UNIVERSIDADE ESTADUAL PAULISTA JÚLIO MESQUITA FILHO

PROGRAMA DE PÓS-GRADUAÇÃO EM ZOOTECNIA

IMMUNOMODULATION WITH β-GLUCAN IN MATRINXÃ (*BRYCON AMAZONICUS*)

Luz Natalia Franco Montoya

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CURRICULUM DATA

LUZ NATALIA FRANCO MONTOYA – born in Colombia, on 25/09/1979. In 2005, she graduated from Caldas University in Colombia with a degree in Veterinary Medicine and Animal Science. She continued her studies at Caldas University and in 2006 she completed postgraduate work, specializing in Higher Education. In August 2006, she attended the *Universidade Federal de Viçosa (UFV)* in Minas Gerais, Brazil, and applied to the Master of Science program in Veterinary Medicine (Animal Comparative Morphophysiology), receiving her master's degree in August 2008. Since then, she has taught comparative anatomy and animal physiology in various universities in her country until 2012. In August of that year, she joined the PhD program in Animal Science in the Faculty of Agricultural and Veterinarian Sciences at *Universidade Estadual Paulista (UNESP)* in Jaboticabal, Brazil. There, she worked to develop her thesis on fish physiology and metabolism under the guidance of Professor Elisabeth Criscuolo Urbinati.

A mi madre e hija

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List of Abbreviations

ACTH	Adrenocorticotropic hormone
APP	Acute phase protein
APR	Acute phase response
βGR	β-glucan receptor
CRH	Corticotropic release hormone
HHI	Hypothalamus / Pituitary (Hypophysis) / Inter-renal axis
hpi	Hours post infection
IP	Intraperitoneal
MTP	Metyrapone
PAMP	Pathogen-associated molecular pattern
RAL	Respiratory activity of leukocytes
ROS	Reactive oxygen species

IMMUNOMODULATION WITH β-GLUCAN IN MATRINXÃ (*BRYCON AMAZONICUS*)

Abstract

This study was performed through three experiments and is presented in six chapters. In the first, we present a general introduction on the subject of the thesis and in the last chapter we present the final considerations and thesis conclusions. The other four chapters were written as scientific papers to be submitted to specialized journals. In the first experiment, we tested the innate immune response of juvenile matrinxã *Brycon amazonicus* fed with two concentrations of β-glucan (β-G) derived from *Saccharomyces cerevisiae* (0.5% and 1.0%) during 10 days. After feeding, we experimentally infected the fish with an intraperitoneal (IP) injection of *Aeromonas hydrophila* and sampled the animals prior to and 6 h post infection (hpi). The results showed that β-glucan (0.5%) improved the pre-infection cortisol and lysozyme serum levels and modulated some acute phase proteins on the acute phase response of fish. In addition, we observed that β-glucan (1.0%) led to the exhaustion of innate immune response and can be detrimental to health of fish. In the second experiment, we evaluated two generations of β-glucan, with different levels of purity: MacroGard (β-G 1° 71% pure) and a new generation "R&D β-glucan" (β-G 2° 62% pure). Two groups of fish were supplemented during 15 days with 0.1% of respective generations of β-glucan and at the end of trial fish were challenged with IP injection of *A. hydrophila.* Fish were sampled prior to, 6, 24, and 72 hpi. Data from this experiment is presented in chapters 3 and 4 of this thesis. The results showed that β-G 2° was more efficient to stimulate both humoral and cellular innate immune responses in fish. However, both β-glucan generations showed an ability to increase pre-infection cortisol and lysozyme serum levels as well as increase the number of circulating neutrophils and monocytes. In addition, the use of β-G 2° was observed to modulate the serum protein profile during acute phase response by bacterial infection of fish. In the third experiment, we discussed the role of serum cortisol levels on the immunostimulant effect of β-glucan. To this end, we fed fish during 15 days with diets containing β-glucan 0.1% only (β-G) or β-glucan 0.1% + metyrapone 30mg kg^{-1} fish (β-G+MTP). Dietary MTP was used to block the cortisol production. The fish were then submitted to 3 min of air exposure as an acute stressor and, following that, challenged with an IP injection of *A. hydrophila*. Fish were sampled prior to stress conditions, 30 min after stressor exposure and 24 hpi. The results showed that β-G modulated the cortisol profile prior to and after stress response, and increased both the number and activity of leukocytes. Furthermore, cortisol was shown to be a strong modulator of both humoral and cellular innate immune mechanisms, since it increased the lysozyme and complement activity as well as the neutrophils and monocytes populations. Our results suggest that β-glucan-induced cortisol level is one important mechanism to improve the innate immune response to β-glucans in matrinxã. Finally, we propose a protocol of immunestimulation for juvenile matrinxã. The addition of β-glucan derived from the cell wall of *Saccharomyces cerevisiae* can be offered to the fish before management practices. The proposed protocol aims to strengthen fish defense mechanisms, reduce disease outbreak and enhance fish resistance, generating a nutritional product of high quality and safety.

Keywords: Humoral and cellular immunity, early immune defense, stress, infection

IMMUNOMODULATION WITH β-GLUCAN IN MATRINXÃ (*BRYCON AMAZONICUS*)

Resumo

Este estudo foi feito por meio de três experimentos e é apresentado em seis capítulos. No primeiro, apresentamos uma introdução geral sobre o tema da tese e no último as considerações finais. Os capítulos restantes foram escritos como artigos científicos. No primeiro experimento, foi avaliada a resposta imune inata de juvenis de matrinxã *Brycon amazonicus* alimentados com duas concentrações de β-glucano (β-G) derivado de *S. cerevisiae* (0,5% e 1,0%) durante 10 dias. Depois da alimentação, os peixes foram infectados experimentalmente com injeção intraperitoneal (IP) de *A. hydrophila*. Os peixes foram amostrados antes e 6 h após a infecção (hpi). Os resultados mostraram que o β-G (0,5%) aumentou os níveis de cortisol sérico e da lisozima antes da infecção e modulou algumas proteínas de fase aguda na resposta imune do matrinxã. Além disso, observamos que 1,0% de β-G na dieta provocou exaustão da resposta imune inata e pode ser prejudicial para a saúde de peixes. O segundo experimento avaliou a inclusão de duas gerações de β-G na dieta, com diferentes níveis de pureza: MacroGard (β-G 1° 71% puro) e uma nova geração do produto "R&D β-glucano" (β-G 2° 62% puro). Os peixes foram alimentados por 15 dias com 0,1% de cada tipo de β-G e depois desafiados com injeção IP de *A. hydrophila*. Os peixes foram amostrados antes, 6, 24, e 72 hpi. Com os dados deste experimento foram escritos os capítulos 3 e 4. Os resultados mostraram que o β-G 2 ° foi mais eficiente para estimular a resposta humoral e celular do sistema imune inato. No entanto, ambas as gerações de β-G aumentaram o cortisol pré-infecção os níveis séricos de lisozima, e o número de neutrófilos e monócitos circulantes. A utilização do β-G 2 ° modulou o perfil de proteínas de soro durante a infecção bacteriana. No terceiro experimento, avaliamos o papel do cortisol na ação imunestimulante do β-glucano. Neste sentido, peixes foram alimentados por 15 dias com dietas que continham Macrogard β-G (0,1%) ou β-G (0,1%) + metirapona (MTP). A MTP foi usada para bloquear a produção do cortisol. Em seguida, os peixes foram submetidos a 3 min de exposição aérea como estressor agudo e logo após desafiados com *A. hydrophila*. Os peixes foram amostrados antes do estresse, 30 min após a exposição ao estressor e 24 hpi. Os resultados mostraram que o β-G modulou o perfil de cortisol antes e depois da resposta de estresse e aumentou o número e a atividade dos leucócitos. Observamos um forte efeito modulador do cortisol nos mecanismos humorais e celulares da imunidade inata do matrinxã, pois aumentou os níveis de lisozima e a atividade do sistema complemento, bem como as populações de neutrófilos e monócitos. O nosso resultado sugere que o aumento do cortisol induzido pelo β-G é um importante mecanismo para melhorar a resposta imune inata promovida por β-G em matrinxã. Finalmente, nós propomos um protocolo de imunestimulação para juvenis de matrinxã pela suplementação com o β-G de *S. cerevisiae* fornecido dias antes das práticas de manejo estressantes. Este protocolo visa reforçar os mecanismos de defesa immune, reduzir surtos de doenças e aumentar a resistência dos peixes, gerando um produto nutricional de alta qualidade e segurança.

Palavras-chave: Imunidade humoral e celular, defesa precoce, estresse, infecção

CHAPTER 1

General considerations

1. Introduction and justification

Fish farming plays an important role in food safety and nutrition worldwide (ALLISON, 2011). Fish have unique nutritional benefits to human health; they are key elements of a healthy diet and are an important source of essential nutrients, e.g., proteins of high value, micronutrients and essential fatty acids (FAO, 2012). Aquaculture is often viewed as the main solution to provide more fish products, given that extractive fishing of wild populations has reached its upper limit (NAYLOR et al., 2000; FAO, 2009), which has led to an increase in fish farming. In addition, aquaculture has progressively played an important sociocultural role in the provision of animal protein, gourmet cuisines, job opportunities, and as a foreign currency for developing countries (LIAO; CHAO, 2009; ALLISON, 2011). Moreover, the rapid and exponential growth of fish farms has led to the introduction of potential hazards such as uncontrolled growth of unwanted seaweeds, fish, invertebrates, parasites, pathogens, and microorganisms that represent serious risks to the aquatic ecosystem and human health (NAYLOR; WILLIAMS; STRONG, 2001; ERONDU; ANYANWU, 2011).

Furthermore, the rise in fish production has led to intensified management practices in the search for increased profitability and efficiency. Therefore, in intensive fish farming there are common stressful situations for fish e.g., confinement at high densities, capture, handling, reproductive management, and transport (URBINATI; CARNEIRO; CYRINO, 2004). In fish, stressful situations by handling affect the health status of the animal and generate losses to the producer, which lead to the use and abuse of antimicrobians as therapeutics to treat several diseases caused by pathogenic microorganisms (ROMERO, 2012).

In order to improve conditions for fish production and avoid economic losses, several alternative products have been tested in fish. The list includes vaccines (LORENZEN; LAPATRA, 2005; SOMMERSET et al., 2005; BRUDESETH et al., 2013); probiotics (MARTEAU et al., 2001; NAYAK, 2010; MARTÍNEZ CRUZ et al., 2012; LAZADO; CAIPANG, 2014), and prebiotics (GATESOUPE, 2005; RINGØ et

al., 2010; GANGULY et al., 2013; SONG et al., 2014). The use of immunostimulants or immunomodulators in aquaculture has been also described in detail (BRICKNELL; DALMO, 2005; MAGNADOTTIR, 2010; MEENA et al., 2013; MASTAN, 2015; MEHANA; RAHMANI; ALY, 2015). Immunomodulators are considered the main tool of modern aquaculture to directly increase the animal's resistance to infectious diseases (MEENA et al., 2013). One of the most important group of immunomodulators is the glucans family, considered to be able to improve the health status in a wide number of animal species, the glucans family are direct stimulants for the innate immune system and do not represent any risk to human health (JØRGENSEN et al., 1993; JENEY; JENEY, 1997; DALMO; BØGWALD, 2008; VETVICKA et al., 2011; MEENA et al., 2013).

The rapid growth of production and consequent intensification of productive practices in modern aquaculture, are responsible for direct and indirect effects of stress on disease resistance in fish. Thus, promoting appropriate management practices and encouraging the use of immunostimulants in fish is necessary to strengthen their defense mechanisms. In this sense, we evaluated the dietary β-glucan effects on the early innate defense mechanism, as well as on the cortisol serum levels in matrinxã prior to and after an acute infection. Our results led to a better understanding of β-glucan effects on cortisol serum levels and on the immune system with the aim to establish new dietary protocols that lead to the strengthening of the early immune response in teleost fish.

