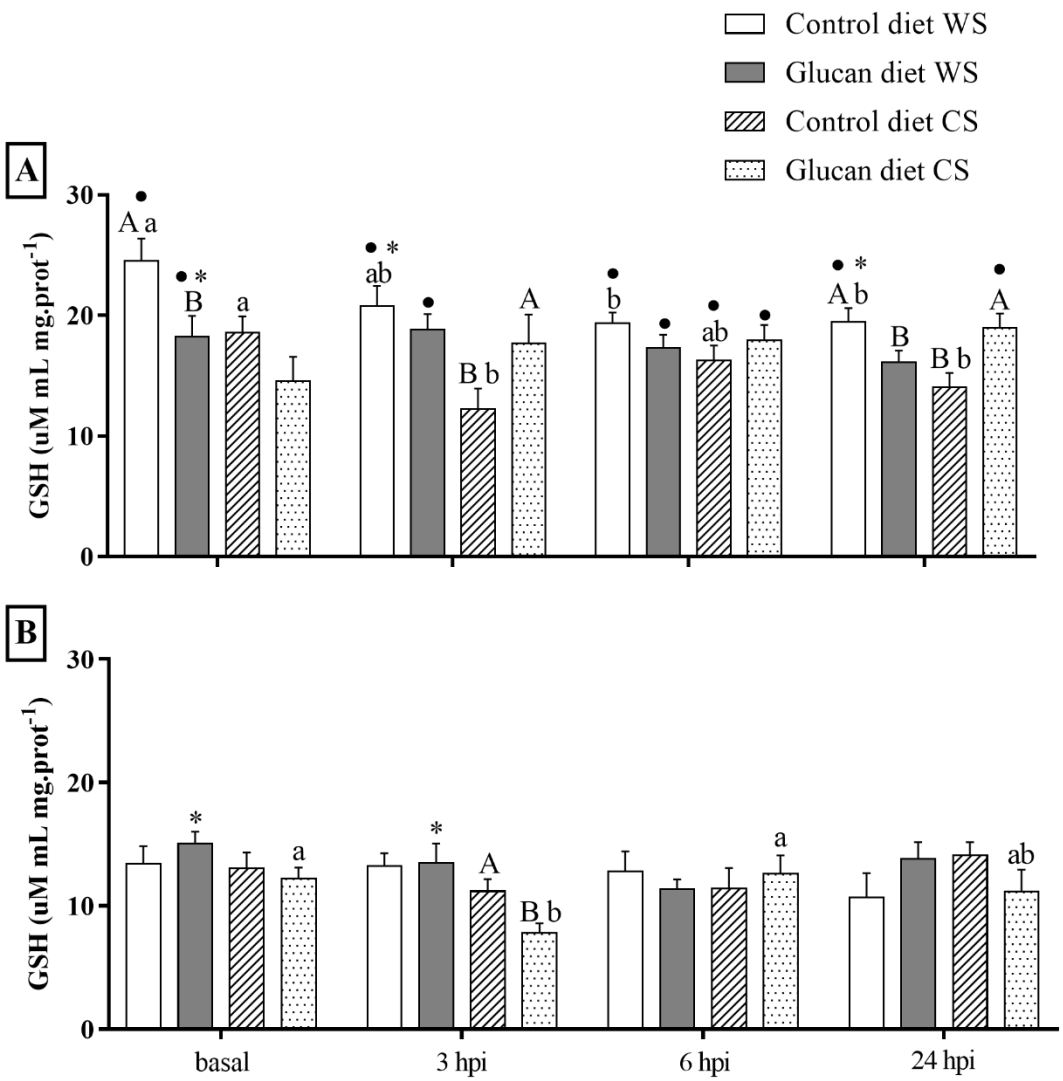

Fig. 12. Reduced glutathione (GSH) concentration in not transported (A) or transported (B) pacu fed with control diet and diet containing 0.1% β-glucan, without stress and after chronic stress (basal) and 3, 6 and 24 h post inoculation with heat-killed *Aeromonas hydrophila*. Different capital letters indicate difference between treatments at particular group in same sampling time. Different lower letters indicate differences between sampling times within the same group. Asterisk indicates difference between stress condition (without stress or chronic stress). Balls indicates difference between not transported and transported groups. Symbols were omitted when there was no statistical difference. Values are means ± standard error (n=8).

3.5. Spleen somatic index (SSI)

The SSI increased after inoculation in CS and WS group, independent of transport in fish fed with both diets (WS-control diet not transported – p=0.0183; WS-control diet transported – p=0.0007; CS-control diet not transported – p=0.0005; CS-control diet transported – p<0.0001; WS-glucan diet not transported – p=0.0346; WS-

glucan diet transported – p=0.0429; CS-glucan diet not transported – p=0.0391; CS-glucan diet transported – p=0.0003, a). CS transported fish showed higher (control –p=0.0047; glucan – p=0.0146, ●) SSI than those not transported fish at 6 hpi. At this time, WS fish fed with glucan diet, and transported, had SSI higher compared with those fed with control diet (p=0.0219, A).

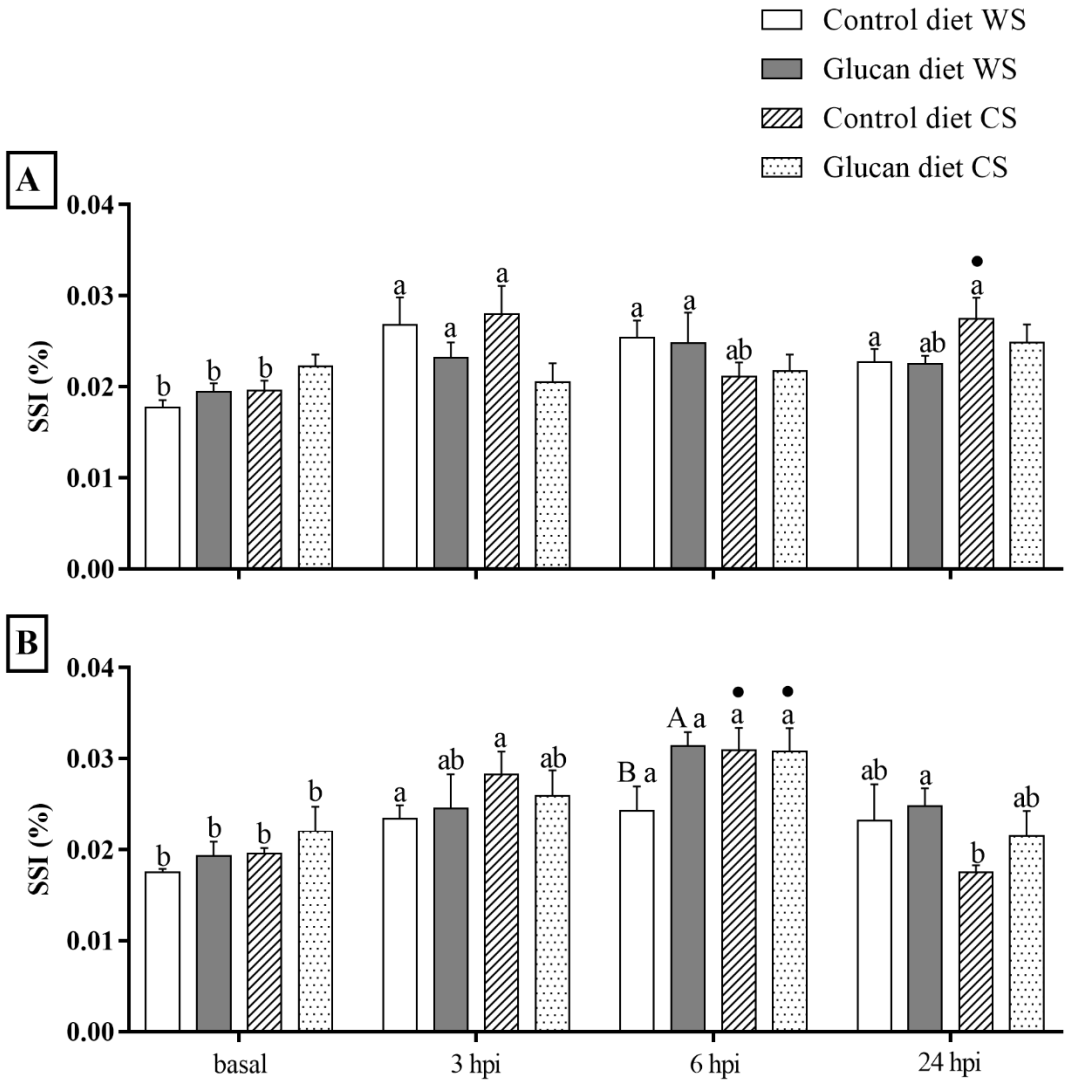

Fig. 13. Spleen somatic index in not transported (A) or transported (B) pacu fed with control diet and diet containing 0.1% β-glucan, without stress and after chronic stress (basal) and 3, 6 and 24 h post inoculation with heat-killed *Aeromonas hydrophila*. Different capital letters indicate difference between treatments at particular group in same sampling time. Different lower letters indicate differences between sampling times within the same group. Asterisk indicates difference between stress condition (without stress or chronic stress). Balls indicates difference between not transported and transported groups. Symbols were omitted when there was no statistical difference. Values are means ± standard error (n=8).

3.6. Hepatosomatic index (HSI)

At basal sampling, fish fed with glucan diet had higher (WS – p=0.0252; CS – p=0.0039, A) HSI than fish fed with control diet for both stress conditions. At this time, WS group showed higher (control – p=0.0251; glucan – p=0.0396, *) HSI than CS group. In CS not transported group, glucan diet increased HSI at 6 (p=0.0039, A) and 24 hpi (p=0.0048, A). The HSI decreased in CS fish fed with glucan diet, and not transported, at 3 hpi (p=0.0487, b) and in CS fish fed with control diet, not transported, at 6 hpi (p=0.0003, b) while in WS, a decrease was observed at 24 hpi (control – p=0.0006; glucan – p=0.0002, b). In transported fish, the HSI decreased at 24 hpi (WS-control diet – p=0.0010; WS-glucan diet – p=0.0011; CS-glucan diet – p=0.0214, b).

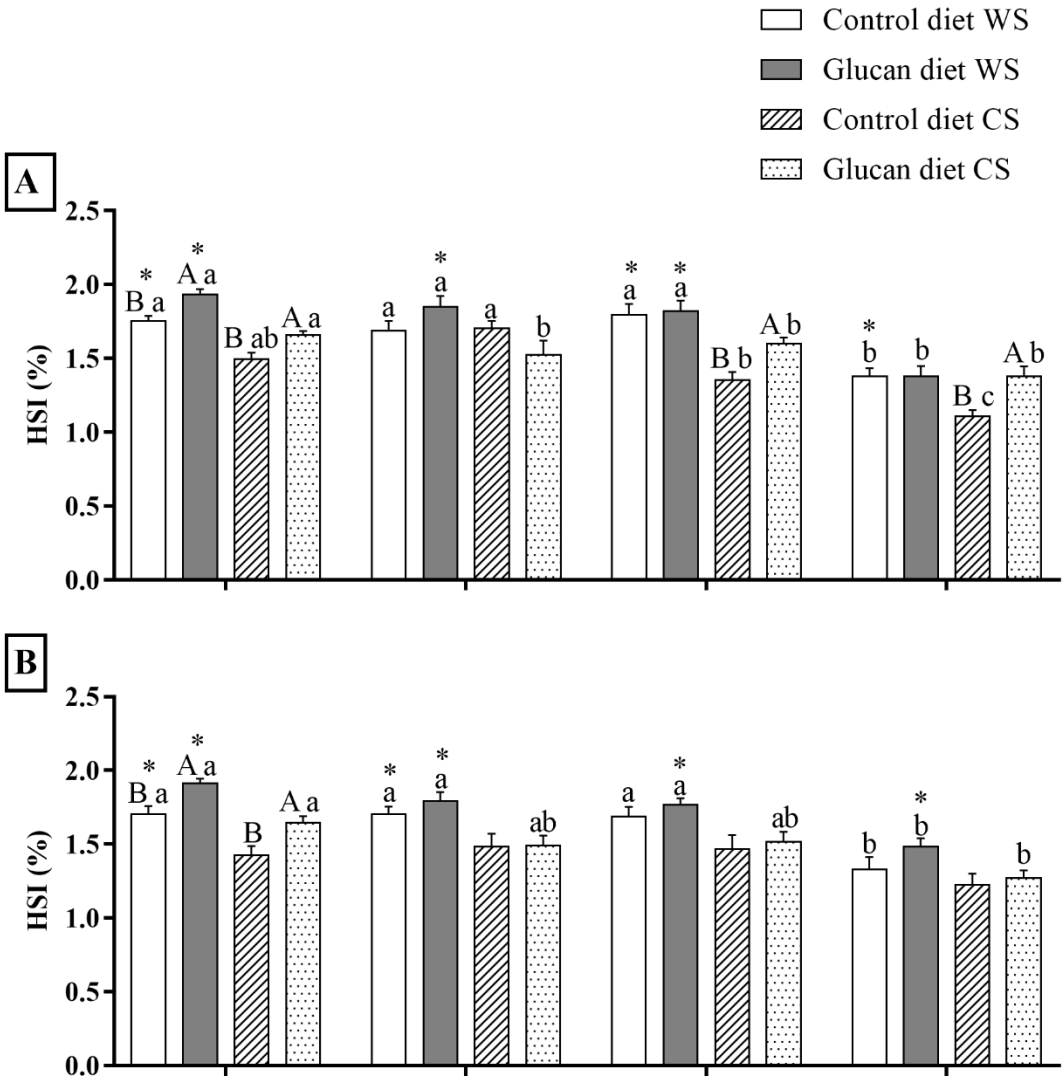

Fig. 14. Hepatosomatic index in not transported (A) or transported (B) pacu fed with control diet and diet containing 0.1% β-glucan, without stress and after chronic stress (basal) and 3, 6 and 24 h post inoculation with heat-killed *Aeromonas hydrophila*. Different capital letters indicate difference between treatments at particular group in same sampling time. Different lower letters indicate differences between sampling times within the same group. Asterisk indicates difference between stress condition (without stress or chronic stress). Balls indicates difference between not transported and transported groups. Symbols were omitted when there was no statistical difference. Values are means ± standard error (n=8).

4. Discussion

This study investigated the effect of dietary β-glucan on stress, innate immunity, and antioxidant system responses of pacu under different stress conditions. Fish were fed with β-glucan fish for 15 days and submitted to 3 conditions: chased daily along the 15-day period (chronic stress), only transported for 4 h after the 15-day period (acute stress) or both chased and transported (chronic plus acute stress), being then challenged by an inoculation of bacterin of *A. hydrophila*. The results indicate a protector effect of glucan on stress response and against damage caused by oxidative stress.

The acute stress (transport) applied highly increased plasma cortisol and glucose concentrations, evidencing the stress induction in fish, confirming the statement of Wendelaar Bonga (2011), that transport of live fish involves capture, handling, high densities and alterations in water quality, which can induce stress response. Chronic stress (chase) and chronic plus acute stress did not affect the cortisol levels. This result may indicate the desensitization of adrenocorticotropic hormone producing cells in the pituitary gland caused by the continuous stressor. This phenomenon is a result of prolonged hyperactivity of the system and include feedback regulation of the HPI axis and cortisol catabolism (Barton *et al*., 1987; Teles *et al*., 2013). Desensitization was observed in fine flounder (*Paralichthys adspersus*) exposed to 7 weeks of crowding (Valenzuela *et al*., 2018) and pacu (*Piaractus mesopotamicus*) exposed to 8 days of handling (Biller *et al*., 2020). During a chronic stressor, the basal levels of cortisol is considered a protective mechanism, preventing an excessive response, which reflects the adaptability of fish to the chronic condition (Pottinger, 1998).

The *A. hydrophila* injection showed to be an additional stressor. Bacterial infection is known to promote high levels of circulating cortisol and the stressful condition imposed by the inoculation in this study, increased the energy expenditure,

which reflected in the decrease in plasma glucose concentration after inoculation in transported fish. The bacterial injection induced increase in plasma cortisol levels independent of dietary treatment. While fish fed with control diet showed more than two-fold of increase, in fish fed with glucan diet the hormone levels increased moderately. We assume that the β-glucan prevented an overshooting of the hormone after bacterial injection as a protective response. A previous study, in which pacu were fed with β-glucan, transported and injected with heat-killed *A. hydrophila*, showed similar effect of glucan (Mello *et al*., 2019). The present results confirm that glucan prevents additional increase in circulating cortisol levels after bacterial challenge in stressed fish, but not described in other fish species yet. In not stressed fish, β-glucan also showed beneficial effect in stress response, since it delayed the increase of plasma cortisol concentrations after immune stimulation. In fish fed with control diet cortisol levels increased at 3 hpi, while in glucan fed fish the increase was observed only at 24 hpi. Together, the results endorse the protective effect of glucan, since high levels or prolonged periods of exposure to cortisol can decrease the responsiveness capacity of body to respond to unfavorable situation.

The stress conditions applied, themselves, did not have a clear impact on innate immune system. We observed effect only in RAL, with reduction in CS fish fed glucan and increase in transported fish independent of diet. The immunomodulatory effect of glucan has been widely observed and it is generally considered to enhance fish health (Meena *et al*., 2013). However, we observed that the combination of chronic stress with glucan administration for 15 days reduced the RAL. Atlantic salmon (*Salmo salar*) injected intraperitoneally with high doses of glucan showed higher mortality than control group when phagocytes were overloaded with glucan particles, what impaired their phagocytosis capacity (Robertson *et al*., 1990). Castro *et al*. (1999), using in vitro techniques, demonstrated that glucan stimulated the respiratory burst of fish leucocytes, but after a period of time, the cells became exhausted and did not respond to the second stimulus. It is probably that the same process happened in our study in fish chased and glucan-fed for 15 days. The relationship between stressor and the immune system in fish has suggested that acute stressors, in general, cause increase in circulating neutrophils (Wendelaar Bonga, 1997), which can explain improvement of RAL in transported fish even at 3 hpi. In rainbow trout (*Oncorhynchus mykiss*) the circulating monocytes/neutrophil

percentage increased in post-transportation period, promoting enhanced phagocytic activity (Volpatti *et al*., 1998). However, after bacterial injection, that demands high energetic input, the immune suppression effect, caused by stress, was evident. The heat-killed *A. hydrophila* injection decreased RAL, primarily in CS not transported fish and following in WS and CS transported fish and HA-AP and lysozyme in all stress conditions. According to Wendelaar Bonga (1997), stress promotes increase of glycemia to meet the increased energy demand. In this study, acute stress increased blood glucose in WS and CS fish. After bacterial injection, plasma glucose decreased and we hypothesized that the consumption of glucose after the injection to energy production was not enough to support the innate immune system activation, causing the immune suppression observed after *A. hydrophila* injection in stressed fish. The impairment on innate immune system of stressed fish after bacterial injection was reported in carp (*Cyprinus carpio*) (Yin *et al*., 1995), pacu (*Piaractus mesopotamicus*) (Zanuzzo *et al*., 2017; Sabioni *et al*., 2020), matrinxã (*Brycon amazonicus*) (Franco-Montoya *et al*., 2017) and Nile tilapia (*Oreochromis niloticus*) (Barros *et al*., 2014). The immune stimulatory properties of glucan were not clear in this study. While glucan reduced lysozyme concentration in WS fish even after bacterial injection, RAL in CS and transported CS fish and HA-AP in WS and transported WS injected fish, the compound anticipated lysozyme increase in WS injected fish and increase of HA-AP in CS injected fish. Other studies did not describe stimulatory effect of glucan in immune system (Aakre *et al*., 1994; Thompson *et al*. 1995; Duncan and Klesius, 1996; Baulny *et al*. 1996; Jeney *et al*., 1997; Sahoo and Mukherjee, 2001; Couso *et al*., 2003). It is known that optimal effects of immune stimulants are dependent of dose, period of administration and fish species. Lysozyme is an important bactericidal molecule of the innate immune system and, in this study, we observed increase in lysozyme concentrations 24 hpi in both diets and stress conditions. Lysozyme breaks the cell wall linkages of gram-positive bacteria, but gram-negative bacteria, as *A. hydrophila*, are not directly destroyed by lysozyme, as it requires the rupture of the external cell wall by complement system (Saurabh and Sahoo, 2008). This fact explains the latter activation of the lysozyme.

Oxidative stress is the result of the oxygen utilization by the cell to energy production, either by body demand or by leucocyte activity increased when the

natural antioxidant mechanisms are inefficient (Aschbacher *et al.,* 2013; Biller and Takahashi, 2018). The transport (acute stress) applied in this study increased the antioxidant system activity, observed by increase of CAT, GSH and GST activities. However, the transport associated with daily chase increased LPO and reduced GSH concentration in glucan-fed fish. Little is known about the relationship between physical stressors and antioxidant status in fish, since the most studies assess the cell redox homeostasis of fish exposed to toxic components (Sinhorin *et al*., 2014; Atli and Canli, 2017; Saddick *et al*., 2017) or environmental changes (Vicente *et al*., 2019; Xavier *et al*., 2020). Acute heat/dissolved oxygen-induced stress in Nile tilapia (*Oreochromis niloticus*) increased SOD and glutathione reductase (GR) activity (Xavier *et al*., 2020) and SOD and CAT activity (Vicente *et al*., 2019). Whereas turbot (*Scophthalmus maximus*) maintained for 120 days at high stocking density the SOD, CAT and GSH activities reduced in liver (Liu *et al*., 2016). Two species of tilapia (*Oreochromis niloticus* and *Tilapia zillii*) exposed to 500 μg L^{-1} of Zinc nanoparticles showed a significant increase in brain GSH, SOD, CAT, GPx, GST and glutathione reductase (GR) activity, although high concentrations (2000 μg L^{-1}) of Zinc nanoparticles induced a decrease of those enzymes (Saddick *et al*., 2017). According to Hao and Chen (2012), studies have confirmed that under certain level of stress, the antioxidant defense system can be remarkably induced and when the time and concentration of exposure increase, they show a tendency of decrease. The *A. hydrophila* injection increased LPO, especially in transported fish. Silver catfish (*Rhamdia quelen*) showed ROS and thiobarbituric acid reactive substances (TBARS) levels increased in liver when experimentally infected by *Aeromonas caviae*, although no difference was found in SOD, GPx, GR and GSh levels (Baldissera *et al*., 2018). African catfish (*Clarias gariepinus*) experimentally inoculated with bacteria *Escherichia coli* or *Vibrio fischeri* had higher levels of LPO, protein carbonylation and GST activity, and the author concluded that bacterial inoculation could result in oxidative stress, mainly on liver, with a higher rate of oxidative stress per mg of tissue when compared to gills and muscle (Adeyemi, 2014). During phagocytosis, leucocytes increase their oxygen consumption, producing reactive oxygen species (ROS), which is a potent oxidant compound and cause lipid peroxidation (Biller and Takahashi, 2018). Since we also observed increase in RAL and decrease in CAT and GSH in those fish, we believe that phagocytosis demand imposed by bacterial injection promoted an increase in ROS

production by leucocytes that the antioxidant system was unable to neutralize, inducing LPO increase. However, transported fish showed increase in GPx after *A. hydrophila* injection, indicating that this enzyme was prioritized to act against LPO increases. Beyond the protective effect on stress response, β-glucan showed to have a protective effect on oxidative stress, since it reduced LPO in WS and transported fish. β-glucan reduced LPO in *Nothobranchius guentheri* (Song *et al*., 2019) and in rats (Suchecka *et al*., 2015). In grass carp (*Ctenopharyngodon idella*), the CAT and SOD activity was higher when fish received glucan by intraperitoneal injection (Kim *et al*., 2009) and were challenged with grass carp hemorrhage virus (GCHV). In this study, the β-glucan also showed stimulatory effect in antioxidant enzymes (GPx and GSH) activity after combination of one stress condition and bacterial injection. However, the *A. hydrophila* injection or the combination of two types of stressors (acute and chronic) reduced GPx and GSH activity in glucan-fed fish.

5. Conclusion

The β-glucan modulates cortisol release in a protector aspect preventing high elevations or delaying the increase of the hormone. Additionally, β-glucan had a protective effect on oxidative stress, reducing lipid peroxidation. Acute, chronic and acute plus chronic stress have immune suppression effect, especially after bacterial injection.

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CAPÍTULO 3

Chronic hypoxia effects on immune responses of Atlantic salmon (*Salmo salar*) injected with formalin-killed *Aeromonas salmonicida*

ABSTRACT

Hypoxia is a common situation in intensive aquaculture, and may lead to negative impact in farmed fish. Chronic exposure to low dissolved oxygen levels is a real condition in aquatic systems and cause impairment on immune responses in fish, making them more susceptible to disease. Knowing the negative effects of chronic hypoxia in farmed fish is important to develop strategies prevent losses on production. To evaluate the effects of chronic hypoxia on immune response of Atlantic salmon (*Salmo salar*), fish were maintained under 40% OD for 6 weeks prior inoculation with formalin-killed *Aeromonas salmonicida* and sampled 6 and 24 hours and 8 weeks post injection. The respiratory burst activity of leucocytes, lysozyme concentration and hemolytic activity of the complement system were determined as innate immune indicators, the blood cells count as hematological parameters, and IgM concentrations as adaptive immune indicator. The expression of immune-related genes [interleukin-1β (*il-1β)*, interleukin-8α (*il-8α)*, cyclooxygenase-2 (*cox-2)*, toll-like receptor 5a (*strl5*), CC chemokine-like 19b (*ccl19b*), serum amyloid A5 (*saa5*), hepcidin anti-microbial peptide a (*hampa*) and cathelicidin anti-microbial peptide b (*campb*)] was also evaluated. We found that 6 weeks of hypoxia caused energy redistribution to immune system and showed an immunosuppressive effect on humoral, cellular and molecular immune response.

KEY-WORDS:

Oxygen, gene expression, immune depression.

1. Introduction

Dissolved oxygen is a critical factor in aquatic environmental (Gallage *et al*., 2016) that limits the optimal growth, health and welfare of fish in intensive aquaculture, including Atlantic salmon (*Salmo salar*), as oxygen is the main factor necessary for fish metabolism and maintenance of homeostasis. Hypoxia is the condition when oxygen concentration is low and cells are deprived of adequate oxygen supply (Hughes, 1973) and can occur in aquaculture systems due to increase of water temperatures, accumulation of organic matter and high stocking density (Rodriguez *et al*., 2016; Gallage *et al*., 2016). In marine sea-cages, which is the primarily culture system for salmon, fish are exposed to a range of environmental conditions that cannot be controlled (Johansson *et al*., 2006, Solstorm *et al*., 2018) and lack of oxygen is one of them, since sea surface temperatures have been higher during the past three decades, increasing the incidence and severity of hypoxia (Levin and Breitburg, 2015).

The negative impact of hypoxia in farmed fish is significant, since they cannot avoid or escape this situation (Gallage *et al*., 2016). When hypoxia is severe enough to alter homeostasis, a stress response is triggered (Rodriguez *et al*. 2016) and the energy is diverted from energy-consuming processes as growth, reproduction or immunity to homeostasis maintenance (Burt *et al*., 2012; Gallage *et al*., 2016). Studies have shown the hypoxia as a stressful factor in aquatic environments, leading to impairment of immune responses. Burt et al. (2012) showed that moderate (~5.0 mg L^{-1}) intermittent hypoxia decreased significantly leucocyte respiratory burst in head kidney of Atlantic salmon. After 24 hours under sublethal hypoxia (1.0 mg DO L^{-1}), Nile tilapia increased the stress response and weakened immune response, reducing their resistance against *Streptococcus agalactiae (*Evans *et al*., 2003)

Many studies have focused in acute, short-term or intermittent hypoxia exposure, but chronic exposure is a condition that most represents the reality of aquatic systems (Gallage *et al*., 2016). Chronic hypoxia reduced or delayed important immune-related gene expression in the Atlantic salmon (Kvamme *et al*. 2013) and decreased the serum bactericidal activity and antibody production against *Vibrio anguillarum* in Nile tilapia (*Oreochromis niloticus*) (Gallage *et al*., 2016).

Understanding the impacts of hypoxia, especially chronically, on salmon immunology will help to predict the salmon response to climate change and prevent production losses. In this study, we evaluated the effects of chronic hypoxia in immunity of Atlantic salmon injected with formalin-killed *A. salmonicida*.

2. Material and Methods

The experimental procedures were approved by the Institutional Animal Care Committee of Memorial University of Newfoundland (Protocol #17-94-KG) and performed in accordance with the guidelines of the Canadian Council on Animal Care.

2.1. Animals and experimental design

Atlantic salmon (*Salmo salar*) were obtained from Cape D'or Sustainable Seafood (Nova Scotia, Canada) and initially held at the Dr. Joe Brown Aquatic Research Building (JBARB; Ocean Sciences Centre, MUN) in a 4,000-L circular tank for 1 month. During this period, fish were fed with a commercial salmonid diet (Optiline MicroBalance, Skretting, St. Andrews, NB, Canada) at a ratio of 1.5% biomass daily (b.w. day^{-1}) and photoperiod was 12 h light:12 h dark. After acclimation, 140 fish were netted, anesthetized in seawater containing tricaine methanesulfonate (AquaLife TMS, 0.1 g L^{-1}; Syndel Laboratories Ltd, Nanaimo, BC, Canada), weighed, and had a PIT (Passive Integrated Transponder) tag implanted in their peritoneal cavity for identification. After tagging, fish (117.4 ± 28.1 g) were randomly distributed in two 3,000-L tanks on Cold-Ocean Deep-Sea Research Facility (CDRF; Ocean Sciences Centre, MUN), with water recirculation to recover for 3 weeks before to start the experiment. After recovery time, a WitroxCTRL Oxygen System (Loligo® Systems) was used to monitor and reduce the dissolved oxygen (DO) levels from one tank (hypoxia treatment) 5% every two days, for two weeks, until they reach 40% DO. For 6 weeks, fish from normoxia tank were kept at 100% DO (normoxia treatment) and fish from hypoxia tank were kept at 40% DO. At the end of 6-weeks period, 8 fish from each tank (8 fish per treatment) were sampled (initial sampling) and the remainders were anesthetized (AquaLife TMS, 0.1 g L^{-1}), injected intraperitoneally with 1µl g^{-1} of formalin-killed *A. salmonicida* or

PBS and returned to the tanks. Four weeks after the inoculation, fish were anesthetized (AquaLife TMS, 0.1 g L^{-1}) to receive a booster (1µl g^{-1} intraperitoneal injection of *A. salmonicida* or PBS). Fish were sampled 6 and 24 hours and 8 weeks after the first injection (n=8). During experimental period, water temperature was 10.3 ± 0.4 °C; photoperiod used was 12 h light: 12 h dark and fish were fed at 1% b.w. day^{-1}.

2.2. Bacteria preparation and inoculation

The *Aeromonas salmonicida* strain was cultured in complex KDM-2 (Gibco, Thermo Fisher Scientific®) for 4 weeks. The bacteria grown were harvested and re-suspended in PBS (pH 7.2, Gibco). The bacterial cell suspension was washed three times with PBS by centrifugation at 4200×*g* for 10 min at 4 °C. *A. salmonicida* cells were inactivated with 6% formaldehyde for 3 days at room temperature with gentle agitation. Formalin was removed by centrifugation at 4200×*g* for 10 min at 4 °C. The bacterial cell pellet was re-suspended in PBS for inoculation. The bacterin cell concentrate was quantified using Bacteria Counting Kit (Invitrogen, Thermo Fisher Scientific®) for flow cytometry based on the manufacturers' instructions.

2.3. Samplings

In each sampling, fish were quickly euthanized in seawater containing tricaine methanesulfonate (TMS, 0.4 g L^{-1}), measured and weighed. The blood was collected by puncture of the caudal vessel using a heparinized (1,000 UI ml^{-1}) or not syringe and head-kidney was removed. The blood collected with non-heparinized syringe was maintained in refrigerator overnight and posteriorly centrifuged for 10 min at 10,000 g to separate serum. The serum was stored on microtubes at -80 °C prior to the determination of IgM concentrations. An aliquot of blood collected with heparinized syringe was immediately used in the leukocyte respiratory activity assay and another aliquot was used for blood cell count through flow cytometry. The remainder blood was centrifuged for 1 min at 10,000 g to separate plasma. The plasma was distributed on different microtubes, immediately frozen in liquid nitrogen and stored at -80 °C prior to the determination of lysozyme concentrations, hemolytic activity of the alternative pathway of complement system. The head-

kidney was frozen in liquid nitrogen and stored at -80 °C until determination of the mRNA expression of immune-related genes: interleukin-1β (*il-1β*), interleukin-8α (*il-8α*), cyclooxygenase-2 (*cox-2*), toll-like receptor 5a (*strl5*), CC chemokine-like 19b (*ccl19b*), serum amyloid A5 (*saa5*), hepcidin anti-microbial peptide a (*hampa*) and cathelicidin anti-microbial peptide b (*campb*).

2.4. Assays

2.4.1. Innate immune indicators

Innate immune parameters were measured on initial sampling and 24 hpi in PBS- and formalin-killed *A. Salmonicida*-injected fish, with goal to evaluate the effect of chronic hypoxia and inoculation in innate immune indicators.

2.4.1.1. Respiratory activity of leucocytes

The production of reactive oxygen species by salmon blood leukocytes was measured using chemiluminescence following the methods described by Marnilla et al. (1995) and Nikoskelainen *et al*. (2004). Briefly, whole blood (2 µl ml^{-1}) was added to a mixture of 1 mM luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, Sigma®, #820071) in 0.2 M sodium borate and PBS. Then, 225 µl of this solution was added to triplicate wells of a 96-well white cliniplate (N° 28298-610, VWR International®), followed by 25 µl of 20 mg ml^{-1} zymosan from *Saccharomyces cerevisiae* (Sigma®, #Z4250). Thereafter, chemiluminescence was measured over 60 min using the microplate reader (SpectraMax M5; Molecular Devices, Sunnydale) at room temperature. During this time, a curve of the luminescence counts per second (LCS) was obtained, and peak values were taken to represent the respiratory burst of each sample.

2.4.1.2. Hemolytic activity of the alternative pathway of complement system

The hemolytic activity of the alternative pathway of complement system was determined according to Zanuzzo *et al*. (2017). A kinetic assay determined the time (in seconds) necessary for the serum sample to lyse 50% of a rabbit erythrocyte suspension, with readings every 20 sec for 20 min, at 700 nm.

2.4.1.3. Lysozyme concentration

The plasma lysozyme concentration was determined according to Demers and Bayne (1997) and Zanuzzo *et al*. (2015) based on lysis of the *Micrococcus lysodeikticus* suspension (Sigma®, M3770). The samples were incubated for 30 min at 40°C to inactivation of complement proteins and addition to *M. lysodeikticus* suspension. The reduction of the absorbance of this solution was measured at 450 nm for 10 min.

2.4.2. Cellular blood counting

The cellular blood counting was performed on initial sampling and 24 hpi in PBS- and formalin-killed *A. Salmonicida*-injected fish, using the flow cytometry. Stock solution of 3,3-dihexyloxacarbocyanine ($DiOC_6$, ThermoFisher Scientific®, D273) was diluted using 1:10 Hanks balanced salt solution (HBSS) and added to whole blood aliquot diluted in deionized water (1:19). The solution was filtered in 100 µM mesh, centrifuged for 5 min at 500 g and the pellet ressuspended in Fluorescence-activated cell sorting (FACs) buffer. The samples were read in flow cytometry (BD FACSCalibur® Flow Cytometer) using the BD FACSDiva software, which identifies each blood cell population by its typical location in a Forward scatter (FSC) vs. Side scatter (SSC).

2.4.3. Gene expression

To evaluate the effect of chronic hypoxia and inoculation in immune-related gene expression, this parameter was measured on initial, 6 and 24 hpi samplings in PBS- and formalin-killed A. Salmonicida-injected fish. Head kidney samples (approximately 100 mg tissue) were homogenized in 400 µL of TRIzol Reagent (Invitrogen/ ThermoFisher Scientific®, #15596-018) using a motorized Kontes RNase-Free Pellet Pestle Grinder (Kimble Chase, Vineland, NJ). An additional 400 µL of TRIzol Reagent (Invitrogen/ ThermoFisher Scientific®, #15596-018) was added, mixed by pipetting, and the homogenates frozen on dry ice and stored at -80°C. Frozen homogenates were further processed by slowly thawing them on ice and then passing them through a QIAshredder (QIAGEN, Mississauga, ON) spin

column following the manufacturer's instructions. Two-hundred µL of TRIzol (Invitrogen/ ThermoFisher Scientific®, #15596-018) was then added to each sample to make a total homogenate volume of approximately 1 mL, and the TRIzol total RNA extractions were then completed following the manufacturer's instructions. Total RNA samples were treated with 6.8 Kunitz units of DNaseI (RNase-Free DNase Set, QIAGEN, #79254) with the manufacturer's buffer (1X final concentration) at room temperature for 10 min to degrade any residual genomic DNA. DNase-treated RNA samples were column-purified using the RNeasy Mini Kit (QIAGEN, #74106) following the manufacturer's instructions. RNA integrity was verified by 1% agarose gel electrophoresis, and RNA purity was assessed by A260/280 and A260/230 NanoDrop UV spectrophotometry for both the pre-cleaned and the column-purified RNA samples. Column-purified RNA samples had A260/280 ratios between 2.0 and 2.3 and A260/230 ratios between 1.9 and 2.3. First-strand cDNA templates for qPCR were synthesized in 20 µL reactions from 1 µg of DNaseI-treated, column-purified total RNA using random primers [250 ng (Invitrogen/Thermo Fisher Scientific®, #48190-011)], dNTPs (0.5 mM final concentration ; Invitrogen/Thermo Fisher Scientific®, #10297-018) and M-MLV reverse transcriptase (200 U; Invitrogen/Thermo Fisher Scientific®, #28025-013) with the manufacturer's first strand buffer (1X final concentration) and DTT (10 mM final concentration) at 37 °C for 50 min.

For the qPCR analysis, we selected 9 genes (cathelicidin anti-microbial peptide b – *campb*, CC chemokine-like 19b – *ccl19b*, cyclooxygenase-2 – *cox2*, hepcidin anti-microbial peptide a – *hampa*, Interleukin-1β – *il1b*, Interleukin-8α – *il8a*, serum amyloid A5 – *saa5* and toll-like receptor 5a – *strl5*), which primers were designed using Primer3 software (http://frodo.wi.mit.edu/primer3), and were based on cDNA sequences for the genes of interest (GOI) and the normalizer [(60S ribosomal protein L32 (*rpl32*) and polyadenylate-binding protein 1 (*polyapb*)] that were available for Atlantic salmon in GenBank (see **Table 1** for accession numbers). Each primer set for each gene was tested for quality before use (Caballero-Solares *et al*., 2017). All PCR amplifications were performed in 13 µL reactions using 1X Power SYBR Green PCR Master Mix (Applied Biosystems/ ThermoFisher Scientific®, #A25776), 50 nM of both the forward and reverse primers, and cDNA representing 4 ng of input total RNA. Amplifications were performed using the ViiA 7 Real Time

PCR system (384-well format) (Applied Biosystems/Thermo Fisher Scientific®). The real-time analysis program consisted of 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min and 40 cycles of 95°C for 15 sec and 60°C for 1 min, with fluorescence detection at the end of each 60°C step, and was followed by dissociation curve analysis. .

Transcript expression levels of the genes of interest (GOI) were normalized to 60S ribosomal protein L32 (*rpl32*) and polyadenylate-binding protein 1 (*polyapb*) transcript levels. These genes were chosen as the endogenous control (i.e. normalizer gene) due to its relatively stable expression in this study. These endogenous controls were selected from six candidate normalizers [60S ribosomal protein L32 (BT043656), β-actin (BG933897), elongation factor 1-alpha 1 (AF321836), elongation factor 1-alpha 2 (BT058669), eukaryotic translation initiation factor 3 subunit D (GE777139) and polyadenylate-binding protein 1 (EG908498)]. Normalizer testing was performed separately 6 hpi and 24 hpi samplings. At 6 hpi, the fluorescence threshold cycle (C_T) values of 16 samples from the control group [PBS-normoxia (n=8), PBS-hypoxia (n=8)] were measured; at 24 hpi, the C_T values of 32 samples from each of the four groups [PBS-normoxia (n=8), PBS-hypoxia (n=8), *A. sal*-normoxia (n=8), *A. sal*-hypoxia (n=8)] were measured. They were analyzed separately using geNorm (Vandesompele 2002). The qPCR analyses of expression levels of the 8 GOIs in the 80 head kidney samples were then performed using the ViiA 7 Real Time PCR system (384-well format) (Applied Biosystems by ThermoFisher Scientific®). On each plate, for every sample, the GOIs and endogenous controls were tested in triplicate, and a plate linker sample (i.e. a sample that was run on all plates in a given study) and a no-template control were included. The relative quantity (RQ) of each transcript was determined using the ViiA 7 Software Relative Quantification Study Application (Version 1.2.3) (Applied Biosystems/Life Technologies®), with normalization to both *rpl32* and *polyapb* and amplification efficiencies incorporated. For each GOI, the sample with the lowest normalized expression (mRNA) level was set as the calibrator sample (i.e. assigned an RQ value = 1).

Table 1. Primers used in the quantitative reverse transcription-polymerase chain reaction analyses.