2. Review

2.1. Stress physiology in teleost fish

Stress is defined as a condition in which the homeostasis of the animal body is threatened or disturbed by intrinsic or extrinsic stimuli, commonly defined as stressors (CHROUSOS, 1992). In teleost fish, stress response shows many similarities to that of the terrestrial vertebrates in both the neuroendocrine systems from the brain to chromaffin cells (catecholamine secretion) and to inter-renal cells (cortisol secretion), as well as to the metabolic and ionic adjustments (WENDELAAR BONGA, 1997). The stressors display coordinated compensatory or adaptive physiological and behavioral responses, allowing the animal to overcome the threat (IWAMA, 1998).

The neuroendocrine network that modulates the stress response in fish is compounded by the central nervous system – chromaffin cells axis and the hypothalamus / hypophysis / inter-renal (HHI) axis (Fig. 1). The perception of stressors stimulates primarily the central nervous system pathways to generate the neuroendocrine response (WENDELAAR BONGA, 1997). The first activated pathway is the sympathetic nervous system that stimulates the release of catecholamines (adrenaline and noradrenaline) by the chromaffin tissue, located on the head kidney in teleost fish. The second pathway consists in the activation of the HHI axis, starting in the hypothalamus, which secretes the corticotrophin releasing hormone CRH, to stimulate the hypophysiary synthesis and the release of the adrenocorticotropic hormone – ACTH, which will act directly on the inter-renal tissue by stimulating the release of cortisol (BARTON; IWAMA, 1991; URBINATI; CARNEIRO; CYRINO, 2004).

The overall effect of stress can be differentiated into three categories (BARTON; IWAMA, 1991). The primary component includes neuroendocrine changes such as increased circulating catecholamine and cortisol. The secondary component includes metabolic changes such as increased glucose, glycogen mobilization and restoration, lactate production, higher oxygen consumption, among others: ionic changes in order to keep the osmorregulatory balance and hematological changes, such as the increasing of red and white blood cells. The tertiary component is established by chronic stimulation of the stress system which can impair growth, reproductive rate and immune response, and promote behavioral changes such as decreased food intake and increased aggression (BARTON; IWAMA, 1991; WENDELAAR BONGA, 1997; URBINATI; CARNEIRO; CYRINO, 2004; TORT, 2011)

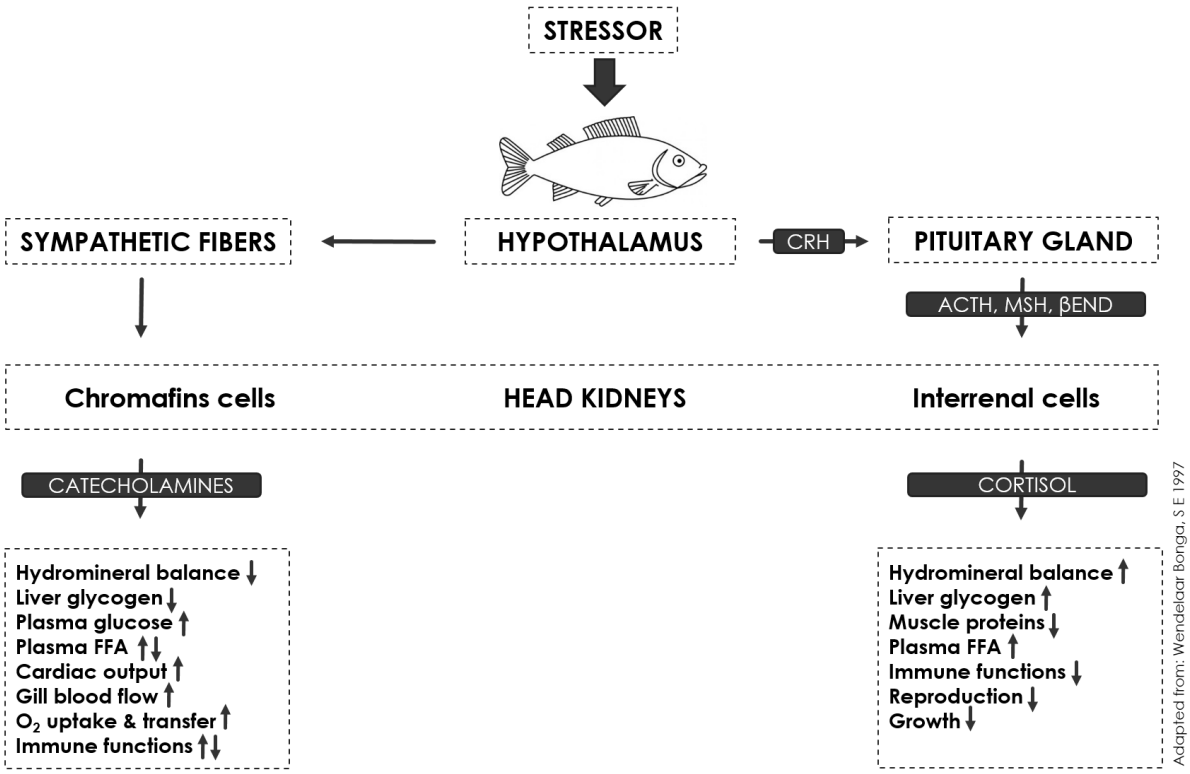

Figure 1. Stress physiology in teleost fish. Adapted from Bonga WSE 1997.

The primary and secondary stress responses have adaptive value for the organism, however the tertiary responses can become maladaptive and detrimental to the health status and welfare in animals subject to severe or prolonged stressors (PRUNET et al., 2008). Comparatively, the exposure to an acute stress factor activates the immune system by an increase of the innate response, leukocytes mobilization, and lymphocytes T helper response. On the other hand, chronic exposure to stressful stimuli can lead to immunosuppression measured by the decrease of immune innate response, distribution and differentiation of leukocytes that make fish more vulnerable to pathogens (Fig. 2) (TORT, 2011).

2.1. Cortisol biosynthesis and metyrapone mechanism

Cortisol is the main circulating glucocorticoid in teleost fish, whose concentration increases dramatically during stress response (ALURU; VIJAYAN, 2009). However, cortisol is always present in vertebrates, even under unstressed conditions, playing metabolic maintenance or “housekeeping” roles; nevertheless, cortisol functions tend to be appreciated when its concentration and actions go well beyond housekeeping range (MOMMSEN; VIJAYAN; MOON, 1999).

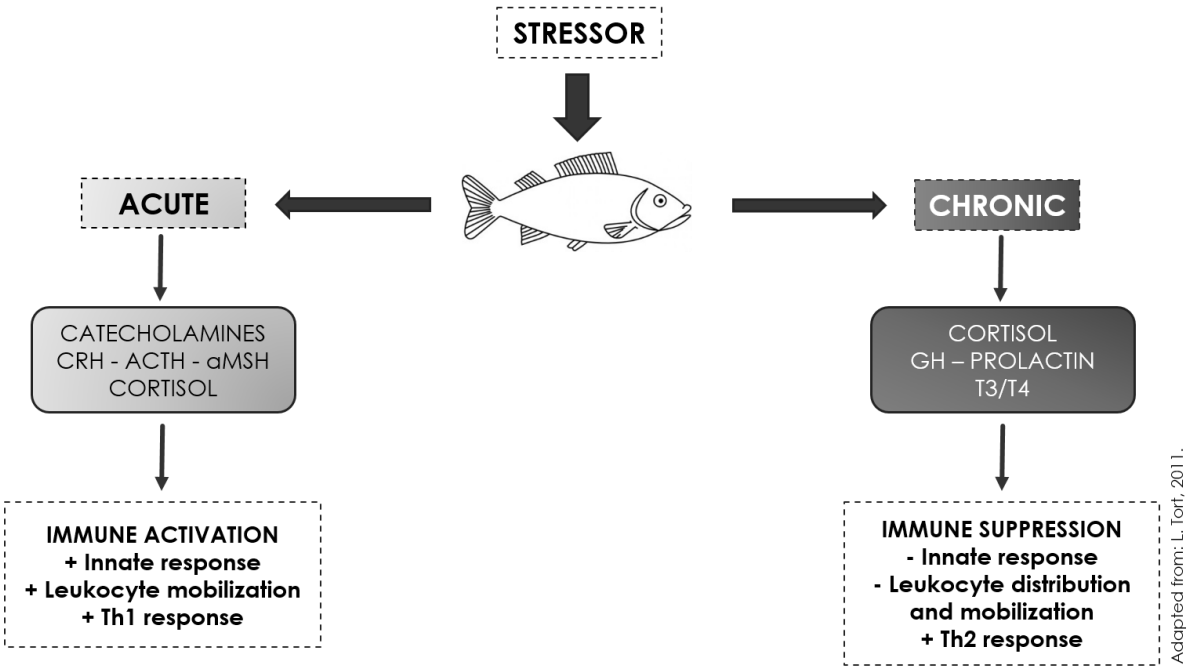

Figure 2. Acute and chronic stress effects on the immune response in teleost fish. Adapted from Tort, 2011.

Teleost fish do not have an adrenal gland like mammals, in its place the steroidogenic cells called inter-renal cells are distributed in the head-kidney region (ALURU; VIJAYAN, 2009; MOMMSEN; VIJAYAN; MOON, 1999). The cortisol biosynthesis in fish is similar to that in mammals and involves microsomal enzymatic pathways, including 21-hydroxylation (P450c21), 17α-hydroxylation (P450c17), and 3β-hydroxy steroid dehydrogenation (3β-HSD). In addition, fish possess the mitochondrial inner membrane monooxygenase enzymes, such as the cholesterol side-chain cleavage enzyme (cytochrome P450scc, desmolase) and the 11β-hydroxylase that catalyze the 11β-hydroxylation of deoxycortisol/deoxy-corticosterone (cytochrome P450c11), (MOMMSEN; VIJAYAN; MOON, 1999).

Cortisol receptors were described in both fish hepatic and extra-hepatic tissues e.g., gonads, brain, gills, and intestine, which can explain the multifaceted role of cortisol during stress adaptation response (ALURU; VIJAYAN, 2009; PRUNET et al., 2008). In addition to stimulating gluconeogenesis and lipolysis (BARTON, 2002) cortisol in fish and mammals, has metabolic effects by increasing plasma glucose levels to supply the homeostatic mechanisms activated during exposure to stressors (LEACH; TAYLOR, 1980; MARTINEZ-PORCHAS; MARTINEZ-CORDOVA; RAMOS-ENRIQUEZ, 2009). Cortisol effects also include osmotic- and ionic-regulation, growth, reproduction, immune responses, and behavior (BARTON;

IWAMA, 1991; WENDELAAR BONGA, 1997; MOMMSEN; VIJAYAN; MOON, 1999; ELLIS et al., 2012).

To form a better understanding of the regulation of the immune response by cortisol, different experimental protocols have been used and include stimulus or inhibition of cortisol biosynthesis. Several substances used to inhibit secretion of cortisol are utilized therapeutically, such as metyrapone, ketoconazole and fluconazole. These drugs act by inhibiting the steroidogenesis (SCHTEINGART, 2009). Because of their action, they have been used as a tool to elucidate the effects of cortisol over the immune system. The metyrapone (MTP) (2-methyl-1, 2-di-3-pyridyl-1-propanon) is a competitive inhibitor of the conversion of 11-deoxycortisol to cortisol by the 11-beta-hydroxylase during the cortisol biosynthesis (BROADLEY et al., 2005). As the biosynthesis of cortisol in fish is similar to that of mammals (MOMMSEN; VIJAYAN; MOON, 1999), metyrapone use can result in a reduction of high cortisol levels under stress condition even in fish (BENNETT; RHODES; RHODES III, 1986; ZANUZZO; URBINATI, 2015). MTP was used in different experimental designs in fish, to study the role of cortisol in metabolism, as a regulator of the muscle glycogen production after exercise in rainbow trout *Oncorhynchus mykiss* (MILLIGAN, 2003), and also to assess the effect of cortisol on the metabolism of catfish, *Clarias batrachus* (TRIPATHI; VERMA, 2003). In addition, MTP was used to determine the physiological role of cortisol in the regulation of estrogen biosynthesis and masculinization of *Japanese flounder* (YAMAGUCHI et al., 2010). However, to our knowledge there are no reports of the use of MTP to evaluate the cortisol role on the immune response during acute stress situations in fish.