Gene of interest	Primer name	Nucleotide sequence (5' → 3')	Efficiency (%)	Amplicon size (bp)	GenBank acc. no.	References
polyadenylate-binding protein 1 (normalizer)	*polyabp* fwd	TGACCGTCTCGGGTTTTTAG	102	108	EG908498	Caballero-Soares *et al.*, 2017
	polyabp rev	CCAAGGTGGATGAAGCTGTT				
60S ribosomal protein L32 (normalizer)	*rpl32* fwd	AGGCGGTTTAAGGGTCAGAT	99.6	119	BT043656	Eslamloo *et al.*, 2019
	rpl32 rev	TCGAGCTCCTTGATGTTGTG				
cathelicidin anti-microbial peptide b	*campb* fwd	AGACTGGCAACACCCTCAAC	101.8	112	AY360357	Eslamloo *et al.*, 2019
	campb rev	TTGCCTCTTCTTGTCCGAAT				
CC chemokine-like 19b	*ccl19b* fwd	CTGCTTGACAACGACCGATA	90.1	151	BT058161	Caballero-Soares *et al.*, 2017
	ccl19b rev	GTTGTTCTTGGTGGCAGGAG				
cyclooxygenase-2	*cox2* fwd	ACCTTTGTGCGAAACGCTAT	104.7	113	AY848944	Caballero-Soares *et al.*, 2017
	cox2 rev	GAGTAGGCCTCCCAGCTCTT				
hepcidin anti-microbial peptide a	*hampa* fwd	ATGAATCTGCCGATGCATTTC	96.3	134	BT125319	Eslamloo *et al*., 2019
	hampa rev	AATGGCTTTAGTGCTGGCAG				
Interleukin-1β	*il1b* fwd	GTATCCCATCACCCCATCAC	97.3	119	AY617117	Soto-Dávila *et al.*, 2020
	il1b rev	TTGAGCAGGTCCTTGTCCTT				
Interleukin-8α	*il8a* fwd	GAAAGCAGACGAATTGGTAGAC	103.1	99	BT046706	Soto-Dávila *et al.*, 2020
	il8a rev	GCTGTTGCTCAGAGTTGCAAT				
serum amyloid A5	*saa5* fwd	AGGAGCTGGAAGTTTGTTGC	100.6	143	BT057477	Unpublished
	saa5 rev	TATGCACGCCACATGTCTTT				
toll-like receptor 5a (soluble)	*stlr5a* fwd	ATCGCCCTGCAGATTTTATG	104.8	103	AY628755	Smith *et al.*, 2018
	stlr5a rev	GAGCCCTCAGCGAGTTAAAG				

2.4.4. IgM assay

The salmon IgM purification was performed according to Vasquez *et al.* (2020) with minor modifications. IgM was purified from pooled serum (~100 mL) collected from ~2 kg salmon, using an immobilized mannan binding protein (MBP) column kit (Pierce™ 44897, Thermo Scientific, USA) according to the manufacturer's instructions. The integrity and purity of IgM in the elution fractions were evaluated by 10% SDS-PAGE (Sambrook and Russell, 2001) against a marker (PageRuler Plus Prestained Protein Ladder, Thermo Scientific) followed by Coomassie staining,

while the concentration was estimated with DirectUV (absorbance at 280 nm) using a Genova-Nano spectrophotometer (Jenway, UK) and with a bicinchoninic acid (BCA) assay (BCA protein assay kit, Pierce™, Thermo Scientific) using a plate reader (SpectraMax M5). Highly quality IgM fractions were pooled and dialyzed twice against 20 mM Tris buffer (pH 8.0) at 4°C with gentle stirring using a dialysis cassette or dialysis bag (3-20 kDa molecular weight cut-off, Thermo Scientific). Subsequently, the purified salmon IgM was lyophilized (EdwarDS-Super Modulyo, Boc Ltd, UK), resuspended in 20 mM Tris buffer (pH 8.0), and re-evaluated for integrity and concentration using 10% SDS-PAGE (**Erro! Fonte de referência não encontrada.**A) and DirectUV. Finally, salmon IgM was stored at -20°C in aliquots to protect against repeated freezing-thawing cycles. The production of chicken anti-salmon-IgM IgY was done commercially at Somru BioScience Inc. (Charlottetown, PEI, Canada), and involved affinity purification of the antibodies from eggs using the antigen, followed by biotinylation of the antibodies. We measured total IgM titres using dELISA according to Lin (2015) and Vasquez *et al*. (2020) with some modifications. Serum samples were initially diluted 100,000x in carbonate-bicarbonate coating buffer (15 mM Na_2CO_3; 35 mM $NaHCO_3$; pH 9.8), and heat-treated at 45ºC for 30 min to inactivate the complement. Then, 100 µL of each sample was pipetted into triplicate wells in high protein-binding polystyrene 96-well plates (Nunc MaxiSorp™, Thermo Fisher Scientific). For each plate, a pool of samples was diluted further 30x in carbonate-bicarbonate coating buffer, and then added into duplicate wells (final dilution of 3,000,000x). The optical density (OD) of the pool was used to account for the non-specific background signal, and this value was deduced from each sample OD. Each plate included a standard curve with purified salmon IgM diluted in coating buffer to 3, 1.5, 0.75, 0.375, 0.1875, 0.0937 and 0.04687 µg mL^{-1}, and a negative control with only coating buffer, in duplicate wells. The plates were incubated at 4°C overnight and washed 4 times with 200 µL of 0.1% v/v Tween 20 in PBS (PBS-T 0.1%) using a microplate washer (BioTek™ 50TS, Thermo Fisher Scientific) on the next day. Subsequently, plates were blocked by adding 150 µL of ChonBlock™ (Chondrex Inc. 9068, WA, USA) in each well for 1 h at 37°C, followed by washing according to the procedure described previously. Then, plates were incubated with 100 µL of chicken anti-salmon-IgM IgY per well (diluted 1,000x in PBS-T 0.05%) for 1 h at 37°C. After washing, 100 µL of

streptavidin-horseradish peroxidase (BioLegend 405210, CA, USA; diluted 500x in PBS-T 0.05%) was added to each well, and plates were incubated for 1 h at 37°C. Following washing, plates were incubated with 100 μL of TMB solution (Kementec Solutions Inc. 4850, NH, USA) per well for 30 min at RT in the dark. The color/reaction development was stopped adding 100 μL of 0.3 M H_2SO_4. The bottom of the plates was wiped and the plate read at 450 nm. Based on the standards OD, the samples were calculated using a 4-parameter sigmoidal regression by the SoftMax Pro.

2.5. Statistical analysis

The data were submitted to normality (Shapiro-Wilk) and homoscedasticity (Brown-Forsythe) tests and were transformed prior to statistical analysis if they failed in those tests. Data of cellular blood count was transformed in square root of arcsine since represent a proportion of total cells. We used a 2 (hypoxia and normoxia) x 2 (PBS and *A. salmonicida*) x 2 (initial and 24 hpi) factorial to innate immune response and cellular blood count, 2 (hypoxia and normoxia) x 2 (PBS and *A. salmonicida*) x 2 (initial and 8 wpi) factorial to adaptive immune response and 2 (hypoxia and normoxia) x 2 (PBS and *A. salmonicida*) x 3 (initial, 6 and 24 hpi) factorial to gene expression, followed by Duncan's post-hoc tests. To compare the gene expression at each treatment and inoculation group in each sampling point with initial values, t-tests was used. The statistical analysis was made on SAS software (version 9.0) considering P value < 0.05 as the level of statistical significance.

3. Results

In this study, we evaluated immune responses in Atlantic salmon exposed to 6 weeks of hypoxia (40% DO) followed by inoculation with formalin-killed *A. salmonicida*. Fish under hypoxia condition tended to be slower, mouthing and going to the surface to breath with gill ventilation increased. Two fish from hypoxia treatment died after injection with formalin-killed *A. salmonicida*, without external disease signals.

3.1. Innate immune response

At initial sampling, fish under hypoxia showed higher RAL than those under normoxia (p=0.0158, B) (Fig. 1 A). Under normoxia, the respiratory activity of leucocytes increased 24 hours after inoculation, either with PBS or bacterin injection (p=0.0048, b). Under hypoxia, the RAL decreased 24 hpi (p=0.0483, a) in fish injected with *A. salmonicida*.

No statistical differences were found in hemolytic activity of complement system (Fig. 1 B).

The lysozyme concentration was higher in fish under hypoxia at initial point (p=0.0403, B) and in fish PBS-inoculated at 24 hpi compared to those under normoxia PBS-inoculated (p=0.0042, B) (Fig. 1 C). At 24 hpi, fish under hypoxia PBS-inoculated showed higher lysozyme concentration than those *A. salmonicida*-injected at the same oxygen condition (p=0.0179, *).

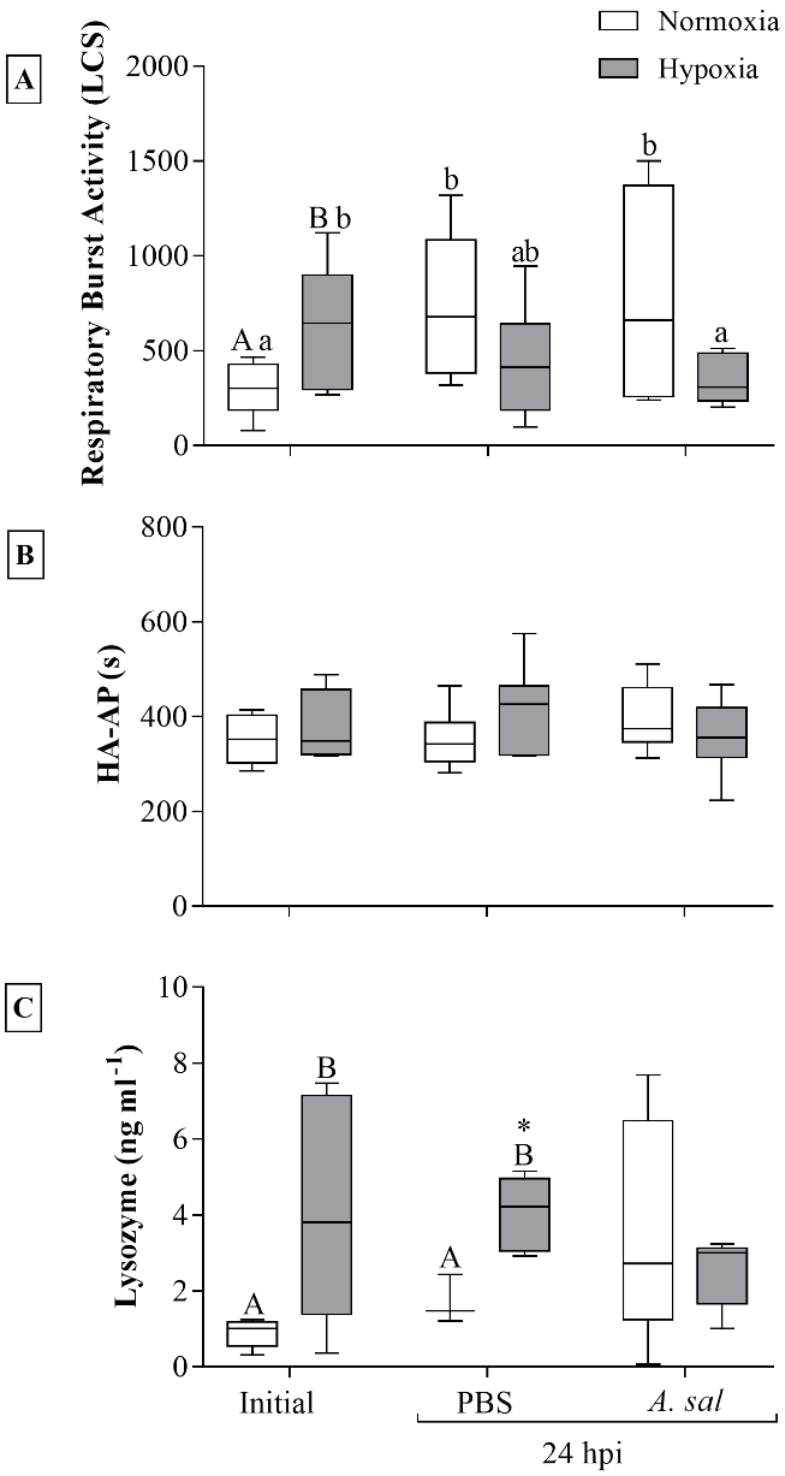

Fig. 1. Respiratory activity of leucocytes (A), hemolytic activity of the alternative pathway of the complement system (B) and plasma lysozyme concentration (C) of Atlantic salmon after 6 weeks under hypoxia and 24 hours post inoculation with PBS or formalin-killed *A. salmonicida*. Different capital letters indicate difference between treatments at same sampling time. Different lower letters indicate differences between sampling times within the same treatment. Asterisk indicates difference between inoculation groups (PBS and *A. salmonicida*). Symbols were omitted when there was no statistical difference Values are means ± standard error (n=8).

3.2. Cellular blood counting

The percentage of erythrocytes increased 24 hpi in fish from both levels of oxygen (normoxia – p=0.0246; hypoxia – p<0.0001; a) independent of inoculation group (Fig. 2 A). After inoculation, fish under hypoxia showed more circulating erythrocytes than those under normoxia (PBS – p=0.0359; *A. salmonicida* – p=0.0003, A). In both conditions, percentage of erythrocytes was higher in fish inoculated with bacterin than in those PBS-injected (normoxia – p=0.0116; hypoxia – p=0.0286; *). The circulating immature erythrocytes increased 24 hpi in fish under hypoxia independent of inoculation group (p=0.0051, a) (Fig. 2 B).

The percentage of circulating monocytes and granulocytes decreased after inoculation in fish under hypoxia (p=0.0337, b) (Fig. 2 C). In PBS-injected group, fish under normoxia showed more circulating monocytes and granulocytes than those under hypoxia, at 24 hpi (p=0.0125, A). Although not statistically different, the percentage of monocytes and granulocytes in initial sampling was 58% higher in fish under hypoxia.

The circulating lymphocytes decreased in both treatments 24 hpi (normoxia – p=0.0007, hypoxia – p<0.0001; b) for both inoculation group (Fig. 2 D). Independent of inoculation group, fish under normoxia had higher percentage of lymphocytes than those under hypoxia, at 24 hpi (PBS – p=0.0436; *A. salmonicida* – p=0.0012, A). Among fish under normoxia, those injected with PBS showed more circulating lymphocytes than those injected with *A. salmonicida* (p=0.0057, *).

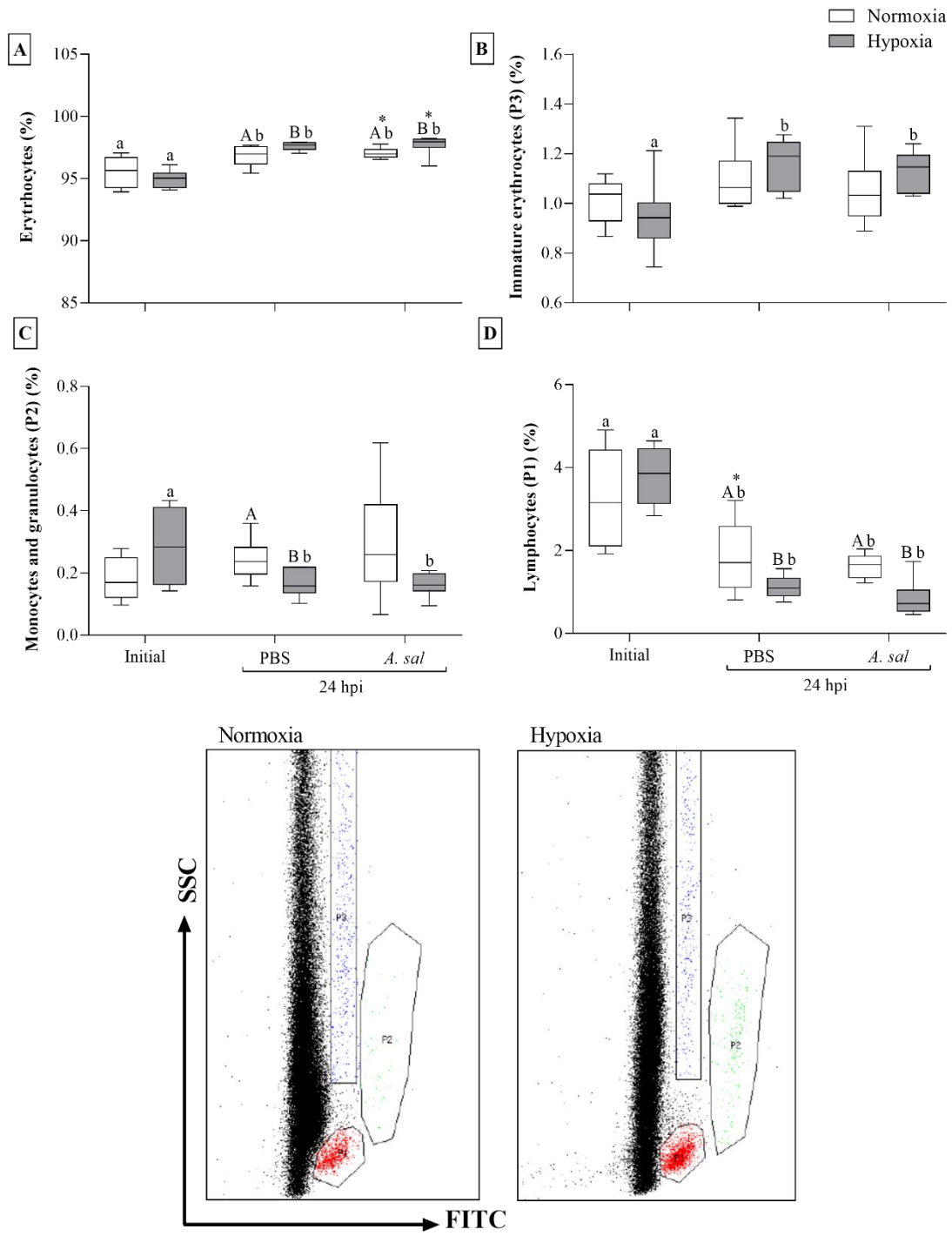

Fig. 2. Percentage of erythrocytes (A), immature erythrocytes (B), monocytes and granulocytes (C) and lymphocytes (D) of Atlantic salmon after 6 weeks under hypoxia or normoxia and 24 hours post inoculation with PBS or formalin-killed *A. salmonicida*. Different capital letters indicate difference between treatments at same sampling time. Different lower letters indicate differences between sampling times within the same treatment. Asterisk indicates difference between inoculation groups (PBS and *A. salmonicida*). Symbols were omitted when there was no statistical difference Values are means ± standard error (n=8). The image (E) represents different populations of leucocytes obtained through flow cytometry on normoxia and hypoxia samples.

3.3. Gene expression

At initial sampling, the hypoxia up-regulated the mRNA expression of *il-8α* (p=0.0004, B), *strl5* (p=0.0002, B), *ccl19b* (p=0.0060, B), *hampa* (p=0.0017, B) and *campb* (p=0.0018, B) (Fig. 3 B, D, E, G and H, respectively).

The *A. salmonicida* injection up-regulated the expression of the all analyzed immune-related genes independent of oxygen condition. At 6 hpi, the expression of *il-1β* (normoxia – p<0.0001; hypoxia – p<0.0001, ↑), *il-8α* (normoxia – p<0.0001; hypoxia – p=0.0005, ↑), *cox-2* (normoxia – p<0.0001; hypoxia – p<0.0001, ↑), *strl5* (normoxia – p=0.0008, ↑), *saa5* (normoxia – p=0.0124, ↑) and *campb* (normoxia – p=0.0127; hypoxia – p=0.0268, ↑) was up-regulated, and at 24 hpi all genes (*il-1β:* normoxia – p<0.0001; hypoxia – p<0.0001; *il-8α:* normoxia – p=0.0036, *cox-2:* normoxia – p=0.0084; hypoxia – p=0.0008, *strl5:* normoxia – p<0.0001, hypoxia – p=0.0007, *ccl19b:* normoxia – p=0.0069; hypoxia – p=0.0002, *saa5:* normoxia – p<0.0001; hypoxia – p<0.0001, *hampa:* normoxia – p=0.0004; hypoxia – p<0.0001, and *campb:* normoxia – p<0.0001; hypoxia – p<0.0001, ↑) were up-regulated in *A. salmonicida*-injected fish (Fig. 3)

Among fish injected with *A. salmonicida,* the mRNA expression of *il-1β* (p=0.0461, B), *il-8α* (p=0.0096, B) and *saa5* (p=0.0285, B) was down-regulated by hypoxia at 6 hpi (Fig. 3 A, B e F, respectively). At 24 hpi, hypoxia down-regulated the expression of *strl5* (p=0.0217, B), *saa5* (p=0.0495, B) and *campb* (p=0.0422, B) (Fig. 3 D, F and H, respectively). Among fish injected with PBS, hypoxia up-regulated the *cox-2* expression (p=0.0252, B) at 6 hpi and the expression of *il-1β* (p=0.0264, B), *cox-2* (p=0.0038, B) and *strl5* (p=0.0340, B) at 24 hpi (Fig. 3 A, C and D, respectively).

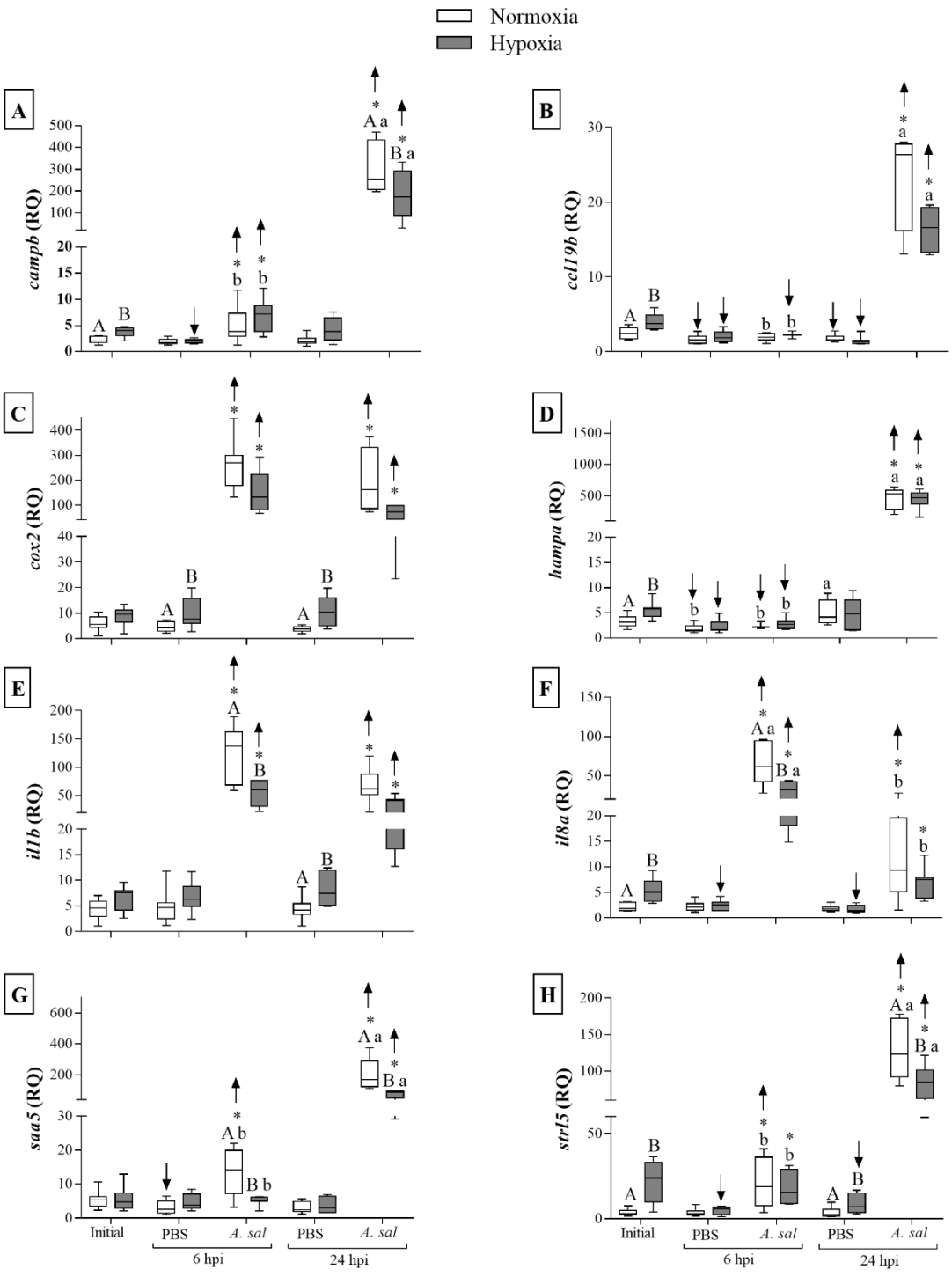

Fig. 3. mRNA expression of 8 antibacterial biomarkers genes of Atlantic salmon after 6 weeks under hypoxia or normoxia and 6 and 24 hours post inoculation with PBS or formalin-killed *A. salmonicida*. Different capital letters indicate difference between treatments at same sampling time. Different lower letters indicate differences between sampling times within the same treatment. Asterisk indicates difference between inoculation groups (PBS and *A. salmonicida*). Arrows indicates difference between initial sampling and each inoculation group at each sampling time. Symbols were omitted when there was no statistical difference. Values are means ± standard error (n=8).