2.2. *Immune system in teleost fish*

The immune system is largely affected by the stress mechanism, whose responsiveness is suppressed under chronic stress (WEENDELAR BONGA, 1997). This can make animals vulnerable to the attack of pathogens present in the environment. The fish immune system response presents two components, which differ in speed and specificity: innate or non-specific and acquired or specific (LEVRAUD; BOUDINOT, 2009; URIBE et al., 2011; WATTS; MUNDAY; BURKE, 2001).

The first line of defense of the body is the innate response, which serves to prevent infection, eliminate potential pathogens, and initiate the inflammatory response (CHARLES A JANEWAY et al., 2001; URIBE et al., 2011). The innate immune system generates a relatively quick response, but does not promote immune memory (URIBE et al., 2011; WATTS; MUNDAY; BURKE, 2001).

The innate immune response involves distinct defense mechanisms, commonly divided in three components: 1) The epithelial mucosal barrier e.g., skin and mucous-membranes (GOMEZ; SUNYER; SALINAS, 2013; ROMBOUT JAN et al., 2011); 2) The humoral factors, that may be cellular receptors or molecules, soluble in plasma and other body fluids (MAGNADOTTIR et al., 2005), which alter the metabolism of the host to inhibit bacterial growth, i.e. the acute phase proteins (CRAY, 2012); 3) The cellular component, composed by phagocytic barriers i.e., tissue base cells that can attack, ingest, and destroy pathogens (URIBE et al., 2011).

Humoral factors include agglutinins, precipitins, primarily lectins, natural antibodies, cytokines, chemokines and antibacterial peptides, among others that can promote inflammatory responses, detect antigens, and stimulate the activity of the adaptive immune response (HOLLAND; LAMBRIS, 2002; MAGNADÓTTIR, 2006; SAURABH; SAHOO, 2008; URIBE et al., 2011). The cellular components of the innate immune response include phagocytic cells such as granulocytes, monocytes, macrophages, and natural killer cells (BILLER-TAKAHASHI et al., 2013). The main functions of these cells are the phagocytosis of tissue debris and microorganisms, secretion of humoral factors, as well as being a bridge between the innate and adaptive immune system (LEVRAUD; BOUDINOT, 2009).

A second line of defense of the immune system is the acquired / specific or adaptive response, an array of humoral and cellular responses that generate a specific response against the pathogens (LITMAN; RAST; FUGMANN, 2010). Specific cells involved T and B lymphocytes, which are mediators of cellular and humoral responses, e.g., recognition of pathogens and antibodies production (LIESCHKE; TREDE, 2009; URIBE et al., 2011). However, there are distinct populations of lymphocyte called natural killers or T cytotoxic that have been classified as an innate immune compound and rely on the ability to destroy injured somatic cells (tumor or viruses infected cells) and produce immune modulation cytokines (see review BILLER-TAKAHASHI; URBINATI, 2014; RAULET, 2004).

Unlike mammals, teleost fish have no separate bone marrow hematopoietic tissue, instead, the primary site of hematopoiesis is the cranial head kidney (LEVRAUD; BOUDINOT, 2009; PRESS, 1999). Other organs and tissues of the fish's immune system include the thymus, spleen, gut associated lymphoid tissue (GALT) and excretory interstitial tissue in the kidneys (URIBE et al., 2011; LIESCHKE; TREDE, 2009; PRESS, 1999).

2.3. Acute phase response and acute phase proteins in teleost fish

The first line of defense of a vertebrate body in immunological challenges is the innate immune system, or non-specific response, composed by several reactions which precede the specific immune response (AOKI et al., 2008). One of these reactions is known as the acute phase response (APR), an early and synchronic response that leads to metabolic and physiologic changes in the host after local or systemic homeostatic disturbances, caused by tissue injury or infection (BAYNE; GERWICK, 2001; GRUYS et al., 2005; SALGADO; ARIAS, 1994). APR involves a wide variety of steps that aim to prevent tissue damage, attempt to isolate and eliminate the cause of inflammation or lesion, and begin the repair process to restore normal body function (GRUYS et al., 2005; CRAY, 2012). In general, local reactions are accompanied by a systemic response characterized by a fast alteration of several macromolecules' concentration in body fluids, with metabolic changes in many organ systems (BAYNE; GERWICK, 2001; JENSEN et al., 1997; TÎRZIU, 2009). The start of APR is marked by the migration of tissue macrophages, blood monocytes, and dendritic cells to the site of tissue damage (Fig. 1). These cells' activation leads to the production of cytokines IL-1, IL-6 and TNF-α, which not only initiate the APR, but also result in chemotactic recruitment of additional cells to the affected area to rapidly augment the response (CRAY, 2012).

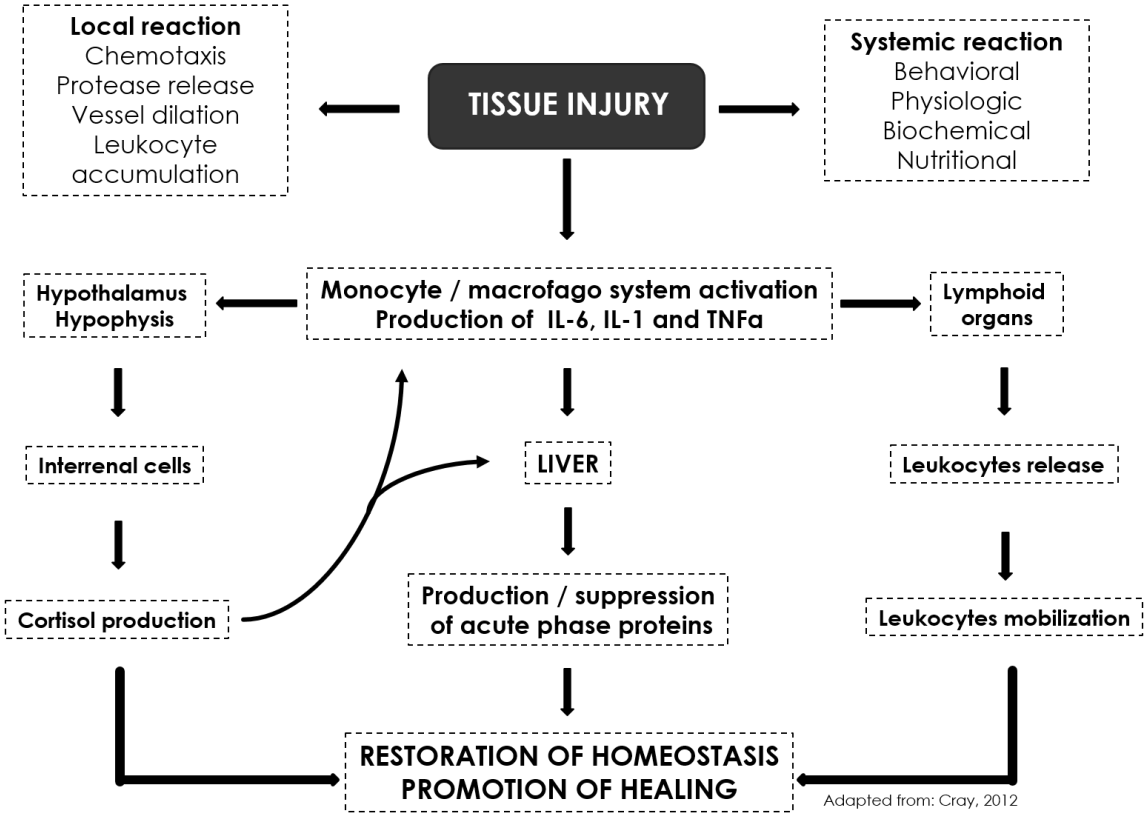

Figure 3. The acute phase response in fish. Adapted from CRAY (2012)

One clear indication of the acute phase response is the increase or decrease of the synthesis and secretion of several blood proteins, called acute phase proteins - APPs (BAUMANN; GAULDIE, 1994). APPs have been observed in many organisms from fish to mammals, but the type of APPs and their behavior during the acute phase response differ by species (CRAY, 2012). APP synthesis takes place mainly in the liver, but can also occur in other cell types e.g., monocytes, endothelial cells, fibroblasts and adipocytes (CRAY, 2012; ECKERSALL; BELL, 2010). Hepatic mRNA upregulation of the APPs is associated with a decrease in synthesis of normal blood proteins known as negative APPs, e.g., transthyretin or thyroxine-binding prealbumin (TTR), retinol-binding protein (RBP), and cortisol-binding globulin, whose decrease indicates a temporal increase of the availability of free hormones bound to these proteins (INGENBLEEK; YOUNG, 1994). The transferrin, iron transporter protein (CHUNG, 1984; STAFFORD; BELOSEVIC, 2003; WOJTCZAK; DIETRICH; CIERESZKO, 2005) and albumin, transporter protein of endogenous and exogenous ligands as nutrients or hormones, also represent negative APPs (ECKERSALL, 2008; GRUYS et al., 2005; ECKERSALL; BELL, 2010).

APPs are released by the hepatocytes after cytokine stimulation (HEINRICH; CASTELL; ANDUS, 1990). They play an important role in the optimization and modulation of the innate immune response, by acting in several steps of the response, such as trapping the microorganisms and their products, activating the complement system, binding to cellular remnants, neutralizing enzymes, and scavenging free hemoglobin and radicals (JAIN; GAUTAM; NASEEM, 2011). The positive APPs consist of mainly the C-reactive protein (CRP), which binds to microbes and enhances phagocytosis by macrophages, promoting complement system activation and opsonization. In addition, CRP modulates the cytokine production by monocytes and macrophages (BAYNE; GERWICK, 2001; JENSEN et al., 1997; SEO et al., 2012). The serum amyloid A (SAA) helps the immune response by modulation of fever induction, platelet activation, neutrophil oxidative burst, and chemotaxis. The haptoglobin (Hp) which binds to hemoglobin has a bacteriostatic effect (GRÖNLUND et al., 2003; WICHER; FRIES, 2006). The ceruloplasmin (CP) is a serum ferroxidase that carries more than 90% of the copper in plasma and helps in iron homeostasis (LIU et al., 2011). The CP molecular mass is ≈132 kDa, and participates in iron metabolism by oxidizing Fe^{2+} to Fe^{3+}, oxidizing biogenic amines, and serving as an antioxidant (LIU et al., 2011; SOKOLOV et al., 2005).

Positive APPs also include the lysozymes, the complement system protein complex (JENSEN et al., 1997; CRAY, 2012; BAYNE; GERWICK, 2001; ECKERSALL, 2000), and lysozyme serum concentrations (ABREU et al., 2009; SAURABH; SAHOO, 2008; WATTS; MUNDAY; BURKE, 2001). Lysozymes are lytic enzymes that hydrolyze glycosidic bonds and are widely distributed in body fluids, e.g., blood serum, mucus, and saliva (MAGNADOTTIR et al., 2005; SAURABH; SAHOO, 2008). In fish, lysozymes are capable of attacking peptidoglycans in both Gram-positive and Gram-negative bacteria. They are also known to activate the complement system and promote phagocytosis processes (SAURABH; SAHOO, 2008).