3.4. Adaptive immune response

Fish injected with PBS under normoxia showed higher IgM concentrations than those under hypoxia (p=0.0262, A) at 8 wpi (Fig. 4).

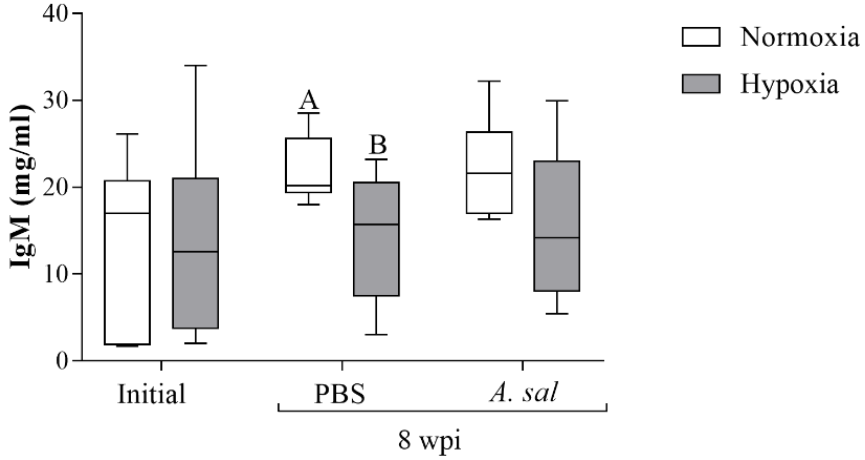

Fig. 4. IgM concentration of Atlantic salmon after 6 weeks under hypoxia or normoxia and 8 weeks post inoculation with PBS or formalin-killed *A. salmonicida*. Different capital letters indicate difference between treatments at same sampling time. Values are means ± standard error (n=8).

4. Discussion

The present study evaluated chronic hypoxia impact in Atlantic salmon immune system. Following 6 weeks of 40% D.O. fish were subjected to in vivo immune stimulation with formalin-killed *Aeromonas salmonicida* injection, mimicking bacterial infection. After chronic hypoxia and immune stimulation, fish showed changes in important immune parameters, demonstrating that are unable to respond effectively and may affect susceptibility to infection in salmon.

The immune system is a highly complex defense mechanism that uses a wide range of individual systems. Because of this complexity, the immune system is often divided into innate/adaptive immunity or nonspecific/specific immunity (Tort, 2011; Bowden, 2008). To evaluate the effects of hypoxia on the immune response of Atlantic salmon, we measured innate and adaptive immune parameters besides immune-related gene expression and blood count cells.

While in the terrestrial environment, the oxygen is rarely limiting, in aquatic environments can become a limiting factor due to a wide variety of factors (Kvamme *et al*., 2013; Magnoni *et al*., 2019) and is especially important because it is closely related to disease outbreaks and mortality increase (Gallage *et al*., 2016). In this

3370 study, fish under hypoxia showed behavioral changes typically related to lack of oxygen as slow swimming, mouth, surface breath and increase of gill ventilation. After injection with formalin-killed *A. salmonicida*, two fish from hypoxia treatment died, without external disease signals. Six weeks of hypoxia is a long-term adverse condition and bacterin inoculation could exhausted the mechanisms to deal with the lack of oxygen to maintain homeostasis, causing death.

Some studies showed that hypoxia may prejudice the immune system (Boleza *et al*., 2001; Ortuño *et al*. 2002; Welker *et al*., 2007; Burt *et al*., 2012; Kvamme *et al*., 2013, Singh *et al*., 2015). The innate or nonspecific immune functions provide the first line of defense due react at short time scale to non-specific antigens (Tort, 2011). The leucocytes activity is a central element in *in vivo* and *in vitro* assays elucidating innate immune mechanisms in teleosts following stimulation. One hour of *in vitro* hypoxia (0.82 mg L^{-1} for 3 min) affected the response of anterior kidney cells of mummichog (*Fundulus heteroclitus*), reducing the production of reactive oxygen species (Boleza *et al*., 2001). In a gilthead seabream (*Sparus aurata*) study, the hypoxia induced by air exposure of 2 min depressed the respiratory burst activity of head-kidney leucocytes (Ortuño *et al*. 2002). In Atlantic salmon (*S. salar*), Burt *et al*. (2012) showed that intermittent hypoxia (3.5 times day^{-1} with a mean duration of 69 min) decreased head-kidney leucocyte respiratory burst, while Zanuzzo *et al*. (2020) showed high temperature (20°C) significantly increased RAL and the combination of this with moderate hypoxia further increased this response.

In this study, salmon under 6 weeks of 40% D.O. showed increase in respiratory burst activity. Since the RAL assays was measured in blood, the higher leucocyte activity observed in the hypoxia group is due to the greater amount of circulating leucocytes in the initial sampling. However, 24 hrs after immune stimulation with formalin-killed *A. salmonicida*, RAL decreased in fish under hypoxia, while increased in fish under normoxia. Again, a direct relationship was found between the RAL and circulating leucocytes count, especially granulocytes and monocytes. We hypnotized that decrease in circulating leucocytes and consequently RAL was due reduction on leucocytes production/activation not due to migration of white blood cells to the inflammation site. Primarily because the reduction was observed in the two inoculation groups (i.e., PBS and *A. salmonicida*) and then because the results of immune-related gene expression in head-kidney, as interleukins, corroborate the

fact that fish under hypoxia decreased the leucocyte production/activation after *A. salmonicida* inoculation. Interleukins are proteins produced mainly by leucocytes with different functions, most of which involved in immune system modulation (Tort, 2011). Moreover, oxygen consumption is necessary in innate immune system, to maintain the NADPH oxidase level in phagocytes and activate biochemical reactions that generate reactive oxygen species (ROS) (Boleza *et al*., 2001)

In this study, we did not observe differences in hemolytic activity of the complement system, which are consistent with previously studies with acute and chronic hypoxia. Ortuño *et al*. (2002) did not found change in alternative complement activity after hypoxia induced by air exposure of 2 min in gilthead seabream (*Sparus aurata*) and Magnoni *et al*. (2019) did not observed alternative complement activity alterations in rainbow trout (*Oncorhynchus mykiss*) maintained under hypoxia (4.5 mg O_2 L^{-1}).

Lysozyme concentration can used as indicator of innate immune response (Tort, 2011), since is rapidly induced in fish exposed to stressors. Interestingly, in this study, as the RAL results, salmon under chronic hypoxia showed higher concentrations of lysozyme than those under normoxia. The expression of important immune-related genes was also up-regulated after chronic hypoxia, demonstrating that the immune response was prioritized in face of a severe chronic stressor. However, lysozyme concentration decreased after bacterin injection. Those results assume that after long-term stressful condition, salmon prioritized the energy to immune function, but in extra energy demand, as infection, the response was depleted. Admittedly, if fish's energy budget is redirected to maintain homeostasis in face of stressor, a higher energy demand to deal with a pathogen may not be able (Douxfils *et al*. 2017). Ni *et al*. (2014) subjected amur sturgeon (*Acipenser schrenckii*) to acute hypoxia (5 mg L^{-1} and 3 mg L^{-1}) and observed significant increase in total protein levels 6 hrs after hypoxia exposure. The authors speculated that fish increase specific proteins such as lysozyme to enhance the immunity level to cope with stress. In study with Nile tilapia (*Oreochromis niloticus*), Abdel-Tawwab *et al*. (2015) evaluated the effect of low (0.1-1.5 mg L^{-1}), medium (3.0-3.5 mg L^{-1}) and high (6.5-7.0 mg L^{-1}) D.O. levels for 12 weeks on the innate immunity after injection with *A. hydrophila*. They found that RAL and lysozyme activity reduces as the D.O. level decreased. Singh *et al*. (2015) exposed *Catla catla* to six different

levels of D.O (1, 3, 5, 7, 9 and 11 mg L^{-1}) and observed reduction on serum lysozyme and RAL in fish challenged at 1 and 3 mg L^{-1} for 1 or 48 hrs. The results indicates impaired immune system of *C. catla* exposed at these treatments.

The number of erythrocytes is an important parameter associated with the oxygen carrying capacity of the blood. The increase in erythrocytes count is a typical response to lack of oxygen, since may help to acquire more oxygen and optimize oxygen transport capacity to the tissues. The present study, we observed increase in circulating erythrocytes in fish under hypoxia after injection with PBS or *A. salmonicida*. Salmon formalin-killed *A. salmonicida*-injected showed higher count of erythrocytes than those PBS-injected and we hypothesized that is due improved energy demand to oppose the pathogen. Fish after 6 weeks under hypoxia showed increase on white blood cells as monocytes and granulocytes, which can explain enhance on RAL as discussed before. However, after injection, independently of the group, the number of circulating leucocytes decrease mainly on hypoxia treatment. Others studies showed the same change profile in blood cells after hypoxia. Affonso *et al*. (2002) and Ni *et al*. (2014) observed red blood cells elevated significantly after hypoxia stress in tambaqui (*Colossoma macropomum) and* amur sturgeon (*A. schrenckii*). Valenzuela *et al*. (2005), Choi *et al*. (2007) and Zanuzzo *et al.* (2020) also showed that hypoxia promote increase in the number of leukocytes in raibow trout (*O. mykiss*), Nile tilapia (*O. niloticus*) and Atlantic salmon (*S. salar*).

To address the hypoxia effect on immune system at molecular level, we measured the expression of immune-related genes in head kidney, as cathelicidin anti-microbial peptide b (*campb)*, CC chemokine-like 19b (*ccl19b)*, cyclooxygenase-2 (*cox2)*, hepcidin anti-microbial peptide a (*hampa)*, Interleukin-1β (*il1b)*, Interleukin-8α (*il8a)*, serum amyloid A5 (*saa5)* and toll-like receptor 5a (*strl5).* The gene expression results confirm the safe status of immune response after 6 weeks under hypoxia, as discussed before on humoral and cellular innate immune parameters. The up-regulated transcripts observed after salmon exposed to chronic hypoxia are related to a number of important aspects of immune response, including antibacterial activities (*campb*), lymphocytes recruitment and proliferation (*ccl19b)*, inflammatory process (*il8a)*, bacterial recognition (*strl5*) or are antimicrobial peptide (*hampa)*. The formalin-killed *A. salmonicida* injection up-regulated the transcript of all genes, indicating that inoculation was able to stimulate the immune response in

fish. However, after bacterin injection, the immune-related genes tested were down-regulated in hypoxia treatment, 6 or/and 24 hpi. The inhibitory effects modulated by immune-related genes were further detected in peripheral blood cells, RAL and lysozyme concentration in formalin-killed *A. salmonicida*-injected salmon under hypoxia. Wang *et al*. (2018) investigated the effect of intermittent hypoxia under different temperatures on the immunomodulation in vaccinated with *Streptococcus agalactiae* Nile tilapia (*O. niloticus*) and showed that IL-1β, TNF-α and IFN-γ mRNA levels were strongly down-regulated in hypoxic fish independently of temperature. IL-1β and others immune-related genes also was down-regulated in Atlantic salmon (*S. salar*) subjected to 58 days under hypoxia and injected with poly I:C or water-based *Vibrio anguillarum* vaccine (Kvamme *et al*., 2013). The authors suggested that long-term hypoxia either reduces or delays the expression of immune-related genes in head kidney.

The influence of environmental changes in immune response can be dubious (Bowden, 2008; Tort, 2011). Usually acute stressors have a greater effect on the innate immune system, whereas chronic stressors involve mainly adaptive immune response. In a chronic hypoxia study, Cecchini and Saroglia (2002) reported that specific antibody titer reduced on European sea bass (*Dicentrarchus labrax*) under lower D.O. and injected intraperitoneally with human-γ-globulin when compared with hyperoxia group. Welker *et al*. (2007) investigated the effect of 2 hours of sublethal D.O. (2 mg L^{-1}) exposure and challenged with a high or low dose of *Edwardsiella ictaluri* on immune responses in juvenile channel catfish (*Ictalurus puncatus*). The authors found that antibody response to bacteria was significantly reduced in fish exposed to hypoxia and, when challenged with the high dose of *E. ictaluri*, fish exposed to hypoxic conditions had significantly higher cumulative mortality. In this study, since we injected pure/intact bacteria (bacterin) and not a vaccine, we did not expected an increase in IgM concentration after 8 weeks of formalin-killed *A. salmonicida* injection. However, 6 weeks of hypoxia affect the total IgM concentration, showing that chronic hypoxia may depress not only innate, but also adaptive immunity, since reduced IgM production in fish PBS-injected.

Overall, collectively, these data suggest that the low energy available after long-term hypoxia was directed towards the maintenance of humoral, cellular and molecular immune components. However, in an extra energy demand, fish was not

able to keep the immune system components activity. The reduction in RAL, lysozyme concentration, circulating leucocytes count and immune-related gene expression after immune stimulation in hypoxia fish is an indicator of poor innate system in that treatment and may have resulted from the energetic demand associated with immune and long-term stress responses.

5. Conclusion

After chronic hypoxia, the energy produced on salmon was directed to maintenance of immune response. However, post-injection with formalin-killed *A. salmonicida*, the immune system was not able to respond efficiently, including RAL, lysozyme and IgM reduction and immune-related genes down-regulation.

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CAPÍTULO 4

Chronic hypoxia effects on metabolic and stress responses of Atlantic salmon (*Salmo salar*)

ABSTRACT

Oxygen limitation is a common situation in intensive aquaculture context, which can alter fish homeostasis. Depletion of aerobic energy production during chronic hypoxia makes rapid adaptive changes in metabolic organization necessary. Knowledge about the negative effects of severe chronic hypoxia in farmed fish is important to develop strategies to prevent losses in production, since hypoxia can compromise the growth performance. To evaluate the effects of chronic hypoxia on metabolic and stress responses of Atlantic salmon (*Salmo salar*), fish were maintained under 40% OD for 6 weeks. We measured plasma cortisol as stress biomarker, catalase (CAT) and superoxide dismutase (SOD) activity as antioxidant defense biomarkers, and plasma lactate, and expression of genes [cold-inducible RNA-binding protein (*cirbp*), DNA-methiltransferase-I (*dnmt1*), growth hormone receptor 1 (*ghr1*), hypoxia inducible factor-α (*hif1a*), heat shock proteins 47 and 70 (*hsp47* and *hsp70*), inducible growth factor-I (*igf1*), lactate dehydrogenase (*ldhaa*), phosphofructokinase (*pfk1*), peroxisome proliferator-activated receptor beta 1β (*pparb1b*), peroxiredoxin-6 (*prdx6*) and protein kinase AMP-activated catalytic subunit alpha 2 (*prkaa2a*)] as metabolic biomarkers, and growth performance. Long-term hypoxia lead to low cortisol and lactate concentrations in plasma and impairment of growth. Salmon can adjust some metabolic process for the new environmental condition, reducing their tissue metabolism and antioxidant activity.

KEY-WORDS:

Oxygen, gene expression, metabolism

1. Introduction

In recent years, the increase of water temperatures, accumulation of organic matter, and the use of high stocking density have caused a lack of oxygen (Robertson *et al*., 2014; Rodríguez *et al*., 2016; Gallage *et al*., 2016) in aquaculture systems. The oxygen is the main factor necessary for the maintenance of fish metabolism, since is used in cellular respiration. Hypoxia is the condition when oxygen concentration is low and cells are deprived of adequate gas supply (Hughes, 1973). Dissolved oxygen is a critical factor in aquatic environmental (Gallage *et al*., 2016) that limits the optimal growth, health and physiological state of fish in intensive aquaculture, including in marine sea-cages, which is the primarily culture system for Atlantic salmon (*Salmo salar*).

The negative impact of hypoxia in farmed fish is significant, since they cannot avoid or escape this situation (Gallage *et al*., 2016). When hypoxia is severe enough to alter homeostasis, a stress response is triggered (Rodríguez *et al*. 2016) and the energy is diverted from energy-consuming processes as growth, reproduction or immunity to homeostasis maintenance (Burt *et al*., 2014; Gallage *et al*., 2016).

To cope with hypoxia, a complex process of physiological and biochemical changes is involved in fish (Terova *et al*., 2008), including low metabolic rate, high ventilation and anaerobic respiration (Rahman and Thomas, 2007). Studies have shown the hypoxia as a stressful factor in aquatic environments. In Atlantic salmon plasma lactate increased at oxygen levels below to 60% and plasma cortisol increased at 50% or less of dissolved oxygen (DO) during the first day of exposure (Remen *et al*., 2012). However, cortisol, lactate and oxidative stress parameters (glutathione peroxidase, glutathione reductase, glutathione total, glutathione reduced and lipid peroxidase) in liver remained unaltered when Rainbow Trout (*Oncorhynchus mykiss*) were subjected to 49 days of hypoxia (Magnoni *et al*., 2019).

Studies have focused in acute, short-term or intermittent hypoxia exposure, but chronic exposure is a condition that mainly represents the reality of aquatic systems (Gallage *et al*., 2016). Nevertheless, its consequences and metabolic adjustments are still poorly documented. Li *et al*. (2018) kept Nile tilapia (*Oreochromis niloticus*) juveniles under hypoxia for 4 weeks and observed lower weight gain and feed intake

in hypoxia treatment from the first week. Survival to hypoxia requires a rapid and well-coordinated reorganization of physiological and biochemical systems and consequently cellular adjustments to maximize the oxygen available from environment (Richards, 2011). Therefore, chronic hypoxia can result in large changes in gene expression, which are the basis for acclimation/acclimatization and potentially increase hypoxic survival. Additionally, the stress situation imposed by hypoxia can prejudice the antioxidant system. In Atlantic cod (*Gadus morhua*), long-term hypoxia exposure down-regulated SOD transcription levels (Olsvik *et al*., 2006).

Understanding the impacts of hypoxia, especially chronically, on salmon physiology will help to predict the salmon response to climate change and prevent production losses. In this study, we evaluated the effects of chronic hypoxia in growth performance, stress, metabolism-related genes and antioxidant responses of Atlantic salmon.

2. Material and Methods

The experimental procedures were approved by the Institutional Animal Care Committee of Memorial University of Newfoundland (Protocol #17-94-KG) and performed in accordance with the guidelines of the Canadian Council on Animal Care.

2.1. Animals and experimental design

Atlantic salmon (*Salmo salar*) were obtained from Cape D'or Sustainable Seafood (Nova Scotia, Canada) and initially held at the Dr. Joe Brown Aquatic Research Building (JBARB; Ocean Sciences Centre, MUN) in a 4,000-L circular tank for 1 month. During this period, fish were fed with a commercial salmonid diet (Optiline MicroBalance, Skretting, St. Andrews, NB, Canada) at a ration of 1.5% biomass daily (b.w. day^{-1}) and photoperiod was 12 h light:12 h dark. After acclimation, 140 fish were netted, anesthetized in seawater containing tricaine methanesulfonate (AquaLife TMS, 0.1 g L^{-1}; Syndel Laboratories Ltd, Nanaimo, BC, Canada), weighed, and had a PIT (Passive Integrated Transponder) tag implanted in their peritoneal cavity for identification. After tagging, fish (117.4 ± 28.1 g) were

randomly distributed in two 3,000-L tanks on Cold-Ocean Deep-Sea Research Facility (CDRF; Ocean Sciences Centre, MUN), with water recirculation for 3 weeks to recover before to start the experiment. After this period, a WitroxCTRL Oxygen System (Loligo® Systems) was used to monitor and reduce the dissolved oxygen (DO) levels from one tank (hypoxia treatment) 5% every two days, for two weeks, until they reach 40% DO. For 6 weeks, fish from normoxia tank were kept at 100% DO (normoxia treatment) and fish from hypoxia tank were kept at 40% DO. At the end of 6-weeks period, 8 fish from each tank (8 fish per treatment) were sampled (initial sampling) and the remainders were anesthetized (AquaLife TMS, 0.1 g L^{-1}), injected intraperitoneally with 1µl g^{-1} of PBS and returned to the tanks. Fish were sampled 6 and 24 hours post injection (n=8) and the remainders returned to the tanks to track the weight gain up to 14 weeks of experiment. During experimental period, water temperature was 10.3 ± 0.4 °C; photoperiod used was 12 h light: 12 h dark and fish were fed at 1% b.w. day^{-1}.