Another important component of the innate immune response is the complement system, which is the major humoral system of innate immunity and is comprised of a complex of nearly 30 individual proteins (NAKAO et al., 2011; HOLLAND; LAMBRIS, 2002). The complement system activity is widely recognized as an indicator of innate immune response in fish (BOSHRA; LI; SUNYER, 2006; HOLLAND; LAMBRIS, 2002; NAKAO et al., 2011). Activation of the complement system takes place through

different pathways, e.g., classical and lectin, alternative and cytolytic. These processes allow the activation of the protein complex that plays an important role in the killing and neutralization of microorganism (NAKAO et al., 2011). The complement system has also been described as mediator of phagocytosis, inflammatory reactions, immune complex clearance, and antibody production (HOLLAND; LAMBRIS, 2002; BILLER-TAKAHASHI et al., 2012; BOSHRA; LI; SUNYER, 2006). Both, lysozymes and the complement system, are currently considered to be acute phase proteins in fish, due to their early response to triggers and increased blood concentrations during an acute phase response (BAYNE; GERWICK, 2001).

Recently, it has been described that the action of both lysozyme and the complement system are related (GIMBO et al., 2015). The fish complement system displays bactericidal activity against non-virulent Gram-negative bacteria, but not against Gram-positive bacteria or virulent Gram-negative bacteria (BOSHRA; LI; SUNYER, 2006). Lipopolysaccharide in the cell walls of Gram-negative bacteria directly activates the alternative pathway of the complement system that can lead to lysis of the bacterial cell. The bactericidal action of lysozymes consist in hydrolyzing the β-(1,4) linkages between N-acetylmuramic acid and N-acetylglucosamine in the cell walls of Gram-positive bacteria, thus preventing them from invading. Lysozyme do not directly affect the Gram-negative bacteria, but when the outer cell wall of Gram-negative bacteria is disrupted, i.e. by the complement system, exposing the inner peptidoglycan layer of bacteria, then lysozyme becomes effective (AUSTIN; AUSTIN, 1999).

Functions of APPs include defense activities such as limiting the dispersal of infection agents, repairing tissue damage, inactivating proteases, killing microbes or potential pathogens and restorating health status (BAYNE; GERWICK, 2001). The APPs can be used to assess the innate immune response to infection, inflammation or trauma (CERON; ECKERSALL; MARTÝNEZ-SUBIELA, 2005; MURATA; SHIMADA; YOSHIOKA, 2004). In this sense, the determination of APPs is currently used as a diagnostic tool in human and veterinary medicine to back up the diagnoses of mainly infectious diseases, e.g., babesiosis, leptospirosis, leishmaniosis, parvovirus and erlichia in dogs, as well as, mastitis, viral diarrhea and respiratory disease in cattle, among others (ECKERSALL; BELL, 2010).

In fish, studies have been fruitful and clearly established that homologs of at least some known APPs are present in both elasmobranchs and teleost, and that in teleost

some of these plasma proteins increase in concentration in response to inflammatory stimuli (BAYNE; GERWICK, 2001; DOOLEY et al., 2010). In this sense, APPs are currently studied in commercial fish: *Salmo salar* and *Salmo gairdneri* (JENSEN et al., 1997); *Pargus auratus* (COOK et al., 2003); *Oreochromis mossambicus, Channa punctatus, Cyprinus carpio* and *Cirrhina mrigala* (SHAMSUDDDIN; SHAGUFTA, 2011), and *Oreochromis niloticus* (KUÇCEUKGUL GULEÇ; CENGIZLER, 2012). The measure of alterations in APP levels becomes an interesting diagnostic tool in fish disease as well as an indicator of the activation of the early defense mechanism after immunostimulation protocols.

2.4. Immunomodulation with β-glucan on fish

In an attempt to strengthen the immune system of farmed fish under stress in modern aquaculture, the use of immunostimulants or immunomodulators has been scattered (MEENA et al., 2013; NEWAJ-FYZUL; AUSTIN, 2014; NOVAK; VETVICKA, 2008). Immunostimulants can promote cell activity and proliferation of some leukocytes as monocyte-macrophages and neutrophils, as well as the stimulation of the phagocytic activity and secretion of immune mediators such as cytokines (VETVICKA et al., 2011; MEENA et al., 2013). The β-glucan is one of the several immunostimulants currently used in aquaculture (ALI; ELVEVOLD, 2009; DALMO; BØGWALD, 2008; JØRGENSEN; ROBERTSEN, 1995; MEENA et al., 2013). β-glucans represent part of a group of physiologically active compounds generally called “biological response modifiers” (NOVAK; VETVICKA, 2009). They are highly conserved and non-digestible carbohydrates that form structural components of cell walls of some plants, fungi, yeast, seaweed, and bacteria (VETVICKA et al., 2011). The common β-glucan is derived from the cell wall of baker’s yeast *Saccharomyces cerevisiae*. Glucans are a heterogeneous group of glucose polymers, consisting of a backbone of β-(1, 3)-linked β-D-glucopyranosyl units with β-(1, 6)-linked side chains of varying distribution and length (MEENA et al., 2013; NOVAK; VETVICKA, 2008).

The β-glucan has been shown to be immunostimulatory and has beneficial properties for animals, including increased protection against infections (JØRGENSEN et al., 1993; SELVARAJ; SAMPATH; SEKAR, 2005; VETVICKA et al., 2011), the development of tumors (CHEUNG et al., 2002; HONG et al., 2004) and

sepsis (BABAYIGIT et al., 2005; SENER et al., 2005). Its effect has been attributed to the glucans ability to bind to several types of leukocytes receptors, known as Toll-like receptors (TLR) and/or pattern recognition receptors (PRR) (BRICKNELL; DALMO, 2005; DALMO; BØGWALD, 2008). In fish, the binding ability of β-glucan, results in the stimulation of innate immune response also being adjuvant in the specific immune response. Some direct or indirect effects of β-glucan are an increase of bactericidal activity, (SELVARAJ; SAMPATH; SEKAR, 2005), the modulation of cytokine production (LJUNGMAN; LEANDERSON; TAGESSON, 1998), an increase of blood monocytes (BABAYIGIT et al., 2005), and an increase in number and phagocytic activity of leukocytes (JØRGENSEN; ROBERTSEN, 1995). Additional effects include the increase of serum lysozyme, hemolytic complement activity, as well as an increase of antibody response and resistance to *Aeromonas hydrophila* (VETVICKA; VANNUCCI; SIMA, 2013; JØRGENSEN; ROBERTSEN, 1995; NOVAK; VETVICKA, 2009; MEENA et al., 2013; DALMO; BØGWALD, 2008).

2.5. The Aeromonas hydrophila as bacterial challenge model

In order to prove the effectiveness of different immunostimulants, studies have used bacterial challenges to measure the ability to modulate the immune system response. In this type of test, one of the most studied bacteria is the *Aeromonas* species. Three species are phenotypically defined - *A. hydrophila, A. caviae* and *A. veronii* subtype *sobria*. *Aeromonas* species are gram-negative, and a facultative anaerobic bacterium (ABBOTT; CHEUNG; JANDA, 2003). *A. hydrophila* is present in soil and in all environments from freshwater to saltwater (MONFORT; BALEUX, 1991). Some strains of *A. hydrophila* are capable of causing disease in fish, amphibians, as well as humans, who can acquire the pathogen through open wounds or infections by ingesting a sufficient number of microorganisms in food or water. *Aeromonas* are being increasingly reported, especially *A. hydrophila*; it is responsible for hemorrhagic septicemia, a disease that affects a wide variety of freshwater fish and occasionally marine fish (ASHIRU et al., 2011; WANG; SILVA, 1999). Furthermore, *Aeromonas* is currently reported as an emerging pathogen in humans that causes a variety of diseases, most commonly gastroenteritis, and septicemia infection in children and adults (ABBOTT; CHEUNG; JANDA, 2003; GALINDO et al., 2006).

2.6. Matrinxã as an experimental model

The matrinxã *Brycon amazonicus* is a teleost fish of the genus Brycon and family Characidae, native of the Amazon River basin in Brazil, that has been considered a promising candidate for aquaculture throughout the country (FILHO; REYNALTE-TATAJE; WEINGARTNER, 2006), especially in the Amazonian region. Due to its active and reactive temperament, matrinxã have been studied as a biological model of the stress physiology (ABREU et al., 2008; FERRAZ; GOMES, 2009; SERRA; URBINATI; WOLKERS, 2014; WOLKERS; SERRA; URBINATI, 2015; WOLKERS et al., 2012, 2014). In addition, matrinxã have been used in studies concerning common procedures in aquaculture, such as transport (CARNEIRO; URBINATI, 2001; CARNEIRO; URBINATI, 2002; URBINATI; CARNEIRO; CYRINO, 2004; URBINATI et al. 2004), capture (CARVALHO; URBINATI, 2004; HOSHIBA; GONÇALVES; URBINTATI, 2009), stocking densities (CARNEIRO; URBINATI, 2002; URBINATI et al., 2004) and behavioral responses (FERRAZ; GOMES, 2009; SERRA; WOLKERS; URBINATI, 2015; WOLKERS; SERRA; URBINATI, 2015; WOLKERS et al., 2012, 2014). Therefore, it would benefit a candidate to assess the effects of the immunostimulant β-glucan in both cortisol serum levels and innate immune response, as that information represents valuable knowledge for future applications in fish farming.

Given the rapid growth in production and consumption of fish, and the consequent enhancements in productivity practices in modern aquaculture, it is important to know the direct and indirect effects of stress on disease resistance in fish. Besides promoting appropriate management practices, we should encourage the use of immunostimulants in animals to strengthen their defense mechanisms. Without neglecting the needs of the population, it is necessary to generate high quality nutritional products with low environmental cost. This study aims to provide new knowledge and strategies to lessen the impact of stressful cultivation techniques on fish health.

3. Hypothesis

Our hypothesis is that a juvenile matrinxã is immunologically more prepared to face stressors and bacterial infection when previously fed with dietary β-glucans.

4. Objectives

4.1. General objective

To evaluate the immunomodulatory effect of dietary β-glucans in matrinxã under immunological and physical stressful conditions.

4.2. Specific objectives

To study the immunomodulatory effect of dietary β -glucan, in juvenile matrinxã, prior to and after bacterial challenge.

To compare the immunomodulatory effect of two dietary β-glucans from 1° and 2° generations on acute stress response, innate immune response, and acute phase response by analyzing serum protein profiles in matrinxã.

To evaluate the cortisol role on the immunostimulator effect of dietary β-glucan 2° generation, through the blocking of cortisol secretion by dietary metyrapone.

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CHAPTER 2

Dietary β-glucan can modulate cortisol and acute phase protein levels in matrinxã (*Brycon amazonicus*) prior to and after bacterial infection

(Research paper to be submitted to Fish & Shellfish Immunology)

Highlights

β-glucan modulates the acute phase response in matrinxã.

β-glucan modulates serum cortisol levels prior to and after bacterial infection.

Acute *A. hydrophila* infection alters serum protein profiles in matrinxã.

β-glucan can modulate profiles of serum proteins including lysozyme in matrinxã.

The serum protein profile may provide an important tool for disease diagnosis in matrinxã.