2.2. Samplings

In each sampling, fish were quickly euthanized in seawater containing tricaine methanesulfonate (TMS, 0.4 g L^{-1}), measured and weighed. The blood was collected by puncture of the caudal vessel using a heparinized (1,000 UI ml^{-1}) syringe. The blood was centrifuged for 1 min at 10,000 g to separate plasma. The plasma was distributed on different microtubes, immediately frozen in liquid nitrogen and stored at -80 °C prior to the determination of cortisol and lactate concentrations. In the initial sampling, liver were removed, frozen in liquid nitrogen and stored at -80 °C for further antioxidant enzymes assays and mRNA expression of metabolism-related genes: cold-inducible RNA-binding protein (*cirbp*), DNA-methiltransferase-I (*dnmt1*), growth hormone receptor 1 (*ghr1*), hypoxia inducible factor-α (*hif1a*), heat shock proteins 47 and 70 (*hsp47* and *hsp70*), inducible growth factor-I (*igf1*), lactate dehydrogenase (*ldhaa*), phosphofructokinase (*pfk1*), peroxisome proliferator-activated receptor beta 1β (*pparb1b*), peroxiredoxin-6 (*prdx6*) and protein kinase AMP-activated catalytic subunit alpha 2 (*prkaa2a*).

2.3. Assays

2.3.1. Weight gain

Weight gain was calculated according the formula:

[WG (g) = actual sampling weight (g) – last sampling weight (g)]

2.3.2. Specific growth rate

Specific growth weight was calculated according the formula:

[SGR (%) = {log10(actual sampling weight-g) – log10(last sampling weight]/days}*100]

2.3.3. Plasma cortisol concentration

Plasma cortisol concentration was determined using a commercial kit through an enzyme-linked immunosorbent assay (Neogen® Cortisol ELISA kit, #402710).

2.3.4. Plasma lactate concentration

To determine lactate concentration, plasma samples were deproteinized with 6% perchloric acid (1:4) and centrifuged at 10,000 g for 10 min to collect the supernatant. An aliquot of 25 µl of the supernatant was added to 200 µl medium containing glycine buffer (Sigma-Aldrich, G5418) and 2.5 mmol L^{-1} NAD^{+}, (pH 9.0) at room temperature and read (340 nm) after addition of 30 µl of lactate dehydrogenase (Sigma®, #L2500), several times until the absorbance is stable/plateau (Clow *et al*., 2017).

2.3.5. Gene expression

To determine metabolism-related genes, liver samples (approximately 100 mg tissue) were homogenized in 400 µL of TRIzol Reagent (Invitrogen/ ThermoFisher Scientific®, #15596-018) using a motorized Kontes RNase-Free Pellet Pestle Grinder (Kimble Chase, Vineland, NJ). An additional 400 µL of TRIzol Reagent

(Invitrogen/ ThermoFisher Scientific®, #15596-018) was added, mixed by pipetting, and the homogenates frozen on dry ice and stored at -80°C. Frozen homogenates were further processed by slowly thawing them on ice and then passing them through a QIAshredder (QIAGEN, Mississauga, ON) spin column following the manufacturer's instructions. Two-hundred µL of TRIzol (Invitrogen/ ThermoFisher Scientific®, #15596-018) was then added to each sample to make a total homogenate volume of approximately 1 mL, and the TRIzol total RNA extractions were then completed following the manufacturer's instructions. Total RNA samples were treated with 6.8 Kunitz units of DNaseI (RNase-Free DNase Set, QIAGEN, #79254) with the manufacturer's buffer (1X final concentration) at room temperature for 10 min to degrade any residual genomic DNA. DNase-treated RNA samples were column-purified using the RNeasy Mini Kit (QIAGEN, #74106) following the manufacturer's instructions. RNA integrity was verified by 1% agarose gel electrophoresis, and RNA purity was assessed by A260/280 and A260/230 NanoDrop UV spectrophotometry for both the pre-cleaned and the column-purified RNA samples. Column-purified RNA samples had A260/280 ratios between 2.0 and 2.3 and A260/230 ratios between 1.9 and 2.3. First-strand cDNA templates for qPCR were synthesized in 20 µL reactions from 1 µg of DNaseI-treated, column-purified total RNA using random primers [250 ng (Invitrogen/Thermo Fisher Scientific®, #48190-011)], dNTPs (0.5 mM final concentration ; Invitrogen/Thermo Fisher Scientific®, #10297-018) and M-MLV reverse transcriptase (200 U; Invitrogen/Thermo Fisher Scientific®, #28025-013) with the manufacturer's first strand buffer (1X final concentration) and DTT (10 mM final concentration) at 37 °C for 50 min.

For the qPCR analysis, we selected 12 genes [cold-inducible RNA-binding protein (*cirbp*), DNA-methiltransferase-I (*dnmt1*), growth hormone receptor 1 (*ghr1*), hypoxia inducible factor-α (*hif1a*), heat shock proteins 47 and 70 (*hsp47* and *hsp70*), inducible growth factor-I (*igf1*), lactate dehydrogenase (*ldhaa*), phosphofructokinase (*pfk1*), peroxisome proliferator-activated receptor beta 1β (*pparb1b*), peroxiredoxin-6 (*prdx6*) and protein kinase AMP-activated catalytic subunit alpha 2 (*prkaa2a*)], which primers were designed using Primer3 software (http://frodo.wi.mit.edu/primer3), and were based on cDNA sequences for the genes of interest (GOI) and the normalizer [60S ribosomal protein L32 (*rpl32*) and

elongation factor 1-alpha 2 (*ef1a2*)] that were available for Atlantic salmon in GenBank (see **Table 1** for accession numbers). Each primer set for each gene was tested for quality before use (Caballero-Solares *et al*., 2017). All PCR amplifications were performed in 13 μL reactions using 1X Power SYBR Green PCR Master Mix (Applied Biosystems/ ThermoFisher Scientific®, #A25776), 50 nM of both the forward and reverse primers, and cDNA representing 4 ng of input total RNA. Amplifications were performed using the ViiA 7 Real Time PCR system (384-well format) (Applied Biosystems/Thermo Fisher Scientific®). The real-time analysis program consisted of 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min and 40 cycles of 95°C for 15 sec and 60°C for 1 min, with fluorescence detection at the end of each 60°C step, and was followed by dissociation curve analysis. .

Transcript expression levels of the genes of interest (GOI) were normalized to 60S ribosomal protein L32 (*rpl32*) and elongation factor 1-alpha 2 (*ef1a2*) transcript levels. These genes were chosen as the endogenous control (i.e. normalizer gene) due to its relatively stable expression in this study. These endogenous controls were selected from four candidate normalizers [60S ribosomal protein L32 (*rpl32*) (BT043656), β-actin (*actb*) (BG933897), elongation factor 1-alpha 1 (*efla1*) (AF321836) and elongation factor 1-alpha 2 (*efla2*) (BT058669)]. Normalizer testing were analyzed using geNorm (Vandesompele 2002). The qPCR analyses of expression levels of the 12 GOIs in the 16 liver samples were then performed using the ViiA 7 Real Time PCR system (384-well format) (Applied Biosystems by ThermoFisher Scientific®). On each plate, for every sample, the GOIs and endogenous controls were tested in triplicate, and a plate linker sample (i.e. a sample that was run on all plates in a given study) and a no-template control were included. The relative quantity (RQ) of each transcript was determined using the ViiA 7 Software Relative Quantification Study Application (Version 1.2.3) (Applied Biosystems/Life Technologies®), with normalization to both *rpl32* and *efla2* and amplification efficiencies incorporated. For each GOI, the sample with the lowest normalized expression (mRNA) level was set as the calibrator sample (i.e. assigned an RQ value = 1).

Table 1. Primers used in the quantitative reverse transcription-polymerase chain reaction analyses.

Gene Name (symbol) (GenBank Acc. No.)	Nucleotide sequence (5' → 3')	Amplicon size (bp)	[c]Efficiency (%)	Reference
6-phosphofructokinase, liver type (*pfkl*) (XM_014165779)	F: CCAGTAGACGGATGGGAAGA	119	92.7	This study
	R: ATGGGTTCACCAGCTCTGTC			
AMP-dependent protein kinase subunit alpha 2a (*prkaa2a*) (XM_014169238)	F: CGCTCAAGAGGGCTACCATTA	138	95.7	This study
	R: TGTCACACACCTCTCGGACTG			
Cold inducible RNA binding protein (*cirbp*) (BT059171)	F: TTGAGTACACAGCGGTGAATT	132	98.8	Beemelmanns *et al*., 2020
	R: ACCAATCTGATGCTATGACGAGA			
DNA (cytosine-5)-methyltransferase 1 (*dnmt1*) (XM_014203933)	F: TGTGTCTTAGAGCGGATCAAGG	83	93.5	Beemelmanns *et al*., 2020
	R: TAGTCCGACACCTCCATCTTCT			
Growth hormone receptor 1 (*ghr1*) (NM_001123576)	F: ACACCTGAGGAGCAGAGGAA	120	89.3	This study
	R: GTAGCCTCCCACATCAGCAT			
Hypoxia inducible factor 1 alpha c (*hif1ac*) (NM_001140022)	F: CCCATGTTCACAACAACAGC	100	103.4	Beemelmanns *et al*., 2020
	R: AATGAGAAGGGGCTGAACCT			
Heat shock protein 47 (*hsp47*) (XM_014214963)	F: GACCATTCAAAAATCAACCTCA	129	92.0	Beemelmanns *et al*., 2020
	R: CATGGCTCCATCAGCATTCT			
Heat shock protein 70 c (*hsp70c*) (BT045715)	F: AGTGATCAACGACTCGACACG	151	89.5	Beemelmanns *et al*., 2020
	R: CACTGCATTGGTTATAGTCTTG			
Insulin growth factor 1 (*igf1*) (EF432852)	F: ACTGTGCCCCTGTCAAGTCT	134	80.0	This study
	R: CTGTGCTGTCCTACGCTCTG			
Lactate dehydrogenase A (*ldha*) (BT043598)	F: AGACGAGGTGTTCCTCAGTG	113	96.3	This study
	R: TCTCGGCGCTGTTGATTAGC			
Peroxiredoxin 6 (*prdx6*) (BT047207)	F: GCGTTCATGTTGCATTTGTTGT	126	92.5	Beemelmanns *et al*., 2020
	R: CGCAATTAGAAGTAAGGCAGCA			
Peroxisome proliferator-activated receptor beta 1b (*pparb1b*) (AF342945)	F: CCCGGTAGCGTATCCATATC	94	91.3	Emam *et al*., 2020
	R: CCCGTTGATCAGCTGGTTCC			
[a,b]60S ribosomal protein L32 (*rpl32*) (BT043656)	F: AGGCGGTTTAAGGGTCAGAT	119	92.6	Xue *et al*., 2015
	R: TCGAGCTCCTTGATGTTGTG			
[a]β-actin (*actb*) (BG933897)	F: CCAAAGCCAACAGGGAGAAG	91	90.4	Xue *et al*., 2015
	R: AGGGACAACACTGCCTGGAT			
[a]elongation factor 1-alpha 1 (*ef1a1*) (AF321836)	F: TGGCACTTTCACTGCTCAAG	197	92.8	Caballero-Solares *et al*., 2017

	R: CAACAATAGCAGCGTCTCCA			
[a,b]elongation factor 1-alpha 2 (*ef1a2*) (BT058669)	F: GCACAGTAACACCGAAACGA	132	99.1	Katan *et al*., 2019
	R: ATGCCTCCGCACTTGTAGAT			

[a]Candidate normalizers.

[b]Expression levels of the transcripts of interest were normalized to expression levels of these two transcripts.

[c]Amplification efficiencies were calculated using a 5-point 1:3 dilution series starting with cDNA representing 10 ng of input total RNA. See methods for details.

2.3.6. Antioxidant enzymes

Approximately 100 mg of liver were homogenized with 1 ml of 0.05M phosphate buffer 1% Triton (pH 7.4), sonicated for 3 sec and centrifuged for 10 min at 10,000g. The supernatant was used to evaluate the catalase (CAT) and superoxide dismutase (SOD) activity by Catalase and Superoxide dismutase colorimetric activity species independent kit (Arbor assays®) following the manufacturer instructions. It was necessary dilute the homogenate 20,000x to CAT and 250x to SOD assay. The 50x diluted homogenate was used to determine the liver protein content according to Bradford (1976), based on an absorbance shift of the dye Coomassie Brilliant Blue G-250 (ThermoFisher Scientific®, 23200). The CAT and SOD activity were expressed in units of mg protein^{-1}.

2.4. Statistical analysis

The data were submitted to normality (Shapiro-Wilk) and homoscedasticity (Brown-Forsythe) tests and were transformed prior to statistical analysis if they failed in those tests. To growth performance, we used a 2 (hypoxia and normoxia) x 4 (initial, 6, 10 and 14 weeks of experiment) factorial and to stress response we used a 2 (hypoxia and normoxia) x 3 (initial, 6 and 24 hpi) factorial followed by Duncan's post-hoc tests. To compare antioxidant response and the gene expression at each treatment t-tests was used. The statistical analysis was made on SAS software (version 9.0) considering P value < 0.05 as the level of statistical significance.

3. Results

In this study, we evaluated metabolic and stress responses in Atlantic salmon exposed to 6 weeks of hypoxia (40% DO) followed by inoculation with PBS. Fish under hypoxia condition tended to be slower, mouthing and going to the surface to breath with gill ventilation increased. There was no mortality in this experiment.

3.1. Stress response

The plasma cortisol and lactate concentrations were higher in normoxia group at initial sampling (Fig. 2 A and B).

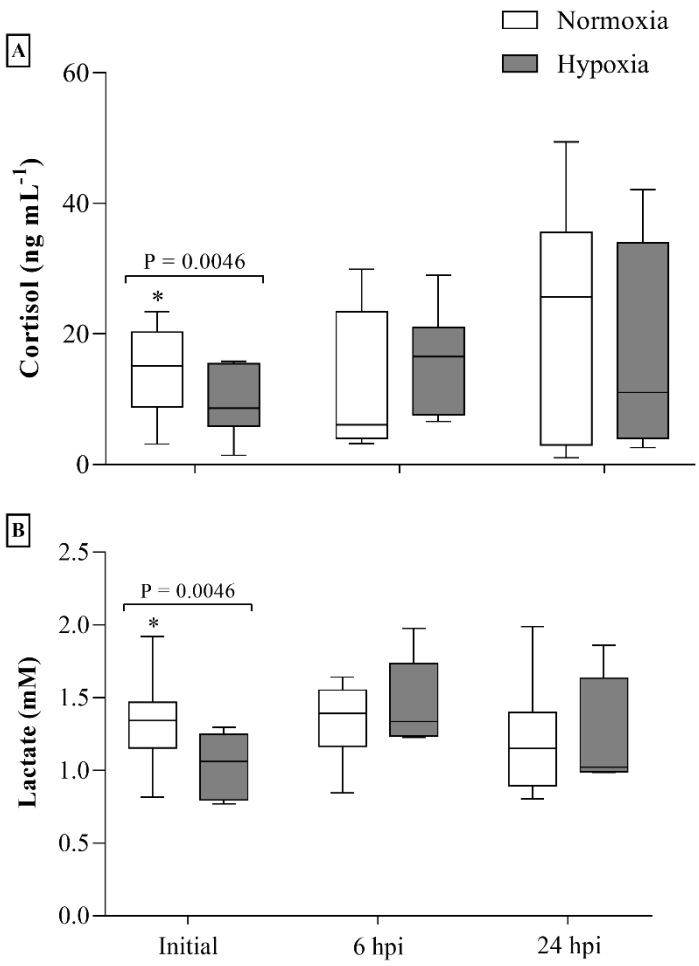

Fig. 1. Plasma cortisol (A) and lactate (B) concentration of Atlantic salmon after 6 weeks under hypoxia or normoxia, and 6 and 24 hours post inoculation with PBS. Asterisk indicate difference between treatments at same sampling time. Values are means ± min. and max. (n=8).

3.2. Gene expression

The hypoxia up-regulated the expression of the lactate dehydrogenase (*ldhaa*), peroxisome proliferator-activated receptor beta 1β (*pparb1b*) and protein kinase AMP-activated catalytic subunit alpha 2 (*prkaa2a*) (Fig. 3). Although no statistical different, the expression of heat-shock protein 47 (*hsp47*) was 51% higher in hypoxia treatment.

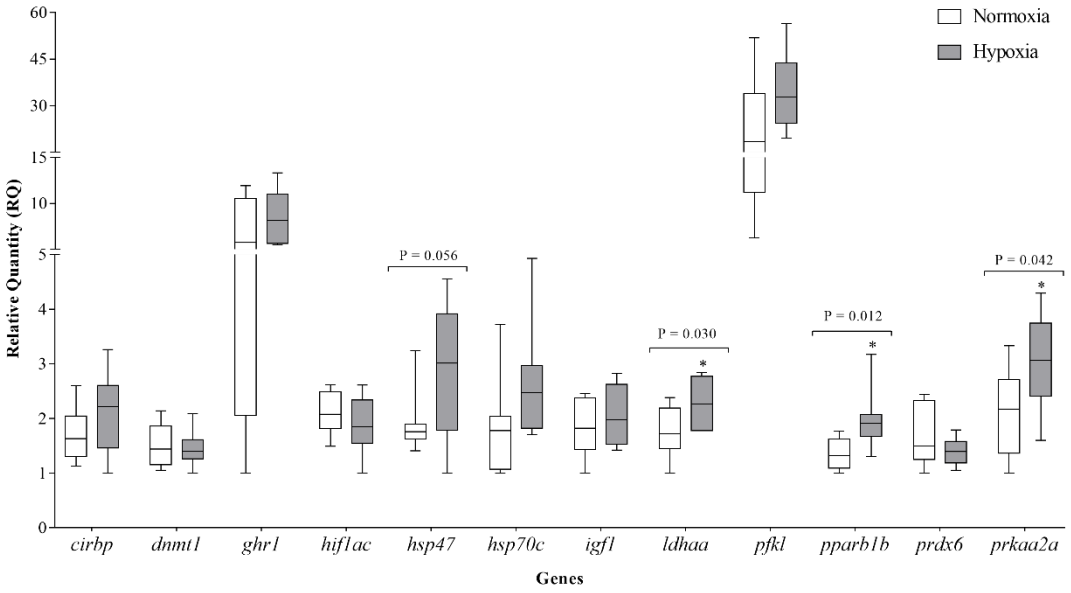

Fig. 2. mRNA expression of cold-inducible RNA-binding protein (*cirbp*), DNA-methiltransferase-I (*dnmt1*), growth hormone receptor 1 (*ghr1*), hypoxia inducible factor-α (*hif1a*), heat shock proteins 47 and 70 (*hsp47* and *hsp70*), inducible growth factor-I (*igf1*), lactate dehydrogenase (*ldhaa*), phosphofructokinase (*pfk1*), peroxisome proliferator-activated receptor beta 1β (*pparb1b*), peroxiredoxin-6 (*prdx6*) and protein kinase AMP-activated catalytic subunit alpha 2 (*prkaa2a*) of Atlantic salmon after 6 weeks under hypoxia or normoxia. Asterisk indicate difference between treatments. Values are means ± min. and max. (n=8).

3.3. Antioxidant response

There was no influence of 6-week hypoxia in catalase and superoxide dismutase activity and protein concentration (Fig. 6 A, B and C, respectively) in liver.

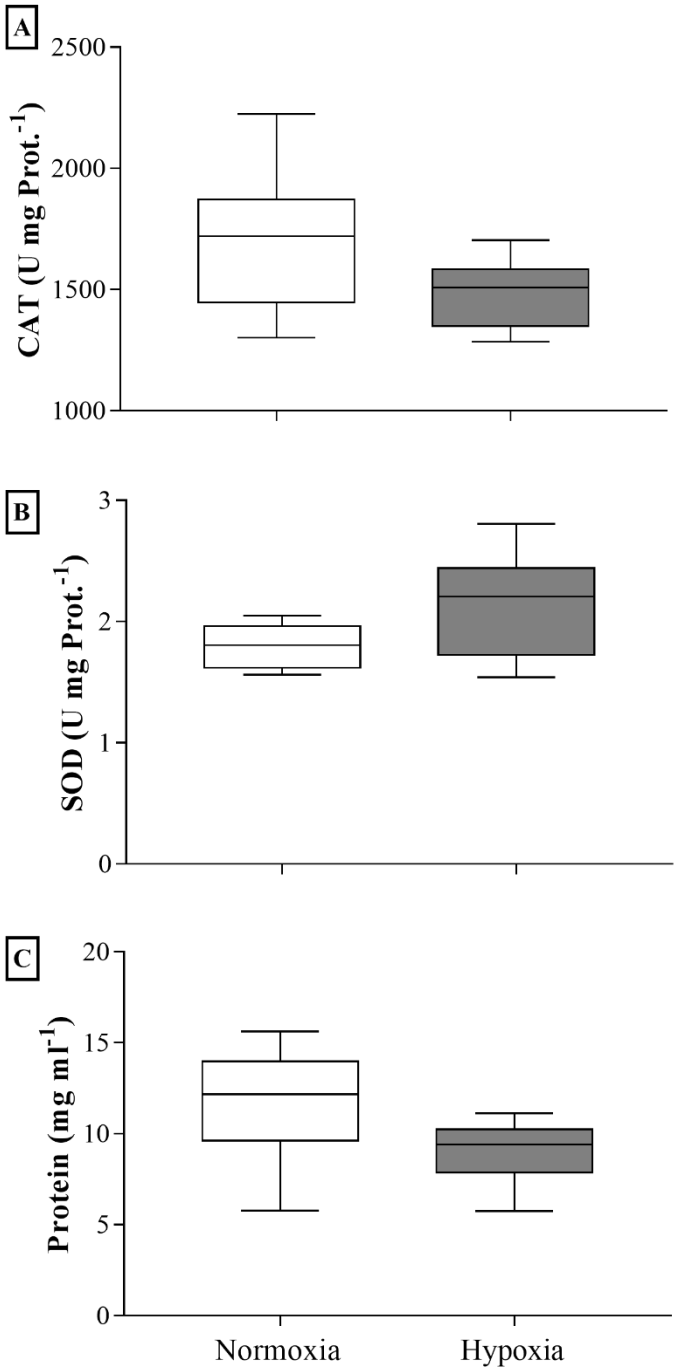

Fig. 3. Catalase (A) and superoxide dismutase (SOD) (B) activity and protein concentration (C) in liver of Atlantic salmon after 6 weeks under normoxia (100% DO) or hypoxia (40% DO). Values are means ± min. and max. (n=8).

3.4. Growth performance

After 14 weeks of feeding at 1% b.w. day^{-1}, fish under normoxia gained more weight than those under hypoxia (Fig. 4A). The weight gain difference is observed at 6 (p=0.0088), 10 (p=0.0037) and 14 weeks (p=0.0010) of experiment. The specific

growth rate was higher in hypoxia fish on initial sampling ($p<0.0001$) and higher in normoxia fish from 6 weeks of experiment (6w – $p=0.0098$; 10w – $p=0.0009$; 14w – $p<0.0001$).

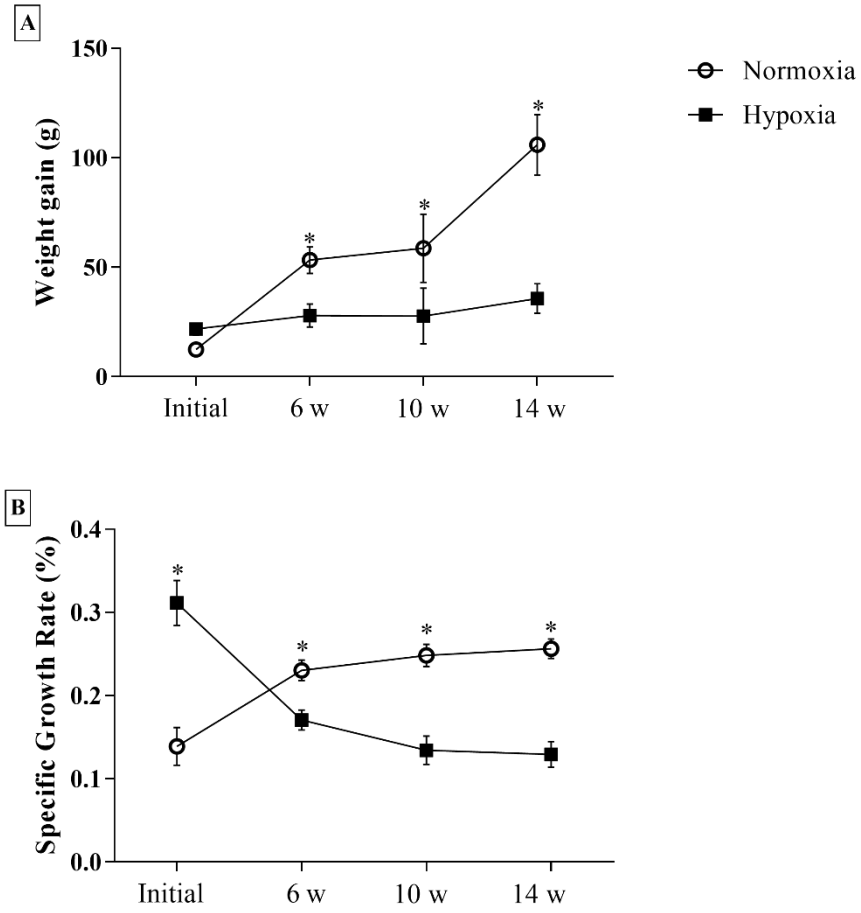

Fig 4. Weight gain (A) and specific growth rate (B) of Atlantic salmon after 14 weeks of feeding under normoxia (100% DO) and hypoxia (40% DO). Asterisk indicate difference between treatments at same sampling time. Values are means ± standard error.

4. Discussion

This study was designed to evaluate the chronic hypoxia effect on Atlantic salmon metabolism, including stress response, metabolic gene expression, antioxidant status and growth performance. To achieve this aim, fish were maintained under severe hypoxia (40% D.O.) for six weeks.

Water D.O. is an important environmental factor for aquatic animals and very relevant under intensive aquaculture (Martos-Sitcha *et al*., 2019). When the D.O. is below which critical for the species, the individual display behavioral signs of distress and physiological signals of stress (Feldmeth and Baskin, 1976). In this

study, Atlantic salmon under chronic hypoxia showed reduction on plasma cortisol concentrations. Fish exhibit a generalized response to acute hypoxia, including sympathetic and hypotalamic-pituitary-interrenal (HPI) axis, which leads to the adrenaline and cortisol release, respectively (Van Raaij *et al*., 1996). However, chronic stress can trigger changes in responsiveness of HPI axis, resulting sometimes in hyporeactivity of the corticosteroid response (Rotllant *et al*. 2000). Additionally, a long-term stress, due prolonged hyperactivity of HPI axis, normally cause a desensitization of adrenocorticotropic hormone produced by pituitary gland cells (Barton *et al*. 1987; Teles *et al*., 2013). Therefore, the decrease in cortisol concentration in fish from hypoxia group could be result of chronic response of stress.

The normal physiological function and metabolic rate cannot be sustained when D.O. is not sufficient to oxygen requirement for aerobic glycolysis (Richards, 2011). Hypoxia is related to anaerobic metabolism activation, given the energy demand of hypoxia stress fish (Bartrons and Caro, 2007; Speers-Roeschb et al., 2010) and leading to lactate accumulation (Van Raaij et al., 1996; Richards, 2011). However, studies with long-term hypoxia showed that fish can adapt and regulates the metabolism to the new oxygen availability condition. Zebrafish (*Danio rerio*) reared under hypoxia produced less lactate than those normoxia-reared (Widmer *et al*., 2006). Three-spined sticklebacks (*Gasterosteus aculeatus*) under 19% D.O. for 21 days (O'Connor *et al*., 2011) and rainbow trout (*Oncorhynchus mykiss*) under 4.5 mg O_2 L^{-1} for 49 days (Magnoni *et al*., 2019) had no significant alterations on cortisol, glucose or lactate levels. According to Gracey *et al*. (2011), fish reprogram your metabolism to adapt to available energy reserve during hypoxia. Despite lactate represents low tissue oxygenation, indicating anaerobiosis, salmon exposed to a chronic hypoxia in this study, showed decrease in plasma lactate concentration, which may be evidence of adjust for the new condition, allocating energy differently and reducing their tissue metabolism.

However, the 6 weeks of hypoxia exposition, up-regulated lactate dehydrogenase-A (*ldhaa*) expression, indicating activation of anaerobic metabolism. There are two predominant isozymes of lactate dehydrogenase, the LDH-A and LDH-B, which differ in their kinetics in the forward and reverse reactions (Nikinmaa and Rees, 2005). While LDH-A is more efficient converting pyruvate into

lactate, LDH-B is better suited converting lactate to pyruvate (Markert, 1975). The proportions of LDH-A and LDH-B vary among tissues and the LDH-A/LDH-B ratio is correlated with environmental oxygen availability (Almeida-Val *et al*., 1995), since fish can change in the isozyme pattern of lactate dehydrogenase (LDH) in response to hypoxia (Nikinmaa and Rees, 2005). *Cichlasoma amazonarum* expressed predominantly LDH-A in hypoxic habitats and LDH-B in normoxic habitats (Almeida-Val *et al*., 1995) and gobies (*Gillichthys mirabilis* and *Gillichthys seta*) exposed to hypoxia increased LDH-A transcript in liver (Gracey *et al*., 2001). Other metabolic biomarkers were up-regulated by chronic hypoxia, as peroxisome proliferator-activated receptor beta 1β (*pparb1b*) and protein kinase AMP-activated catalytic subunit alpha 2 (*prkaa2a*). AMP-activated protein kinase is an important energy sensor for metabolic adaptations in the shortage of ATP, as hypoxia (Zhu *et al*., 2013). We hypothesize that hypoxia lead to up-regulation of *prkaa2a* due to failure to generate ATP enough for cell metabolism. Although without statistical difference, hsp47 transcript was 51% higher in salmon under chronic hypoxia, indicating molecular response of stressor.

Reactive oxygen species (ROS) are formed as by-products of oxygen metabolism. Fish are susceptible to ROS harmful effects and to minimize they developed enzymatic antioxidant defenses, as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) (Martinez-Alvarez *et al*., 2005). Exposure to hypoxia cause oxidative damage (Lushchak and Bagnyukova, 2007) and enhance of antioxidant enzymes activity (Birnie-Gauvin *et al*., 2017). However, studies focused on acute hypoxia, and effects of prolonged period of low D.O. in antioxidant system are poorly investigated. In estuarine killifish (*Fundulus heteroclitus)* chronic hypoxia showed low to none oxidative damage in association with enhance of catalase activity in muscle (Borowiec and Scott, 2020). Similar to our study, rainbow trout (*Oncorhynchus mykiss*) under 49 days of hypoxia did not alter all oxidative stress parameters analyzed (glutathione peroxidase – GPx, glutathione reductase – GR, total glutathione, oxidized glutathione – GSSH, reduced glutathione – GSH and lipid peroxidation – LPO) (Magnoni *et al*., 2019). In our study, Atlantic salmon did not exhibited alteration on CAT and SOD activity after 6 weeks of hypoxia and we hypothesize that hypoxia acclimation led to adjustments in ROS homeostasis and oxidative status that do not reflect antioxidant system modification,

but may instead be part of the suite of responses that salmon use to cope with chronic hypoxia. Additionally, the little oxygen available reduced the tissue metabolism evidenced by reduction in lactate concentration, and consequently the demand for the antioxidant system.

Impaired growth performance due chronic hypoxia was demonstrated in young-of-the-year winter flounder (*Pseudopleuronectes Americanos*) (Bejda *et al.*, 1992), grass carp (*Ctenopharyngodon idella)* (Gan *et al*., 2013), turbot (*Scophthalmus maximus*) (Pichavant *et al*., 2000), southern flounder (*Paralichthys lethostigma)* (Taylor and Miller, 2001) and rainbow trout (Oncorhynchus mykiss) (Magnoni *et al*., 2019). Studies show that long-term hypoxia produces a hypometabolic state with a negative impact on feed intake (Martos-Sitcha *et al*., 2020). In our study, fish under hypoxia feed 32% (data not show) lower than those under normoxia, which explain the loss in growth performance.

5. Conclusion

Prolonged exposure to severe hypoxia could fatigue the HPI axis, leading to a low cortisol state. Salmon can adjust some metabolic process for the new environmental condition, reducing their tissue metabolism and antioxidant activity. The lower feed intake caused by hypoxia impaired growth performance of salmon.

6. Acknowledge

The authors thank to Albert Caballero-Solares for growth hormone receptor 1 (*ghr1*) primer design.

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CAPÍTULO 5

Glucan effects on stress, innate immune system and antioxidant enzymes responses of pacu (*Piaractus mesopotamicus*) injected with LPS after thermal challenge

ABSTRACT

Exogenous factors as low water temperature can be stressful for fish and elicit negative effects on the immune system. The stress and immune responses require an energy overload, which can affect the cellular redox balance, causing oxidative damage. To reduce susceptibility to stressors and diseases, immunostimulants such as β-glucans have been effective tools to enhance the innate immune responses of fish. However, the effects of β-glucans on innate immune and antioxidant responses in stressed fish is not yet explored. In order to evaluate the role of β-glucan in thermal challenged pacu, the present study fed fish with a diet containing Macrogard® (0.1%) for 14 days prior to reduce the water temperature from 29.5°C to 16°C and then inoculated fish with lipopolysaccharide from *Escherichia coli* (LPS). We evaluated indicators of stress, immune and antioxidant system and oxidative stress responses. Our results showed that thermal challenge affected negatively innate immune system, especially humoral responses, and LPS injection impaired the antioxidant enzymes activity. The β-glucan showed no clear immunostimulant effect, however it reduced LPO and avoided reduction of antioxidant enzymes activity in thermal stressed and LPS injected fish.

KEY-WORDS:

Immunostimulant, temperature, fish.

1. Introduction

The success of livestock is closely dependent of animal health status and the negative impact of disease in fish farming is becoming increasingly serious (FAO, 2001). The immune system is the main defense of the body against foreign substances as toxins and pathogens (Magnadottir, 2006; Secombes and Wang, 2012) ensuring animal health. The immune response is modulated by several endogenous and exogenous factors in fish. Among the exogenous factors, water temperature is described as the "abiotic master factor" for fish (Brett, 1971), because it is related disease outbreaks (Soto *et al*., 2014; Guijarro *et al*., 2015, Larsen *et al*., 2018). Temperature affects the development of parasites and pathogenic bacteria and may also alters the immune response and decrease the resistance of fish to disease, for example by influencing the leukocyte activity (Alborali, 2006). Since fish is an ectothermic organism, the environmental temperature limits and control their behavioral and physiological parameters (Donaldson *et al*., 2008). To deal with low temperatures, fish can increase the degree of lipids unsaturation to maintain the biological membranes fluidity. However, this mechanism can increase oxidative damage, since unsaturated fatty acids are highly susceptible to the lipid peroxidation (Crockett, 2008).

Dietary supplementation with immunostimulants in aquaculture is a strategy increasingly employed to improve defense responses against the adversities to which fish are exposed (Dalmo and Bøgwald, 2008; Meena et al., 2013). β-glucan is an immunostimulant widely used in aquaculture, with stimulatory effect on components of aquatic animal immune systems and subsequent protection against pathogens well stablished (Dalmo and Bøgwald, 2008). The repeating patterns of β-glucans structures called pathogen-associated molecular patterns (PAMPs) are also present in pathogens and are recognized by immune cells, causing an inflammatory cascade, which leads to the enhancement of innate immune responses (Brown and Gordon, 2005; Dalmo and Bøgwald, 2008).

The inflammatory response, as well as the stress response, is an energy-intensive process (Magnadottir, 2006), overloading the cellular mechanisms of energy production. During the mitochondrial respiration to energy production, reactive oxygen species (ROS) are produced. In energy-intensive situations, the disproportional ROS production in relation to antioxidant mechanisms can cause

oxidative damage in several cellular molecules (Schwarz, 1996; Nimse and Pal, 2015).

Studies have shown that β-glucans can enhance antioxidant enzymes activity after infection in fish (Kim *et al*., 2009; Guzmán-Villanueva *et al*., 2013), but to the best of our knowledge, there are no studies on β-glucans effects in cellular oxidative system following infection in stressed fish. In addition, studies evaluating thermal stress are needed, specially to assess the antioxidant responses. Thus, our goal is to evaluate the β-glucan action on stress response, innate immune system, antioxidant system and oxidative stress of pacu injected with LPS after thermal challenge, simulating a temperature drop common in the rearing environment in the winter.

2. Material and Methods

The experimental procedures were approved by the Comitê de Ética no Uso de Animais (CEUA) of the Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal campus (Protocol 014928/18).

2.1. Animals and experimental design

A total of 160 juveniles of pacu (114.6 ± 8.8 g and 17.8 ± 0.62 cm) were kept in 16 100-L tanks (10 fish per tank) with constant water renewal and aeration. Fish were fed with an extruded commercial diet twice a day (3% of their body mass) for 5 days to acclimatization to the laboratory conditions. After the acclimation period, fish were distributed into two treatments: fed with a control diet (commercial diet – glucan free) or fed with a glucan supplemented diet (0.1% of β-glucan) for 14 days (8 tanks/80 fish per treatment) with water temperature of 29.5 ± 0.18 °C. The β-glucan concentration was chosen according to previous study (Mello et al., 2019). After the last feeding, on 14th day, we reduced the water temperature in half of the tanks (8 tanks/80 fish) 0.5 °C per hour until it reaches 16 °C using a chiller (Gelaqua® Split). The temperature decrease took 26 hours and fish were maintained at 16 °C for 15 hours (Thermal stressed – TS group and Thermal comfort – TC group). Following the thermal challenge, one fish from each tank (n=8) was sampled to assess the basal condition, and the remainders were anesthetized (MS-

222 – Tricaine methanesulfonate, 0.15 g L^{-1}), injected in the mesenteric cavity with lipopolysaccharide (LPS) (1.5 mg kg^{-1}) and returned to the tanks. Fish were sampled 3, 6 and 24 hours after LPS injection (n=8).

During the experimental period, the water oxygen levels were 5.09 ± 0.26 mg L^{-1}. The photoperiod was 12 hours light: 12 hours dark.

2.2. Experimental diet

The experimental diet was prepared using an extruded commercial feed (Laguna® Onívoros Alevinos 36: 2.6 mm, 36% crude protein), ground and mixed with 0.1% of β-glucan (Macrogard®, batch Q719120(1000423, Biorigin, Brazil). The diet was moistened with 40% of water, granulated in a food processor and dried at 40 °C for 24 hours. The control diet was processed following the same procedure without the inclusion of β-glucan.

2.3. LPS preparation

The lipopolysaccharide from *Escherichia coli* (Sigma Aldrich®, code L3755, batch #046M4084V) was dissolved in 0.6% saline solution at dilution of 10 ml kg^{-1} of body mass. The solution was prepared at the concentration of 1.5 mg kg^{-1} of body mass.

2.4. Samplings

At each sampling, fish were anaesthetized, weighed, and blood samples were drawn from caudal vessels. Then fish were euthanized by prolonged immersion on anesthetic to tissue removal. The total blood was dispensed into microtubes with and without anticoagulant. Blood with Glistab® (EDTA 6 g dL^{-1} and KF 12 g dL^{-1}, Labtest, São Paulo, Brazil, code 29) was used to separate plasma for glucose and cortisol determination; blood with heparin (5.000 UI ml^{-1}) was utilized to measure the respiratory activity of leukocytes, and blood without anticoagulant was centrifuged (10 min at 20000 g) to separate serum and stored at -20 °C for determination of lysozyme activity and at -80 °C for determination of the hemolytic activity of the complement system. The liver was weighed to hepatosomatic index

calculation and stored at -80 °C for determination of the antioxidant enzymes activity. The spleen was weighed to spleen somatic index calculation.

2.5. Stress indicators

2.5.1 Plasma cortisol and glucose concentrations

Cortisol concentration was measured by enzyme-linked immunosorbent assay (ELISA, ThermoPlate Reader MN) with a commercial kit (DRG® Cortisol ELISA, EIA-1887). The glucose concentration was measured by an enzymatic method with a commercial kit (Labtest, São Paulo, Brazil, code 133).

2.6. Immunological indicators

2.6.1. Respiratory activity of leukocyte (RAL)

The production of reactive oxygen species (ROS) was measured by the reduction of NBT (Nitroblue tetrazolium chloride - Sigma Aldrich®, code N6876), following protocol described by Anderson and Siwicki (1995) and modified for pacu by Biller-Takahashi *et al*. (2013). An aliquot of 50 µL of total blood was incubated with 50 µL of 0.2% NBT solution at room temperature for 30 min. Thereafter, 1 mL of DMF (dimethylformamide, Sigma Aldrich®, code 227056) was added to the samples and the moisture was centrifuged at 9000 g for 5 min to read in a spectrophotometer (Thermo Scientific®, Genesys 10S) at 540 nm.

2.6.2. Hemolytic activity of the alternative pathway of the complement system (HA-AP)

The hemolytic activity of the alternative pathway of serum complement was determined according to Zanuzzo *et al*. (2017). A kinetic assay determined the time (in seconds) necessary for a serum sample to lyse 50% of 400 µl of a rabbit erythrocyte suspension in Alséver solution (anticoagulant, pH 6.1), measured in a spectrophotometer (Thermo Scientific®, Genesys 10S), with readings every 30 s for 20 min at 700 nm. The HA-AP in each sample was calculated through the equation of a straight line representing the decay of the absorbance by time.

2.6.3. Lysozyme concentration

The lysozyme concentration was determined according to Demers and Bayne (1997) with modifications by Zanuzzo *et al*. (2015), based on lysis of the gram-positive bacterium *Micrococcus lysodeikticus* suspension (Sigma Aldrich®, code M3770) by serum, using hen egg white lysozyme as a standard (Sigma Aldrich®, code L6876). The absorbance of the solution was measured at 450 nm over 5 min in a microplate reader (ThermoPlate Reader MN) at room temperature. The rate of decrease in absorbance for each sample (ΔOD) was then compared to the standard curve.

2.7. Antioxidant system and oxidative stress indicators

Liver samples were homogenized (1:10 w/v) in a phosphate buffer solution 0.1 M, pH 7.0 (lipid peroxidation - LPO, superoxide dismutase - SOD, catalase - CAT, reduced glutathione – GSH and glutathione-S-transferase - GST) or pH 7.6 (glutathione peroxidase - GSH-Px), centrifuged, and the supernatants were stored at -80ºC for further analysis. For all biochemical biomarkers, the protein content was determined according to Bradford (1976).

2.7.1. Superoxide dismutase activity (SOD)

The activity of superoxide dismutase (SOD) was determined according to McCord *et al*. (1969), based on the inhibition of the reduction rate of cytochrome c by the superoxide radical, at 550 nm, 25ºC. SOD activity was expressed in units of mg protein^{-1}, with one unit of SOD corresponding to the quantity of enzyme that promoted the inhibition of 50% of the reduction rate of cytochrome c.

2.7.2. Catalase activity (CAT)

The activity of catalase (CAT) was determined according to Aebi (1984), based on the consumption of exogenous hydrogen peroxide (H_2O_2) by CAT, generating

water and oxygen. The absorbance of the solution was read at 240 nm, every 15 sec for 5 min, and activity was expressed as nmol / mg protein / min.

2.7.3. Glutathione peroxidase activity (GSH-Px)

The activity of glutathione peroxidase (GSH-Px) was measured according to Hafeman *et al*. (1974), based on the reduction of GSSG (oxidized glutathione) in reduced glutathione (GSH) in the presence of NADPH promoted by glutathione reductase, since the oxidation rate of NADPH is proportional to the production of GSSG, by GSH-Px, in the presence of H_2O_2. The reduction of the absorbance was read every 15 sec for 5 min, at 340 nm, and the results were expressed in mmol / mg protein / min.