Abstract

This study investigated the effects of dietary β-glucan on both cortisol secretion and the acute phase response (APR) of matrinxã (*Brycon amazonicus*) prior to and after bacterial infection by *Aeromonas hydrophila*. Fish were fed with feed supplemented with dietary β-glucan (0.0%, 0.5% and 1.0%) for 10 days before bacterial challenge. We measured serum cortisol and lysozyme concentrations as well as acute phase proteins prior to and 6 hours post infection (hpi). Before bacterial challenge, serum cortisol levels were higher in fish fed with the 0.5% β-glucan diet. At 6 hpi, β-glucan prevented increase in cortisol levels in fish fed with the 0.5% diet compared to fish from the control group. Serum lysozyme concentrations were higher, relative to the control, in fish fed with 0.5% β-glucan prior to bacterial challenge. At 6 hpi, control fish and those fed with 0.5% β-glucan displayed similar levels of blood serum lysozyme, whereas these levels were reduced in fish previously treated with 1% β-glucan. Three serum proteins with molecular weights of ≈190 kDa, ≈36 kDa and ≈26 kDa were characterized as positive acute phase proteins due to increased expressions at 6 hpi, while five serum proteins with molecular weights of ≈228 kDa, ≈212 kDa, ≈168 kDa and ≈90 kDa that had decreased expression at 6 hpi were characterized as negative acute phase proteins. Our results suggest that β-glucan may modulate cortisol levels and APR in matrinxã.

Key-words: immunostimulant, humoral innate immunity, acute infection, early defense.

1. Introduction

In intensive aquaculture, fish often face stressful situations such as confinement at high densities, handling, pathogens and low water quality, among others. These situations affect animal health and reduce productivity [1]. Stress-inducing agents can promote physiological responses such as hormone secretion, especially cortisol. In the long term, this increased hormonal activity suppresses functions that do not improve the chances of fish survival, including those of the immune system [2]. However, previous studies have described a short-term immune stimulation in response to acute stress [3,4]. These previous reports described increased blood levels of lysozyme and C3 proteins, components of the humoral immunity. Furthermore, glucocorticoid receptor number increased in head kidney leukocytes after acute handling stress [5]. In short, the stress activation phase involves the production of acute phase proteins and the release of cytokines, hormones and peptides that are stored or produced on a short-term basis [6]. These stimuli can lead to the start of early immune response in front of physical or immune stress situations.

Techniques used in modern aquaculture to modulate the immune system of fish include the use of several types of immunostimulants that prevent disease by improving fish resistance [7,8]. The β(1-3)-D-glucans, hereafter referred to as "β-glucans", represent an important group of highly conserved and indigestible carbohydrates originally derived from the cell walls of plants, fungi, yeast, seaweed, and bacteria [8]. β-glucans benefit animals and humans by protecting against infections [7,9], reducing the development of tumors [10], and combating sepsis [11]. These effects have been attributed to their capacity to bind specific receptor types, e.g., Toll-like receptors and Dectin-1, among others expressed on monocytes, dendritic cells, neutrophils and natural killer cells [7,12]. Receptor binding results in the stimulation of immune responses, including bactericidal activity [13] and cytokine production [14,15] as well as production of C-reactive protein and complement protein [16]. These immunomodulators can improve animal acute response against pathogens and disease.

The acute phase response (APR) is an early and synchronic reaction of the host to local or systemic homeostatic disturbances caused by pathogens, parasites or tissue injury as well as other factors [17,18]. APR involves a wide variety of reactions that minimize tissue damage and restore normal body function [19]. APR is marked by changes in the production and secretion of liver proteins called acute phase

proteins (APPs) [20]. APP functions include defense activities, such as limiting the dispersal of infection agents, repairing tissue damage, inactivating proteases, killing pathogens, and restoring the healthy state [18, 20]. In fish, studies have described APP levels and activity in response to bacterial and parasite infections in several species: *Salmo salar* and *Salmo gairdneri* [21]; *Pagrus auratus* [22]; *Oreochromis mossambicus*, *Channa punctatus*, and *Cirrhina mrigala* [23]; *Oreochromis niloticus* [24], and *Cyprinus carpio* [23,25]. To date, only one study using *Cyprinus carpio* has described the immunostimulant effects of dietary β-glucan on serum proteins levels during APR caused by infection with *Aeromonas salmonicida*.

Despite this recent report, little is known about the effects of β-glucan on fish cortisol secretion during APR and on the modulation of APP levels. Therefore, we tested the effects of dietary β-glucan on matrinxã (*Brycon amazonicus*), over the cortisol and lysozyme serum levels as well as the electrophoretic proteins profile, prior to and after acute bacterial challenge with *Aeromonas hydrophila*. Our results offer novel information on immunostimulant effects on cortisol and serum proteins profiles during the first hours of an APR.

2. Material and methods

2.1. Experimental animals and lab conditions

This study utilized 42 matrinxã juveniles (104.8 ± 12.7 g and 20.1 ± 0.8 cm) that were kept individually in 40-liter fiber tanks (density ~2.6 g fish L^{-1}). During a 7-day period of acclimatization to laboratory conditions, fish were fed a commercial feed (28% crude protein CP). Temperature and oxygen levels were 29.2 ± 0.5°C and 6.1 ± 0.1 mg L^{-1}, respectively, with a photoperiod of 12 h light: 12 h dark.

2.2. Experimental design and diets

The present study evaluated diets with two levels of β-glucan (0.5% and 1%) added to a commercial feed (28% CP), and their effects were compared to those of a β-glucan-free control diet. After acclimatization, fish were fed with 3% of their body mass twice a day (11:00 and 16:00 h) during 10 days. Twelve fish were used per treatment (0.0%, 0.5% and 1% β-glucan). Six additional fish were fed with control feed to provide a negative control group for infection with *A. hydrophila*. On day 11,

fish were sampled to determine their prior infection condition, after which six fish per treatment were inoculated in the mesenteric cavity with a sublethal concentration of *A. hydrophila* (5 µl g^{-1}), while six fish from the negative control group were inoculated with the same quantity of phosphate buffer saline solution (PBS). Six hours post infection (6 hpi) fish were sampled again in order to evaluate serum cortisol, lysozyme, and APP levels.

Experimental diets were prepared using an extruded commercial feed that was ground, mixed to incorporate the 0.5% or 1.0% of β-glucan, and then in order to re-pelletize the food was moistened with 40% water; the feed was then passed through a food processor, and finally dried in an oven with air extraction at 40 °C for 24 hours. The control feed was β-glucan-free. The immunostimulant used was a commercial preparation of β-glucan (Macrogard®) derived from *Saccaromyces cerevisiae* with 71% activity (Biorigin, São Paulo, Brazil; http://www.biorigin.net/biorigin/macrogard/macrogard_en/index.html).

2.3. Acute bacterial challenge

A bacterial suspension was obtained using an *A. hydrophila* strain isolated from the skin of *Pseudoplatystoma corruscans* (strain A122 - GenBank access: KJ561021). For bacterial growth, 200 µl of the pure strain was added to 200 ml of sterile tryptone soya broth (Himedia M011; http://www.himedialabs.com/TD/M011.pdf). This suspension was incubated at 30°C for 24 h and then centrifuged at 8000 x g for 10 min. The supernatant was discarded and the pellet was washed three times with PBS buffer (0.01 M). Finally, the bacterial PBS suspension lower than the lethal concentration CL-50 used was (1 x 10^4 CFU ml^{-1}) adjusted according to the nephelometric McFarland scale and read in a spectrophotometer (OD_{600}=0.682). The efficacy of the *A. hydrophila* suspension to cause infection and clinical signs in matrinxã was previously determined in order to guarantee the acute phase response in challenged fish. For bacterial inoculation, fish were anaesthetized by immersion in benzocaine water solution (0.1 g L^{-1}), after which six fish per treatment were individually weighed and then inoculated with 5 µl g^{-1} of *A. hydrophila*. Fish from the negative control group were inoculated with PBS as described above. All inoculated fish were immediately returned to their individual tanks.

2.4. Sampling

At each sampling time, six fish per treatment were anaesthetized and a blood sample was drawn from the caudal vessel using a syringe without anticoagulant. Blood was dispensed into 2-ml microtubes that were maintained at room temperature for 3 h and then centrifuged (3000 rpm for 5 min) for serum separation. Serum samples were stored at -20°C and further used to measure total serum protein and acute phase proteins.

2.5. Serum cortisol and lysozyme concentrations

Blood cortisol concentration was measured by enzyme-linked immunosorbent assay (ELISA) with a commercial kit (DRG® Cortisol ELISA, EIA-1887; DRG International, Inc., USA; http://www.drg-international.com). Serum lysozyme concentration was determined according to Demers and Bayne (1997) with modifications by Zanuzzo et al. 2015 [26]. The assay is based on lysis of a *Micrococcus lysodeikticus* (Sigma-Aldrich, São Paulo, Brazil; #M3770) suspension using hen egg white lysozyme (Sigma-Aldrich, São Paulo, SP, Brazil; #L6876) as a standard. The assay was performed in a 96-well plate in triplicate. The rate of decrease in absorbance for each sample (∆OD) was then compared to that obtained with the standard curve so that lysozyme concentration could be expressed in ng μl^{-1}.

2.6. Serum proteins levels by SDS-PAGE

Serum protein profiles were obtained by electrophoretic fractionation using 4%-10% gradient polyacrylamide gels containing sodium dodecyl sulfate (SDS-PAGE) according to Laemmli (1970) [27] and modified for a unidimensional vertical electrophoresis system (BioRad Laboratories Inc.; http://www.bio-rad.com). Serum samples (10 µl) were prepared in 40 µl of Dulbecco phosphate-buffered saline solution and 10 µl of gel mixture (10% water, 2% SDS, 5% 2-mercaptoethanol, 10 mM ethylenediaminetetraacetic acid, 20 mM Tris phosphate [pH 7.4], 5% glycerol, and 0.001% bromophenol blue as the dye). The electric current for the 20 x 20.5 cm vertical gel electrophoresis system was programmed at 35 and 50 mA while samples were in the stacking and running gel, respectively. After fractionation, the gel was

stained for 10 min in 0.2% Coomassie Brilliant Blue and then destained in a solution containing 250 ml methanol, 100 ml acetic acid and 650 ml water until protein fractions appeared clear. Concentrations of these protein fractions were determined according to Fagliari et al.1998 [28] using a digital densitometer (9301PC Shimadzu, Tokyo, Japan). Proteins were identified using reference markers (http://www.sigmaaldrich.com/brazil) with molecular masses of 200, 116, 97, 66, 55, 45, 36, 29, 24 and 20 kDa. The electrophoresis was performed using six fish per treatment per sampling time to ensure constancy of protein pattern. The results were adjusted in relation to the total protein levels for each fish. The total serum protein concentration was measured with a commercial kit (Labtest® kit; http://www.labtest.com.br/reagentes).

2.7. Data analysis

All data was tested for normality (Cramer Von Mises) and homoscedasticity (Brown-Forsythe). A completely randomized design was used with a 3 x 2 factorial arrangement of 3 dietary treatments (control, 0.5 and 1% β-glucan) x 2 sampling times (prior to and 6 h after infection). Duncan's post-hoc test was used for comparing means of immune effects of diet and sampling time. The *t* test was used to compare means of infected and control groups 6 h after inoculation. Statistical significance was defined as $P < 0.05$. Values in the text and figures represent means ± standard error of the mean (SEM).

2.8. Ethical statement

All experimental procedures in this study that involved the use of animals were performed in accordance with the ethical principles for animal experimentation adopted by the Colégio Brasileiro de Experimentação (COBEA), Brasilia, Brazil, submitted and approved by the Comissão de Ética no Uso de Animais (CEUA) protocol n° 014679/14 UNESP - Jaboticabal, Brazil.

3. Results

In order to evaluate the effect of dietary β-glucan on the acute response of matrinxã to bacterial challenge with *Aeromonas hydrophila*, we assessed molecular weight and variation in the concentrations of blood serum proteins as well as blood cortisol levels prior to and 6 hours post infection (hpi).

3.1. *Blood cortisol levels*

We observed that serum cortisol of fish pre-treated with 0.5% β-glucan was higher prior to bacterial infection. However, 6 hpi, fish that received 0.5% dietary β-glucan responded to the challenge with a significantly lower increase in blood cortisol levels when compared to fish that fed with control diet (Figure 1A).