2.7.4. Glutathione-S-transferase (GST)

The glutathione-S-transferase (GST) concentration was determined according to Keen *et al*. (1976). This method is based on glutathione reduction by 1-chloro-2,4-dinitrobenzene substrate (CDNB) producing glutathione-2,4-nitrobezene. The reduction was read at 340 nm and results were expressed in nmol mg protein^{-1}.

2.7.5. Reduced glutathione concentration (GSH)

The concentration of reduced glutathione (GSH) was determined following Beutler *et al*. (1963), using 5,50-dithiobis-2-nitrobenzoic acid (DTNB), at 412 nm, against a GSH standard curve.

2.7.6. Lipid peroxidation (LPO)

Lipid peroxidation (LPO) was determined by thiobarbituric acid reactive substances (TBARS) assay, performed according to Camejo *et al*. (1998). TBARS concentrations were expressed in nmol MDA mg protein^{-1}.

2.8. Statistical analysis

The data were submitted to normality (Shapiro-Wilk) and homoscedasticity (Brown-Forsythe) tests. To adjust lysozyme concentration data for normality, they were transformed using log10. The experiment was set up in a completely randomized design in a factorial scheme 2x2x4 (two diets - control and glucan 0.1%; two thermal conditions - thermal comfort and thermal challenged; four sampling times - basal, 3 hpi, 6 hpi and 24 hpi). Means were compared by Duncan's post-hoc tests using SAS software (9.0). P value < 0.05 was used to estimate the level of significance for statistical differences. N=8.

3. Results

3.1. Stress response

3.1.1. Plasma cortisol concentration

Plasma cortisol concentration was lower (p=0.0709, b) in fish from thermal stressed group (TS group), fed with control diet, 3 hours post LPS injection (hpi) compared to 24 hpi.

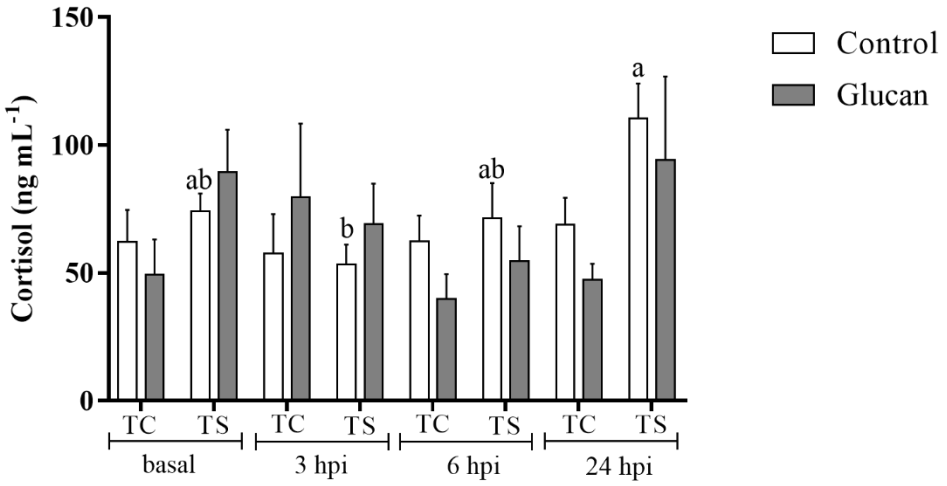

Fig. 1. Plasma cortisol concentration in pacu fed with control diet and diet containing 0.1% β-glucan, after thermal challenge (basal), and 3, 6 and 24 post intraperitoneal LPS injection (hpi). Different capital letters indicate difference among diets at a particular group in same sampling time. Different lower letters indicate differences among sampling times within the same group. Asterisk indicates difference among thermal comfort (TC) and thermal stressed (TS) groups in the same diet and sampling time. Symbols were omitted when there was no statistical difference. Values are means ± standard error (n=8).

3.1.2. Plasma glucose concentration

After the thermal challenge, pacu showed increase in plasma glucose concentration independent of the diet (control – p=0.0055; glucan – p=0.0033, *). The plasma glucose concentration increased 3 hpi in both diets (control – p=0.0457; glucan – p=0.0763, a) and both thermal conditions (TC – p=0.0048; TS – p=0.0457, a) and remained elevated until 6 hpi on both diets of TC group and until 24 hpi on TC group fed with control diet.

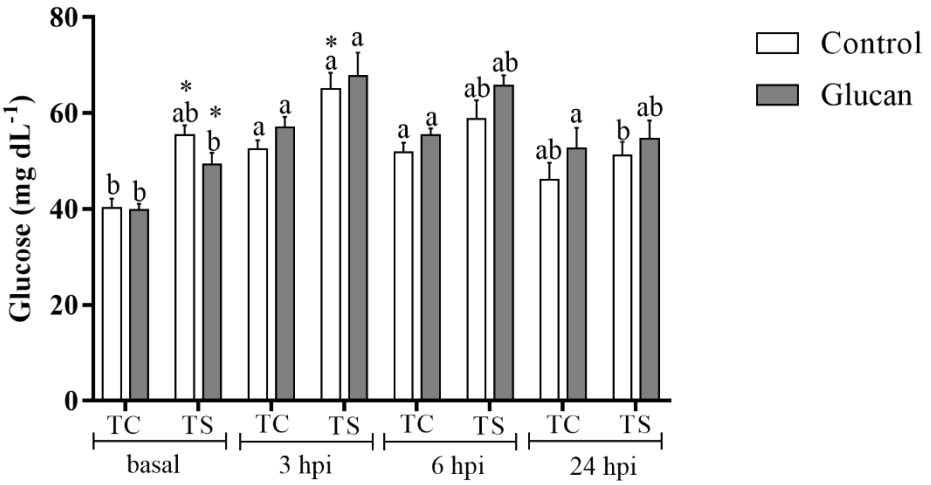

Fig. 2. Plasma glucose concentration in pacu fed with control diet and diet containing 0.1% β-glucan, after thermal challenge (basal), and 3, 6 and 24 post intraperitoneal LPS injection (hpi). Different capital letters indicate difference among diets at a particular group in same sampling time. Different lower letters indicate differences among sampling times within the same group. Asterisk indicates difference among thermal comfort (TC) and thermal stressed (TS) groups in the same diet and sampling time. Symbols were omitted when there was no statistical difference. Values are means ± standard error (n=8).

3.2. Innate immune response

3.2.1. Respiratory activity of leucocytes (RAL)

The respiratory activity of leucocytes (RAL) was not influenced by glucan. At 3 hpi the TS group showed higher RAL than TC group in both diets (control – p=0.0027; glucan – p=0.0071, *). In the TC group, the RAL decreased 3 hpi and most sharply 6 hpi (control – p<0.0001; glucan – p<0.0001, a). In the TS group the decrease was observed only at 6 hpi (control – p<0.0001; glucan – p<0.0001, a). Both groups recovered basal RAL at 24 hpi.

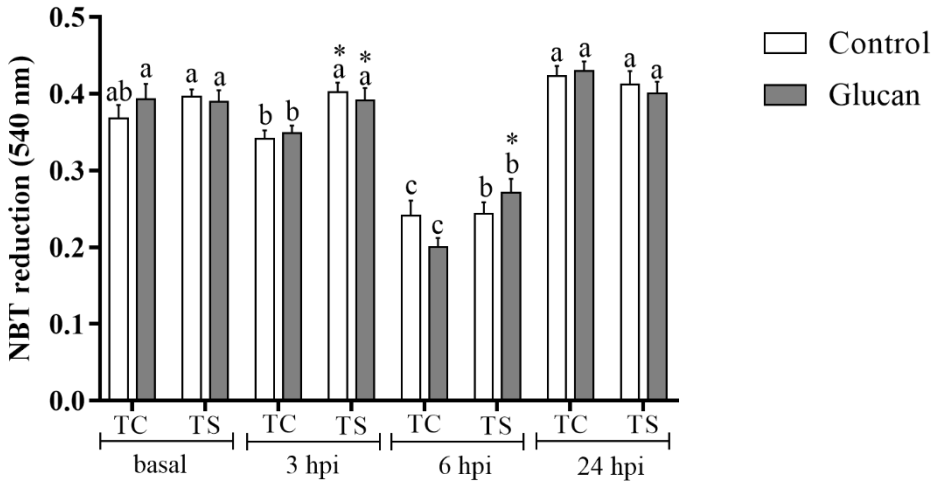

Fig. 3. Respiratory activity of leucocytes in pacu fed with control diet and diet containing 0.1% β-glucan, after thermal challenge (basal), and 3, 6 and 24 post intraperitoneal LPS injection (hpi). Different capital letters indicate difference among diets at a particular group in same sampling time. Different lower letters indicate differences among sampling times within the same group. Asterisk indicates difference among thermal comfort (TC) and thermal stressed (TS) groups in the same diet and sampling time. Symbols were omitted when there was no statistical difference. Values are means ± standard error (n=8).

3.2.2. Serum hemolytic activity of the alternative pathway of the complement system (HA-AP)

After 14 days of feeding, the serum hemolytic activity of the alternative pathway of the complement system (HA-AP) was higher (p=0.0284, B) in fish from TS group fed with glucan diet. However, HA-AP reduced (p=0.0735, a) after LPS injection in those fish.

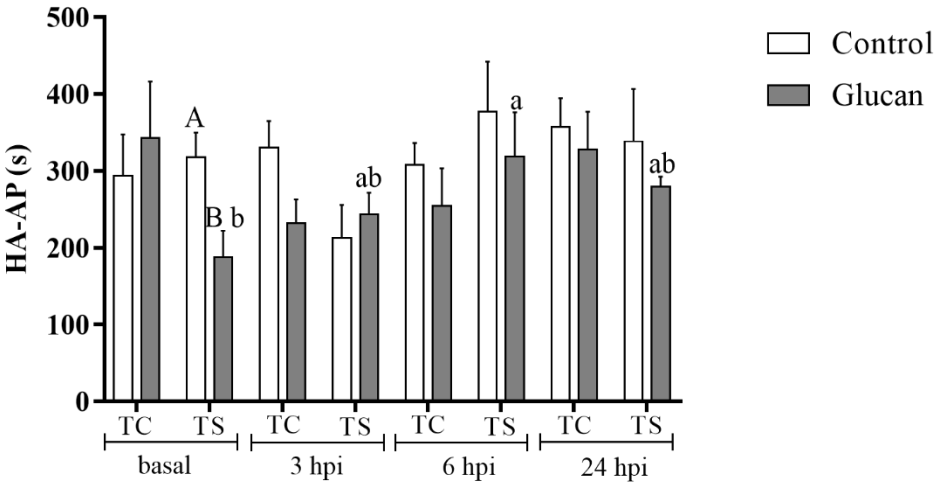

Fig. 4. Hemolytic activity of the alternative pathway of the complement system concentration in pacu fed with control diet and diet containing 0.1% β-glucan, after thermal challenge (basal), and 3, 6 and

24 post intraperitoneal LPS injection (hpi). Different capital letters indicate difference among diets at a particular group in same sampling time. Different lower letters indicate differences among sampling times within the same group. Asterisk indicates difference among thermal comfort (TC) and thermal stressed (TS) groups in the same diet and sampling time. Symbols were omitted when there was no statistical difference. Values are means ± standard error (n=8).

3.2.3. Lysozyme concentration

The lysozyme concentration increased 24 hpi in TC group independent of the diet (control – p=0.0154; glucan – p=0.0029, a). In all sampling times, the TC group showed higher lysozyme concentration than TS group (control – p<0.0001; glucan – p=0.0003, *).

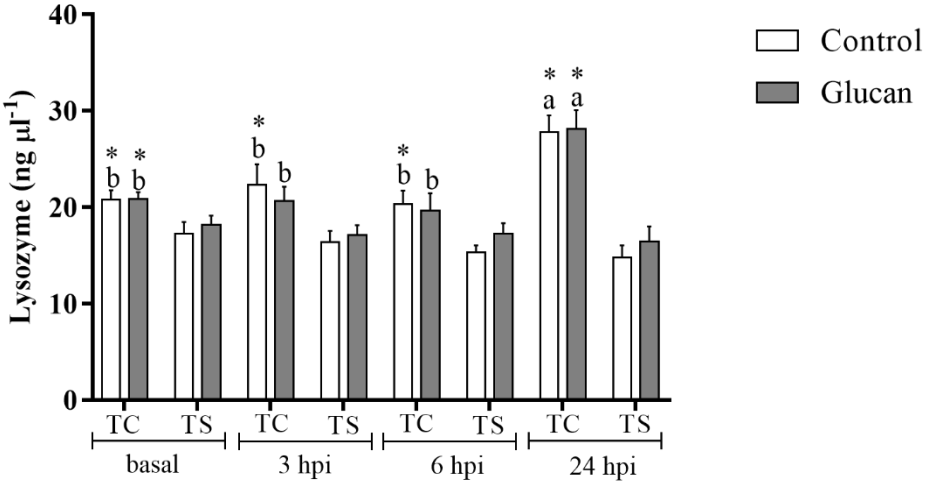

Fig. 5. Serum lysozyme concentration in pacu fed with control diet and diet containing 0.1% β-glucan, after thermal challenge (basal), and 3, 6 and 24 post intraperitoneal LPS injection (hpi). Different capital letters indicate difference among diets at a particular group in same sampling time. Different lower letters indicate differences among sampling times within the same group. Asterisk indicates difference among thermal comfort (TC) and thermal stressed (TS) groups in the same diet and sampling time. Symbols were omitted when there was no statistical difference. Values are means ± standard error (n=8).

3.2.4. Spleen somatic index (SSI)

The spleen somatic index (SSI) was higher in TC fish fed with glucan at basal sampling (p=0.0469, A) and higher in TS fish fed with control diet at 6 hpi (p=0.0241, A). TS fish fed with control diet showed SSI higher compared with TC group at 3 (p=0.0309, *) and 6 hpi (p=0.0094, *).

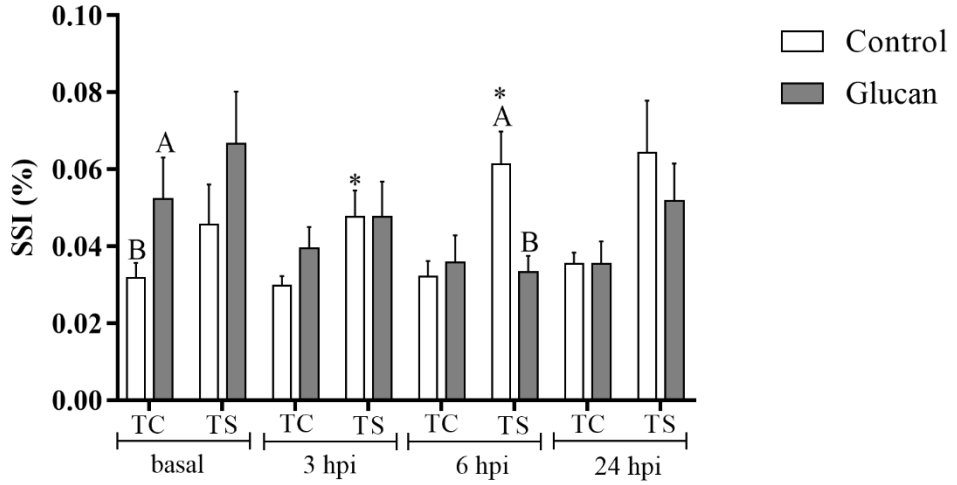

Fig. 6. Spleen somatic index in pacu fed with control diet and diet containing 0.1% β-glucan, after thermal challenge (basal), and 3, 6 and 24 post intraperitoneal LPS injection (hpi). Different capital letters indicate difference among diets at a particular group in same sampling time. Different lower letters indicate differences among sampling times within the same group. Asterisk indicates difference among thermal comfort (TC) and thermal stressed (TS) groups in the same diet and sampling time. Symbols were omitted when there was no statistical difference. Values are means ± standard error (n=8).

3.3. Antioxidant system responses

3.3.1. Superoxide dismutase activity (SOD)

The LPS decreased the SOD activity independent of the diet in TC fish (control – p=0.0728; glucan – p=0.0026) and remained lower until 24 hpi. In TS group, the LPS reduced (p=0.0017) the SOD activity only in fish fed with control diet. At 3 hpi, SOD activity was higher in TC group compared with TS group for both diets (control – p=0.0198; glucan – p=0.0005, *).

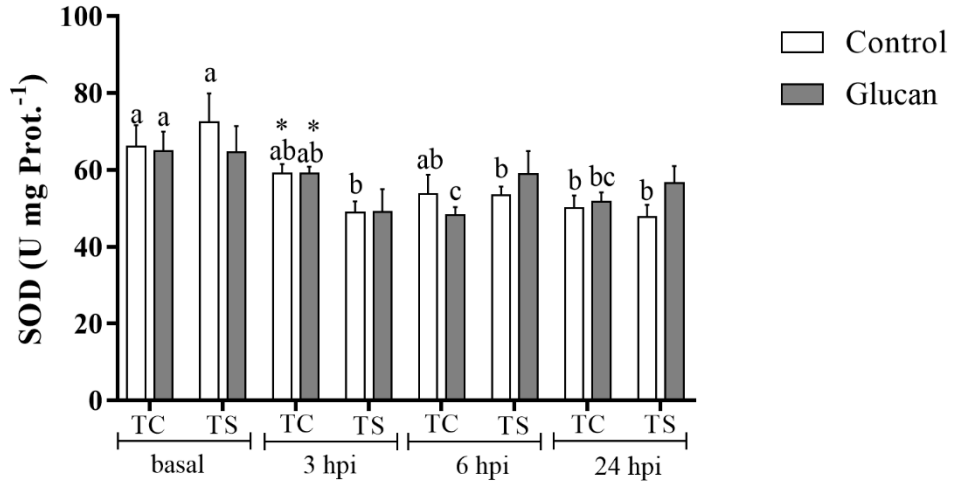

Fig. 7. Superoxide dismutase activity in pacu fed with control diet and diet containing 0.1% β-glucan, after thermal challenge (basal), and 3, 6 and 24 post intraperitoneal LPS injection (hpi). Different capital letters indicate difference among diets at a particular group in same sampling time. Different lower letters indicate differences among sampling times within the same group. Asterisk indicates difference among thermal comfort (TC) and thermal stressed (TS) groups in the same diet and sampling time. Symbols were omitted when there was no statistical difference. Values are means ± standard error (n=8).

3.3.2. Catalase activity (CAT)

The catalase activity decreased in fish from both diets after LPS in TS condition (control – p=0.0370; glucan – p=0.0351). At 3 hpi, the CAT activity was higher in TC fish fed with glucan diet (p=0.0446, *).

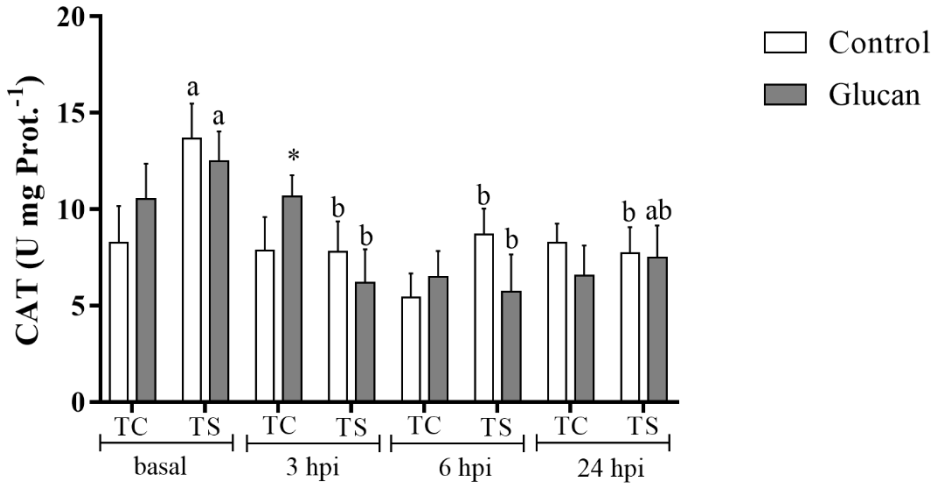

Fig. 8. Catalase activity in pacu fed with control diet and diet containing 0.1% β-glucan, after thermal challenge (basal), and 3, 6 and 24 post intraperitoneal LPS injection (hpi). Different capital letters indicate difference among diets at a particular group in same sampling time. Different lower letters indicate differences among sampling times within the same group. Asterisk indicates difference among thermal comfort (TC) and thermal stressed (TS) groups in the same diet and sampling time. Symbols were omitted when there was no statistical difference. Values are means ± standard error (n=8).

3.3.3. Glutathione peroxidase activity (GSH-Px)

The *GSH-Px* activity decreased (p=0.0072, b) in fish fed with control diet in TS group after LPS inoculation. At basal sampling, *GSH-Px* activity of TS group was higher (p=0.0480, *) than in TC group in fish fed with control diet and at 24 hpi GSH-Px activity of TC group was higher (p=0.0243, *) than in TS group in fish fed with glucan.