3.2. *Lysozyme concentration*

Prior to bacterial challenge, fish fed the 0.5% β-glucan diet, but not the 1% diet, had elevated serum lysozyme concentrations compared to the control. At 6 hpi, control fish and those that fed with 0.5% β-glucan displayed similar levels of serum lysozyme, whereas these levels were reduced in fish previously treated with 1.0% β-glucan (Figure 1B).

3.3. *Concentration of blood serum proteins*

In order to create a comprehensive profile of protein level changes during the acute phase of infection in matrinxã, we separately evaluated lysozyme concentration by an assay based on lysis of *Micrococcus lysodeikticus*, while variations in other serum proteins were visualized by SDS-PAGE (Figure 2).

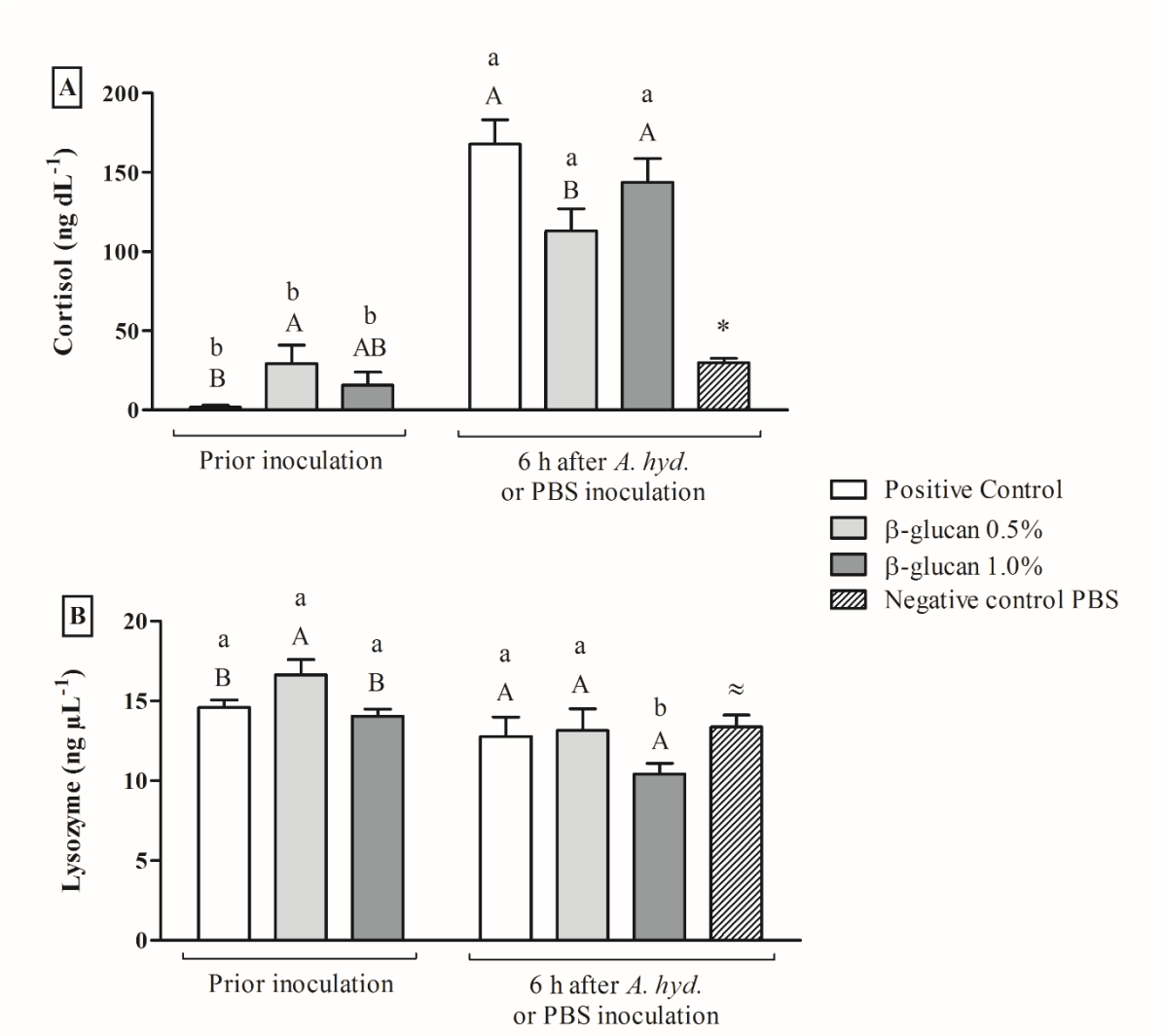

Figure 1. *Serum cortisol (A) and lysozyme concentrations (B), in matrinxã.* Fish fed with dietary β-glucan (0.5% and 1.0%) compared to positive and negative (PBS) control groups prior to and after intraperitoneal injection of *Aeromonas hydrophila.* Different capital letters indicate differences between treatment groups in each isolated sampling time. Different lowercase letters indicate differences between the readings of the same treatment group, collected at all times shown. Symbols, ≈ no difference and * indicates difference between positive and negative control groups 6 h after acute infection. Columns represented the means ± SEM (n = 6; P < 0.05).

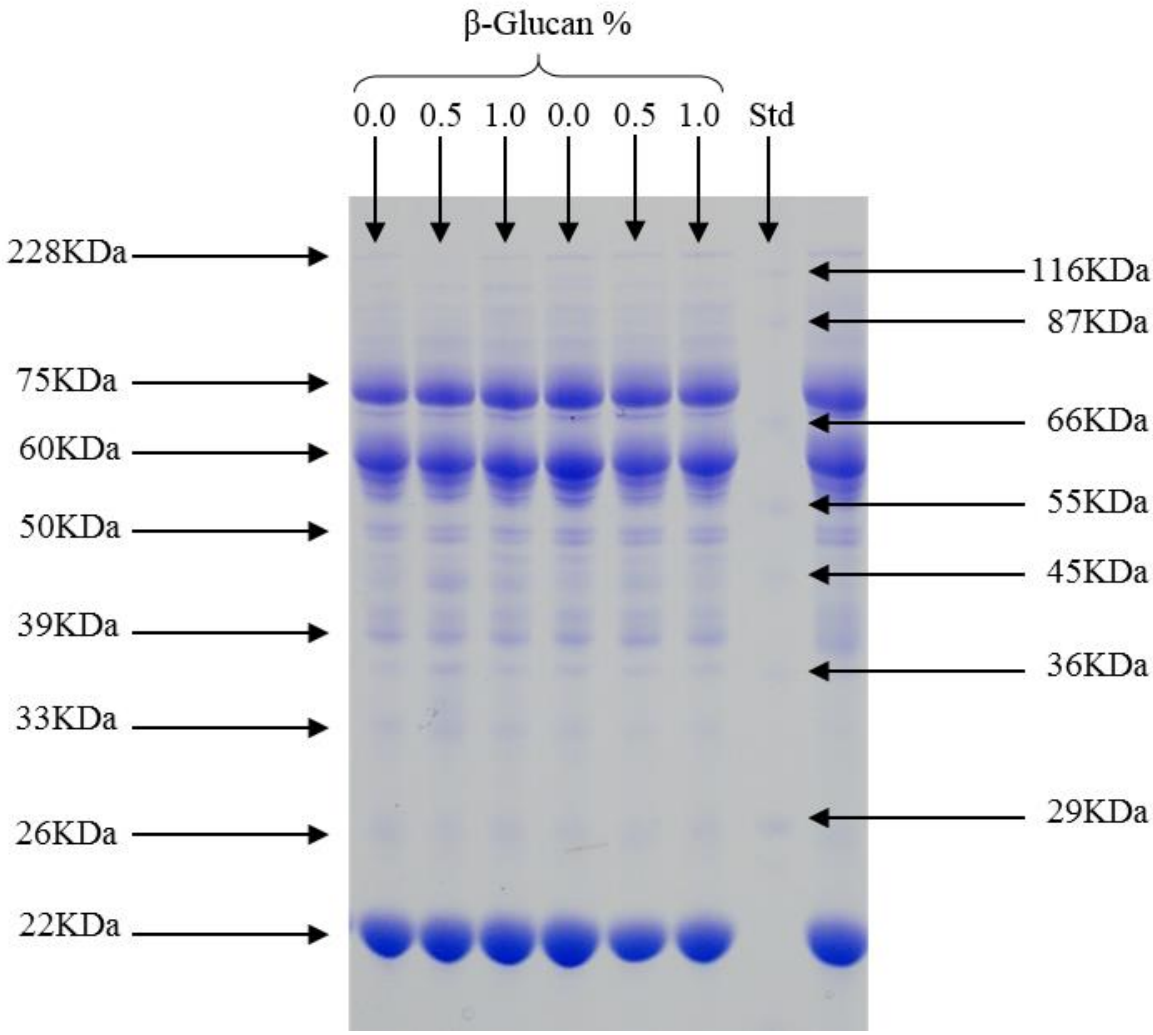

Figure 2. *Serum proteins bands of matrinxã in polyacrylamide gel.* Separating by molecular mass. The proteins were carried in SDS-PAGE polyacrylamide gel to 10%. Molecular weights of size marker proteins (right side) and of matrinxã serum proteins (left side).

3.3.1. *Positive acute phase proteins*

Three serum proteins with molecular weights of ≈190 kDa, ≈36 kDa and ≈26 kDa had increased expression 6 hpi (Figure 3A, B, C).

Prior to infection, serum concentration of the ≈190-kDa protein did not differ among treatments. Levels of this protein increased 127%, at 6 hpi, but only for fish in the positive control group (+infection, −β-glucan). That is, β-glucan prevented induction, albeit to a lesser degree at the 1% β-glucan diet.

Prior to infection, serum concentration of the ≈36-kDa protein was approximately 45% higher in fish fed with the 0.5% β-glucan diet when compared to fish in the control or 1% β-glucan diets. At 6 hpi, serum levels remained the same in fish on the 0.5% β-glucan diet, but increased 108% and 94% in fish fed with the control and 1% β-glucan diets, respectively. The positive control group had 66% higher levels of the protein when compared to the negative control group.

Prior to infection, we observed levels of the ≈26-kDa protein 60% higher in fish fed with the 0.5% β-glucan diet relative to control fish, and an intermediate level in fish fed with the 1% β-glucan diet. At 6 hpi, serum levels in the positive control group were 88% higher compared to the initial time point. For both β-glucan treatments, levels of the protein were similar between the two time points, whereas at 6 hpi, the level in the negative control group did not differ from that of the positive control group.

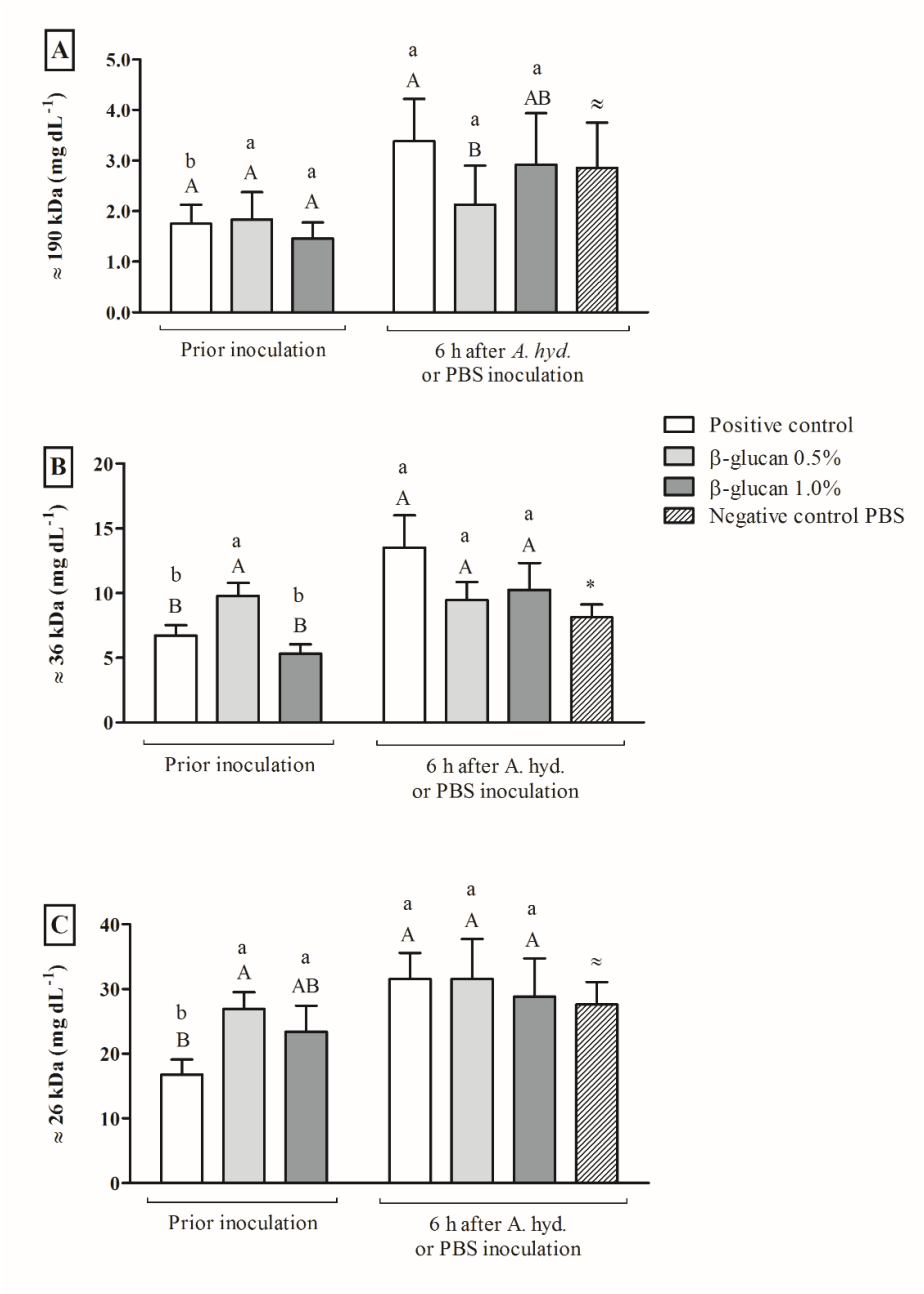

Figure 3. Positive acute phase proteins in matrinxã during the acute phase response. Fish fed with 0.5 and 1.0% β-glucan compared to positive and negative (PBS) control groups prior to and after intraperitoneal injection of Aeromonas hydrophila. The matrinxã serum proteins are identified by their molecular weights: A) ≈190 kDa; B) ≈36 kDa; C) ≈26 kDa. Different capital letters indicate differences between treatment groups in each isolated sampling time. Different lowercase letters indicate differences between the readings of the same treatment group, collected at all times shown. Symbols, ≈ no difference and * indicates difference between positive and negative control groups 6 h after acute infection. Columns represented the means ± SEM (n = 6; $P < 0.05$).

3.3.2. *Negative acute phase proteins*

Five serum proteins with molecular weights of ≈228 kDa, ≈212 kDa, ≈168 kDa and ≈90 kDa had decreased expression at 6 hpi (Figure 4A, B, C, D).

Prior to infection, serum concentrations of the ≈228-kDa protein did not differ among treatments. However, at 6 hpi, levels decreased by 87% in the positive control group, by 56% for fish fed with the 0.5% β-glucan diet, and fish fed with the 1% β-glucan diets only displayed a tendency to decrease. At 6 hpi, levels in the positive control animals were 78% lower than in the negative control group.

Prior to infection, serum concentrations of the ≈212-kDa protein did not differ among treatments. However, at 6 hpi, serum protein concentrations decreased in all groups by approximately 50-60%. Levels in the positive control group were 50% lower than in the negative control group at 6 hpi.

Prior to infection, serum concentrations of the ≈168-kDa protein did not differ among treatments. However, at 6 hpi, serum protein levels decreased by 40% in fish fed with the 1% β-glucan diet. Fish fed with the 0.5% β-glucan diet showed only a tendency towards lower levels of the protein. At 6 hpi, positive control animals had 42% lower levels than negative control fish.

Prior to infection, fish previously fed with the 0.5% β-glucan diet had levels of the ≈90-kDa protein approximately 19% higher than animals on both the control and 1% diets. At 6 hpi, levels of this protein decreased in all fish groups: 64% in fish fed with 0.5% β-glucan and 43% in the other two groups. We did not observe a difference between the positive and negative control groups at 6 hpi.

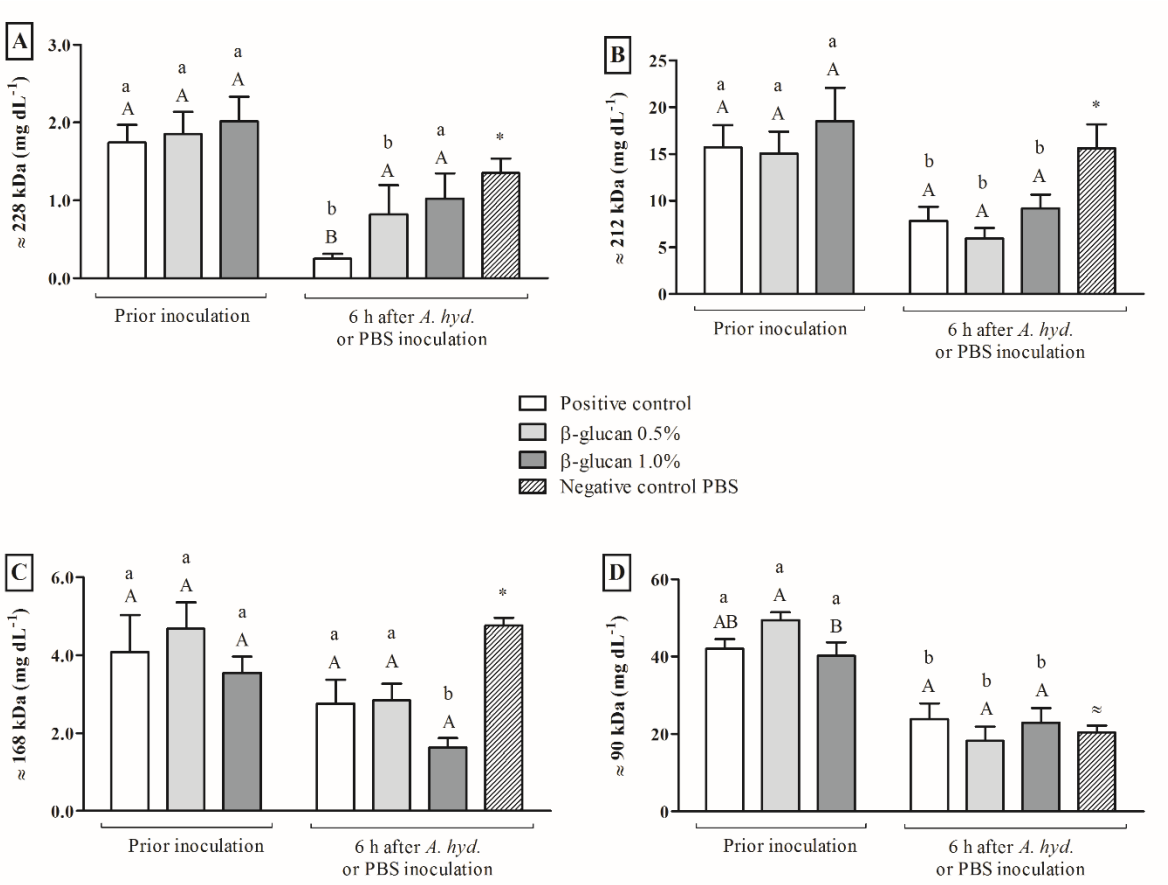

Figure 4. *Negative acute phase proteins in matrinxã during acute phase response.* Fish fed with 0.5 and 1.0% β-glucan compared to positive and negative (PBS) control groups prior to and after intraperitoneal injection of *Aeromonas hydrophila*. The matrinxã serum proteins are identified by their molecular weights: A) ≈228 kDa; B) ≈212 kDa; C) ≈168 kDa and D) ≈90 kDa. Different capital letters indicate differences between treatment groups in each isolated sampling time. Different lowercase letters indicate differences between the readings of the same treatment group, collected at all times shown. Symbols, ≈ no difference and * indicates difference between positive and negative control groups 6 h after acute infection. Columns represented the means ± SEM (n = 6; P < 0.05).

3.3.3. *Other serum proteins*

Of the protein bands, the most intense weighed ≈75 kDa, and ≈60 kDa (Figure 5). This group of proteins was mildly induced by the β-glucan diets prior to infection. At 6 hpi, serum levels of these proteins were reduced in fish on the immunostimulant diets but not in control fish. Other bands were observed, but none of them were significantly altered by treatment or time. These bands weighed: ≈132 kDa (7.8 ± 6.8 mg dL^{-1}), ≈112 kDa (7.4 ± 2.5 mg dL^{-1}), ≈100 kDa (11.8 ± 4.7 mg dL^{-1}), ≈50 kDa (42.9 ± 7.6 mg dL^{-1}), ≈39 kDa (49.0 ± 21.1 mg dL^{-1}), and ≈33 kDa (22.0 ± 2.0 mg dL^{-1}).

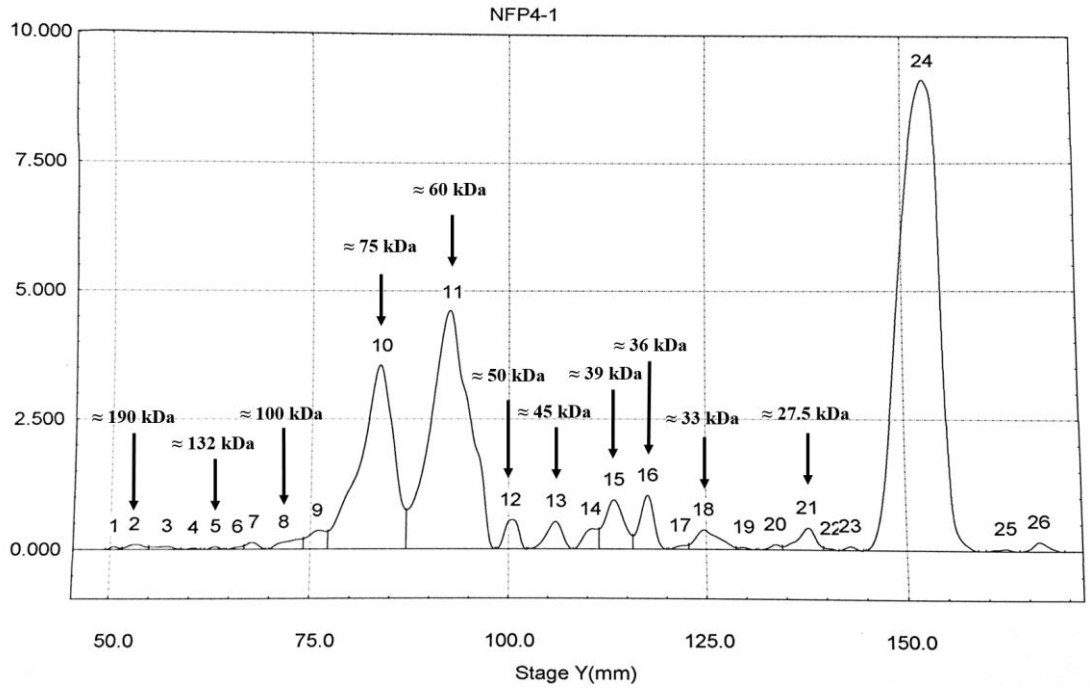

Figure 5. *Normal electrophoretic profile of serum proteins in matrinxã.* Separation by molecular weight. The proteins were carried in SDS-PAGE polyacrylamide gel to 10%.