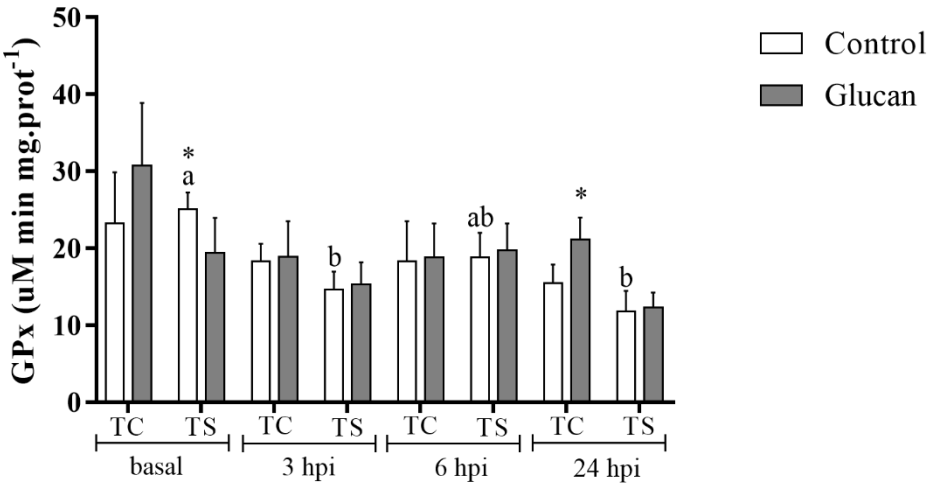

Fig. 9. Glutathione peroxidase activity in pacu fed with control diet and diet containing 0.1% β-glucan, after thermal challenge (basal), and 3, 6 and 24 post intraperitoneal LPS injection (hpi). Different capital letters indicate difference among diets at a particular group in same sampling time. Different lower letters indicate differences among sampling times within the same group. Asterisk indicates difference among thermal comfort (TC) and thermal stressed (TS) groups in the same diet and sampling time. Symbols were omitted when there was no statistical difference. Values are means ± standard error (n=8).

3.3.4. Glutathione-S-transferase activity (GST)

The GST activity decreased (p=0.0114, b) at 3 hpi in TS fish fed with control diet. In TC fish fed with glucan, GST activity decreased (p=0.0858, b) 6 hpi. At 6 hpi, GST activity was higher in TS group independent of the diet (control – p=0.0271, glucan – p=0.0233, *).

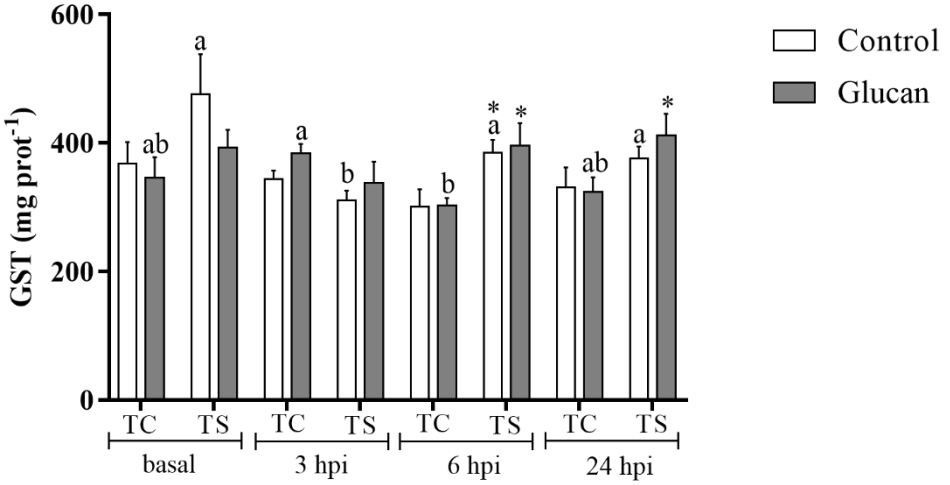

Fig. 10. Glutathione-S-transferase activity in pacu fed with control diet and diet containing 0.1% β-glucan, after thermal challenge (basal), and 3, 6 and 24 post intraperitoneal LPS injection (hpi). Different capital letters indicate difference among diets at a particular group in same sampling time.

Different lower letters indicate differences among sampling times within the same group. Asterisk indicates difference among thermal comfort (TC) and thermal stressed (TS) groups in the same diet and sampling time. Symbols were omitted when there was no statistical difference. Values are means ± standard error (n=8).

3.3.5. Reduced glutathione concentration (GSH)

The GSH concentration decreased after LPS injection in both thermal conditions independent of the diet (control/TC group – p=0.0097; control/TS group – p=0.0073; glucan/TC group – p=0.0017; glucan/TS group – p=0.0069).

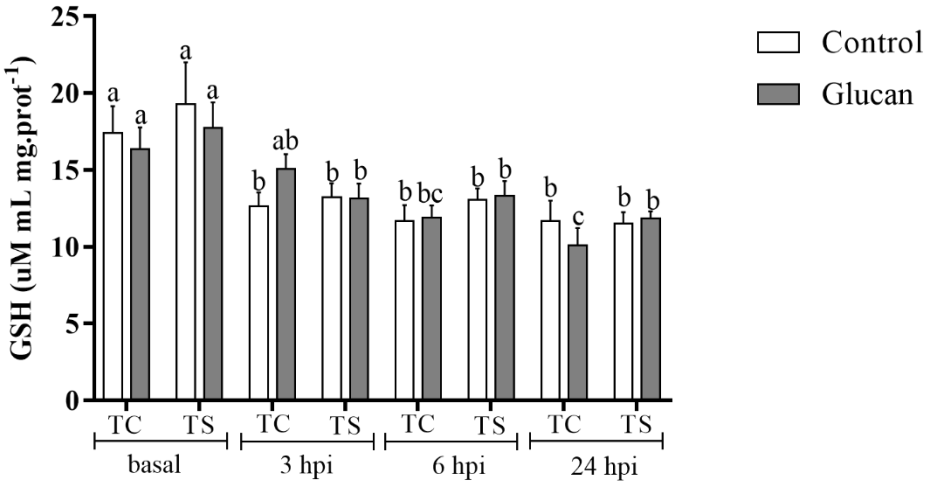

Fig. 11. Reduced glutathione concentration in pacu fed with control diet and diet containing 0.1% β-glucan, after thermal challenge (basal), and 3, 6 and 24 post intraperitoneal LPS injection (hpi). Different capital letters indicate difference among diets at a particular group in same sampling time. Different lower letters indicate differences among sampling times within the same group. Asterisk indicates difference among thermal comfort (TC) and thermal stressed (TS) groups in the same diet and sampling time. Symbols were omitted when there was no statistical difference. Values are means ± standard error (n=8).

3.3.6. Lipid peroxidation (LPO)

At basal sampling, lipid peroxidation was higher (p=0.0028, *) in TS fish fed with glucan compared with TC group. However, at 3 (p=0.0156, *) and 6 hpi (p=0.0478, *) LPO increased in TC group fed with glucan. In TC group LPO was higher at 24 hpi in fish fed with control diet (p=0.0293, a) and 3 hpi in fish fed with glucan diet (p=0.0783, a). The LPO decreased (p=0.0009, B), at 3 hpi, in TS fish fed with glucan diet compared with those fed with control diet.

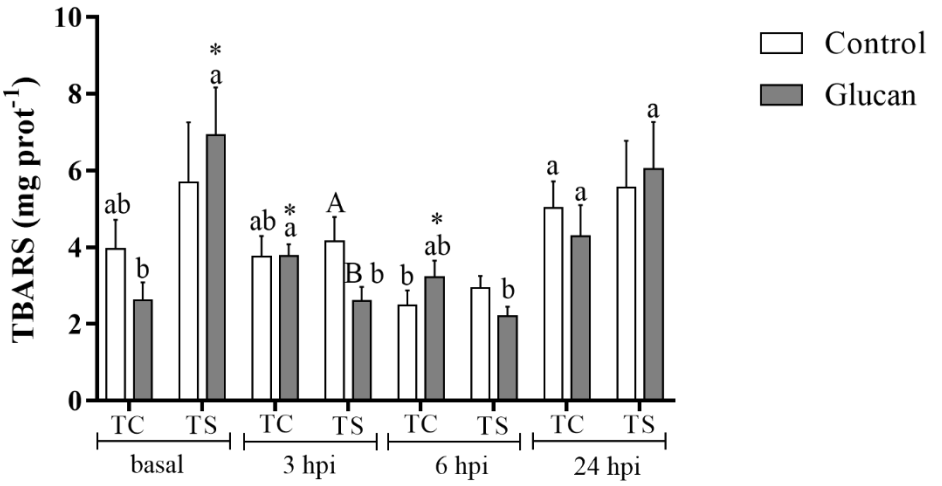

Fig.12. Lipid peroxidation on liver of pacu fed with control diet and diet containing 0.1% β-glucan, after thermal challenge (basal), and 3, 6 and 24 post intraperitoneal LPS injection (hpi). Different capital letters indicate difference among diets at a particular group in same sampling time. Different lower letters indicate differences among sampling times within the same group. Asterisk indicates difference among thermal comfort (TC) and thermal stressed (TS) groups in the same diet and sampling time. Symbols were omitted when there was no statistical difference. Values are means ± standard error (n=8).

3.3.7 Hepatosomatic index (HSI)

The HSI was higher in TS fish fed with control diet at 3 hpi ($p=0.0487$, A). In TC group, fish fed with glucan decreased the HSI after LPS injection ($p=0.0262$, a) and remained until 24 hpi. Independent of diet, the HSI was higher in TS group on all sampling times (control/basal – $p=0.0006$; control/3 hpi – $p<0.0001$; control/6 hpi – $p=0.0012$; control/24 hpi – $p=0.0012$; glucan/basal – $p=0.0387$; glucan/3 hpi – $p=0.0010$; glucan/6 hpi – $p=0.0339$; glucan/24 hpi – $p=0.0286$).

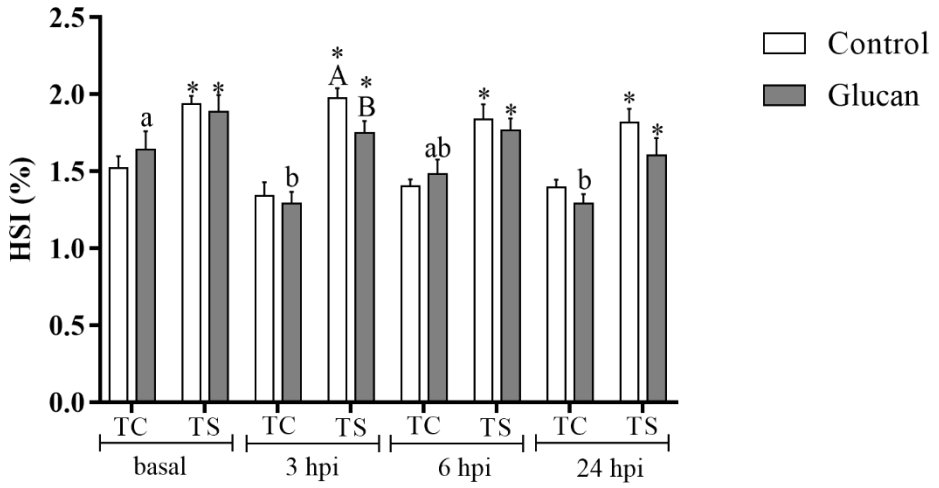

Fig. 13. Hepatosomatic index in pacu fed with control diet and diet containing 0.1% β-glucan, after thermal challenge (basal), and 3, 6 and 24 post intraperitoneal LPS injection (hpi). Different capital letters indicate difference among diets at a particular group in same sampling time. Different lower letters indicate differences among sampling times within the same group. Asterisk indicates difference among thermal comfort (TC) and thermal stressed (TS) groups in the same diet and sampling time. Symbols were omitted when there was no statistical difference. Values are means ± standard error (n=8).

4. Discussion

Global climate change is expected to lead to increased temperatures and more extreme climatic events that may negatively impact the ecosystems and the ectothermic animals, directly exposed to external temperature change (Dittmar *et al*., 2014). While temperatures above the physiological range for a fish species triggers a stress response that can negatively impact immune function (Magnadottir *et al*., 1999), suboptimal temperatures also can have negative impacts on fish health (Abram *et al*., 2019). Temperature shifts outside of the optimal temperature range has profound effects on biological systems of aquatic organisms and this study evaluated the impact on reducing temperature on stress, immune and antioxidant responses of a tropical fish. Pacu were fed with dietary supplementation of glucan, for 14 days, exposed to thermal stress for 24 hours and then immune stimulated with LPS injection. After thermal stress, fish showed changes that indicate that the species can be tolerant to acute decreases of the environmental temperature, as those we tested. The thermal stress was stressful and impaired lysozyme response; however, it did not affect the antioxidant system and the oxidative stress, although in fish thermal stressed and fed with glucan we observed oxidative stress, in a profile similar to that of the thermal stressed fish fed with control diet.

On the other hand, the LPS inoculation potentiated the stress response (glucose levels), increased the respiratory activity of leukocytes, decreased SOD activity, and increased GST activity in thermal stressed fish. By the other hand, β-glucan activated the complement system in thermal stressed fish, reduced the lipid peroxidation, decreased CAT activity after LPS in the same group of fish.

Plasma cortisol and glucose concentrations are commonly used as stress response indicators in fish (Barton and Iwama, 1991; Wendelaar Bonga, 2011) and several studies showed alterations in those parameters due to temperature changes (Takahara *et al*., 2011; Hwang *et al*., 2012; Árnason *et al*., 2013; Arslan *et al*., 2015). In this study, thermal stressed fish showed increase in glucose concentrations, although the increase in cortisol levels was not statistically significant (16.0% and 46.4% higher in control and glucan diets, respectively), suggesting a stress response. Cortisol measurements has been used to indicate the responsiveness of fish to changes in water temperature (Benitez-Dorta *et al*., 2017; Cockrem *et al*., 2019). After LPS injection, the plasma glucose and cortisol in TS control group increased, suggesting that the LPS challenge acted as an additional stressor. The β-glucan did not affect the stress response.

The innate immunity is assumed to be the most important immune component in fish that depend on this system for survival (Rombout *et al*., 2005). Studies have reported immune suppression at low temperatures. Phagocytosis and complement lytic activity were depressed in rainbow trout (*Oncorhynchus mykiss*) acclimatized for 57 days at lower temperatures (5-10 °C) (Nikoskelainen *et al*., 2004) and HA-AP reduced during winter period in gilthead seabream (*Sparus aurata*), when environmental temperature was ~10°C (Tort *et al*., 1998).

In our study, the respiratory activity of leukocytes was not affected by thermal decrease, however it increased after LPS in thermal stressed fish and was not influenced by β-glucan. Previous studies have shown that the adaptation to lower temperature increased leucocytes proliferation and activity in fish (Dexiang and Ainsworth, 1991; Collazos *et al*., 1994; Le Morvan *et al*., 1997; Alcorn *et al*., 2002; Kumari *et al*., 2006). Alcorn et al. (2002) observed that fish reared at 8°C had a greater percentage of phagocytic macrophages than the fish reared at 12°C. Unfortunately, we did not count the white blood cells. However, we can hypothesize that the increased RAL to LPS in fish exposed to thermal challenge

was based on cellular mechanisms. The innate immune system cellular components may be enhanced during exposure to thermal stress to compensate other immune deficit responses.

Cold water negatively affected the lysozyme concentrations and the LPS challenge increased the enzyme levels in fish not exposed to temperature drop 24 hpi. The depression of lysozyme concentrations observed after the thermal drop remained until 24 hpi. According to Saurabh and Sahoo (2008), lysozyme activity has been shown to vary depending on the water temperature. Lysozyme is an enzyme and temperatures above or under the optimal range can cause denaturation and compromise their function. The Asian catfish (*Clarias batrachus*) is a cold water (~18°C) fish and showed the lowest lysozyme level at the highest temperature (32.5 °C) (Kumari *et al*., 2006) whereas a clear reduction in lysozyme level was marked in gilthead seabream (*Sparus aurata*), a warm water (~25°C) fish, at lower temperature (11°C) (Tort *et al*., 1998). Temperature affect all living organisms, however fish, as ectothermic animals, is a thermo-dependent organism and the temperature critically influences your physiological mechanisms.

Despite the suppressive effects of temperature drop on lysozyme concentration, the environmental condition did not affect the HA-AP response. The LPS inoculation also did not affect such response, however β-glucan activated HA-AP response in thermal stressed fish. According to Zarkadis *et al*. (2001), the optimal temperature for complement activation in fish is 20-25°C, although this system has a very wide range of temperatures for activation, which along the diversity of key components and high titers make a very powerful defense system in fish (Boshra and Sunyer, 2006). β-glucan bind specific receptors on immune cells, like toll-like receptors and complement receptor 3, activating a series of process involved in inflammation (Novak *et al*., 2008; Ji *et al*., 2020). Similar to our results, β-glucan stimulated complement system responses in common carp (*Cyprinus carpio*) after *Aeromonas salmonicida* infection (Pionnier *et al*., 2013) and in pacu (*Piaractus mesopotamicus*) after transport followed by *Aeromonas hydrophila* injection (Mello *et al*., 2019). In a recent study, Ji *et al*. (2020) found that β-glucan up-regulated gene expression of complement system, such as c1ql2, c3, c5, c7, c9, cfb and cfh in rainbow trout

(*Oncorhynchus mykiss*), which can explain the enhancement in HA-AP observed in this study.

A decrease in environmental temperature promote an adaptive response of fish with increase in the lipid unsaturation to maintain the biological membranes fluidity (Martínez-Álvarez *et al*., 2005; Crockett, 2008; Sladjan *et al*., 2010). However, this scenario favors an increase in oxidative damage, since unsaturated fatty acids are highly susceptible to lipid peroxidation (LPO) (Crockett, 2008), so LPO content is considered a valuable biomarker of oxidative damage of cellular constituents. In this study, we observed higher LPO in thermal stressed fish (only numerically in control-fed fish) at basal sampling, nevertheless, after LPS injection, fish from TC group showed higher LPO. Drastic thermal alteration is a stressor since may result in several physiological consequences for fish (Donaldson *et al*., 2008), called stress response, which is an energy-intensive process (Magnadottir, 2006). The energy production mechanism produces ROS and the excess of ROS produced in high-energy demand situations cause oxidative damage in several cellular molecules (Schwarz, 1996; Nimse and Pal, 2015). Bacterial LPS is an important component of external cell wall of gram-negative bacteria that triggers rapid and non-specific immune responses in fish, which can increase the oxidative stress (Jiang *et al*., 2017). Senegalese sole juveniles (*Solea senegalensis*) did not show alterations in LPO levels due to rise or drop of temperature. However, when fed with protein-unbalanced diet and exposed to acute colder temperature, fish exhibited a significant decrease on LPO levels (Castro *et al*., 2012). The authors concluded that nutritional condition is important to oxidative status and antioxidant response of fish to thermal challenge. According to this and our results, we hypothesize that homeostasis, including nutritional status or/and immune competence, is determinant to oxidative status and antioxidant response to thermal challenge in fish. Glucan reduced the oxidative damage (LPO) in thermal stressed pacu injected with LPS. In African catfish (*Clarias gariepinus*) glucan reduced another oxidative damage indicator, the malondialdehyde (MDA) content in liver, caused by chlorpyrifos exposure (Mokhbatly *et al.*, 2020). A study with yellow croaker (*Pseudosciaena crocea*) showed that β-glucan decreased ROS during the stress, indicating that β-glucan is efficient to reduce oxidative stress (Zheng *et al*., 2016).

Biological oxidation is a primitive and inevitable process, but organisms have defensive strategies. Several antioxidant enzymes prevent the oxidant reactions cascade, inactivating the reactive intermediates of oxygen and closing the lipid-peroxidation cycle (Martínez-Álvarez *et al*., 2005). Catalase (CAT), superoxide dismutase (SOD), and enzymes dependent on glutathione, as glutathione peroxidase (GPx), have been detected in most fish species (Rudneva, 1997; Martínez-Álvarez *et al*., 2005). SOD is the first enzyme to act against oxygen radicals, converting the superoxide anion (O_2^-) into oxygen and hydrogen peroxide (H_2O_2). The CAT is associated with elevated concentrations of hydrogen peroxide that is converted to water and oxygen and GSH-Px converts the H_2O_2 into water, using GSH as cosubstrate (Nimse and Pal, 2015). GST represents a group of enzymes, which promote detoxification of toxic metabolites produced by oxidative damage (Maltez *et al*., 2018). Despite the thermal challenge has increased the LPO, it did not show a clear effect on antioxidant defenses. Our results suggest differential regulation of the enzymes during the thermal stress response, which is possible considering that these enzymes are responsible for different actions during the redox cycle. According to Kaur *et al*. (2005), antioxidant enzymes can be induced by mild oxidative stress and suppressed or inhibited in the case of severe oxidative stress. Furthermore, adaptation to temperature changes involves adjustments of density and activities of the mitochondria, affecting ROS generation and antioxidant defenses (Martínez-Álvarez *et al*., 2005). Otherwise, the LPS injection reduced antioxidant enzymes activity in liver of pacu, especially combined with thermal stress. We conclude that the additional stressor (LPS injection) imposed in thermal stressed fish compromised the antioxidant response of fish. Studies have shown that β-glucans can enhance antioxidant enzymes activities after infection in fish (Kim *et al*., 2009; Guzmán-Villanueva *et al*., 2013). Glucan increased GSH, GPx and CAT activity in African catfish (*Clarias gariepinus*) intestines exposed to chlorpyrifos (Mokhbatly *et al.*, 2020). However, in this study, glucan-fed fish was LPS injected after thermal challenge and did not exhibit improvement of antioxidant enzymes activities. On the other hand, the reduction caused by combination of thermal stress and LPS injection occurred mainly in control-fed fish (see fig.7 and 9), so we believe that glucan acted with a protector role, preventing the harmful effect of two stressors on antioxidant system. Additionally, as glucan decreased the oxidative damage in

thermal stressed and LPS injected fish, an increase in antioxidant activities would require more energy unnecessarily.

5. Conclusion

Thermal challenge and LPS injection were stressful situations. While the thermal stress affected negatively humoral innate immune system, the LPS injection impaired the antioxidant enzymes activity. Although β-glucan showed no clear immunostimulant effect, this molecule improved oxidative status in pacu thermal stressed and injected with LPS, reducing the lipid peroxidation, and preventing decrease in antioxidant enzymes activity.

6. Acknowledge

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