4. Discussion

We evaluated the immunostimulant effects of dietary β-glucan (0.5% and 1%) on serum cortisol and protein levels prior to and after bacterial challenge with *A. hydrophila,* and compared positive (bacterial inoculated) and negative (PBS inoculated) control groups to isolate responses to infection. Our results show the β-

glucan-induced cortisol and lysozyme serum levels, as well as the normal electrophoretic protein profile as indicators of innate immune response in matrinxã.

We observed that serum cortisol increased in fish that received 0.5% dietary β-glucan prior to bacterial challenge. Moreover, at 6 hpi, the same treatment with β-glucan prevented the increase in blood cortisol levels observed in the control treatment. Previous studies report diverse effects of β-glucan on serum cortisol levels [29–31]. Our results suggest that β-glucan modulates cortisol levels prior to and after pathogenic challenge. To our knowledge, no previous reports have shown β-glucan effects on physiological cortisol levels. However, in contrast to our study, other authors have observed an increase in the serum cortisol of tilapia after transport-induced stress in fish fed dietary β-glucan for 7 days but not in fish fed the immunostimulant for longer periods [31]. Also contrasting with our results, it has been reported that trout previously fed with 0.1% β-glucan exhibited a decrease in serum cortisol level after transport-induced stress [29]. Finally, another study described no effects of dietary β-glucan on serum cortisol level of *Pangasianodon hypopthalmus* after cold stress [30].

Our results are the first evidence that β-glucan can modulate blood cortisol levels in resting conditions, these findings can be explained because β-glucan has been recognized as a major fungal pathogen-associated molecular pattern (PAMP) [12,32]. For this reason, when β-glucan binds to receptors it can be perceived similar to pathogen stimuli that lead to activation of monocyte / macrophages system which increase IL-6 expression and then activate the Hypothalamus / Pituitary / Interrenal (HPI) axis and consequently cortisol secretion. This perception of β-glucan and the responses relative to cortisol, vary between fish species. However, a direct association between bacterial infections or treatment with lipopolysaccharide (LPS; bacterial cell wall components of Gram-negative bacteria) with induction of cortisol levels has been described in fish. For instance, *Oreochromis mossambicus* injected with LPS from *Escherichia coli* showed the modulation of the HPI axis at the level of the cortisol producing tissue, as well as the corticotropic release hormone CRH production on the ventral telencephalon tissue [33].

In the current study, the immunostimulant increased lysozyme serum concentrations prior to infection in fish fed with the 0.5% β-glucan diet, although this effect did not occur in fish fed with the 1.0% diet. Previous work has shown a linear increase in serum lysozyme with increasing β-glucan concentrations of 0.0%, 0.09%

and 0.18% in *Pseudosciaena crocea* [34]. Similar increases in lysozyme level after β-glucan treatment were observed in *Salmon salar* and *Cyprinus carpio* [35–37]. The higher concentration of 1.0% β-glucan employed in the current study far exceeds the ones previously used, and may result in the exhaustion of the immunological response, explaining the lack of effects. At the lower dose, the increase in lysozyme prior to bacterial challenge paralleled the increase in cortisol, suggesting a possible association between these patterns. At 6 hpi, serum lysozyme remained at the level observed prior to infection in fish fed with the 0.5% β-glucan diet. This apparent lack of further response may result from the pathogen agent used. Fish lysozyme does not immediately act against virulent Gram-negative bacteria such as *A. hydrophila* [38]. Only after complement and other enzymes disrupt the outer cell wall of these bacteria does lysozyme become effective [39, 40]. In fish, this process may occur approximately 24 h after infection [41]. Thus, it is possible that levels of the enzyme would rise at later time points not evaluated in the current study.

Acute phase proteins (APPs) include blood proteins that undergo increases (positive APPs) or decreases (negative APPs) in concentration of more than 25% after, for example, inflammatory disorders or infection [42,43]. The SDS-PAGE methodology employed in this study does not allow for the nominal identification of individual proteins; the proteins were identified by means of their molecular weights. However, we observed important alterations in protein bands before and after bacterial inoculation. Pattern changes in APP expression independent of protein identity have been used as biomarkers for inflammation and infection in humans as well as in companion and farm animals [44, 45]. Several recent studies have documented the presence of APPs in exotic and wildlife species, but biomarker assays have yet to be adapted for these animals [43]. Thus the alterations we report here, independently of protein identity, may provide an important tool for disease diagnosis in matrinxã and other fish.

Three protein bands with molecular weights of ≈190 kDa, ≈36 kDa and ≈26 kDa reacted as positive APPs at 6 hpi. Moreover, the ≈36-kDa and ≈26-kDa protein bands were positively modulated by dietary β-glucan. In several fish species immunologically relevant proteins have been identified with molecular weights that coincide with these bands and also respond positively during APR, such as the C3 component (≈185 kDa [46]), the β-chain of haptoglobin protein (≈35 kDa [47]), and the IgM low-chain (≈26 kDa [48]). Based on the biological behavior of these three

proteins, we speculate that they may correspond totally or partially to the observed bands. Regarding the effect of β-glucan, a recent study reported stimulating effects of this immunostimulant on complement activity and on the globulin profile in carp fry [37]. Future studies should confirm the identities of these positive APPs and elucidate their role in APR as well as the mechanism of β-glucan-induced alterations.

On the other hand, fourth protein bands with molecular weights of ≈228 kDa, ≈212 kDa, ≈168 kDa and ≈90 kDa reacted as negative APPs after bacterial infection. A few hours after infection, the APR includes an important decrease in serum copper, zinc, iron, and calcium that limits nutrient availability to pathogens [49]. Moreover, the pattern of protein synthesis by the liver is altered, resulting in decreased production of several blood proteins including albumin, transferrin and cortisol binding globulin, all of them negative APPs [17]. Their down-regulation may reflect a temporary increase in the availability of free hormones [49]. Alternatively, during APR, the system may direct amino acids towards priority protein synthesis to the detriment of what have become known as negative APPs [50]. In light of the lack of available data pertaining to negative APPs in fish, our results provide an important source for future functional characterization and identification of these proteins.

In summary, dietary β-glucan elevates pre-infection cortisol and lysozyme levels. After infection previous treatment with β-glucan maintained elevated lysozyme levels and did not allow the same degree of increase in cortisol as seen in control animals. Furthermore, we report relevant information regarding the patterns of APP expression in fish in our control groups and those fed with β-glucan-containing diets before and after bacterial challenge. These results have important implications for aquaculture, since they represent potential starting points for the future development of diagnostic tools, the identification of positive and negative APPs, and the elucidation of β-glucan-mediated defense mechanisms in matrinxã and other fish.

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Comparison between two generations of β-glucan: effects over the cortisol levels and immunological response in matrinxã (*Brycon amazonicus*)

(Research paper to be submitted to Fish & Shellfish Immunology)

Highlights

β-glucan β-G 2° modulates serum cortisol levels in matrinxã.

β-glucan β-G 2° modulates leukocytes' respiratory activity in matrinxã.

β-glucans β-G 1° and β-G 2° increase the populations of lymphocytes in matrinxã.

β-glucan β-G 2° increases monocyte populations and migration in matrinxã.

Abstract

This study investigated the effects of two generations of β-glucan on the immune response of matrinxã (*Brycon amazonicus*) prior and after bacterial challenge with *Aeromonas hydrophila*. In this sense, we evaluated serum cortisol and plasma glucose levels, the number of leukocytes (lymphocytes, neutrophils and monocytes), as well as the respiratory activity of leukocytes prior to, 6 and 24 hours post infection (hpi). During 15 days, fish were fed with diets containing 0.1% of two β-glucans (β-G 1° and β-G 2°, with 71 and 62% of purity, respectively) and then submitted to bacterial challenge. Results were compared with a positive control group fed with a β-glucan-free diet. A negative control group, also fed with β-glucan-free diet but inoculated with PBS, was established to evaluate the effect of handling during injection. Our results showed that β-G 2° modulated the cortisol profile prior to and after the acute infection with *A. hydrophila*, increased the mobilization and activity of leukocytes. Bacterial infection promoted lymhopenia at 6 hpi and both β-glucans increased the circulating lymphocyte population 24 hpi. Moreover, the β-G 2° prevented the infection-induced neutrophilia at 6 and 24 hpi. Finally, the β-G 2° caused a marked increase in the circulating monocytes prior to infection, and a reduction at 6 hpi that was reversed at 24 hpi. Observation of both reduced neutrophilia and monocytes after infection suggest cell migration to the inflammatory areas. In summary, our study demonstrates that β-G 2° was more efficient on the induction of the cell-mediate immunity in matrinxã.

Key-words: immunostimulation, cell-mediated immunity, early defense, white blood cells.

1. Introduction

Modern intensive aquaculture can adversely affect the health of cultured fish by promoting a potentially stressful environment and the growth of infectious diseases. Unfortunately, the indiscriminate use of antibiotics and other drugs to control diseases has contributed to the emergence of several resistant pathogenic organisms [1]. Therefore, efforts aiming to develop strategies to control the pathogen and immuno-prophylactic measures are needed to support the economic viability of the activity.

Dietary addition of immunostimulants has been shown to enhance fish innate humoral and cellular immune responses against infectious diseases [2,3]. These substances induce the proliferation of leukocytes and their phagocytic activity, as well as secretion of immune mediators such as cytokines [4]. Among these immunostimulants are the β(1-3)(1-6)-D-glucans, hereafter referred to as "β-glucans", which are highly conserved carbohydrates found in the cell walls of plants, fungi, yeast, seaweed, and bacteria [5,6]. They consist of a backbone of β-(1, 3)-linked β-D-glucopyranosyl units with β-(1, 6)-linked side chains of varying distributions and lengths [3,4,7]. The β-glucan from the cell wall of *Saccharomyces cerevisiae* has been shown to have immunostimulatory and beneficial properties, including enhanced protection against infections [8], tumor development [9], and sepsis [10,11]. Its effect has been attributed to the binding to multiple toll-like receptors on leukocytes membrane, resulting in the stimulation of immune responses, such as the increase of bactericidal activity [2] and modulation of cytokine production [12].

Several β-glucans of different purities are now commercially available to be incorporated in the diets of livestock, including farmed fish [4,13]. However, there is a remarkable lack of knowledge about the efficiency of these new generations as immunostimulants able to strengthen fish defense mechanisms. Therefore, we compared the effects of two generations of β-glucan over the immune response in matrinxã, a teleost fish from the Amazon basin, with high economic value for aquaculture in some South American countries [14]. To this end, fish were experimentally inoculated with *Aeromonas hydrophila*, a gram-negative bacterium that is commonly isolated from freshwater environment and used to stimulate immune responses in fish [15,16]. The results presented herein offer new knowledge about the immunostimulant effects of β-glucan generations as well as feed strategies to increase disease resistance and improve fish health.

2. Material and methods

2.1. Experimental animals and lab condition

This study utilized 64 (250.9 ± 45.9 g and 25.7 ± 1.4 cm) juveniles of matrinxã that were kept individually in 64 40-liter fiber tanks (1 fish per tank, density near to 6.2 g fish L^{-1}), during 10 days for acclimatization to the laboratorial conditions being fed with a commercial feed (28% crude protein CP). Temperature and oxygen levels were 30° ± 0.4° C and 5.7 ± 0.2 mg L^{-1}, respectively. Ammonia 71.1 ± 21.2 µg L^{-1} and photoperiod of 14 h light: 10 h dark, during summer season.

2.2. Experimental design and diets

The present study evaluated the individual immunostimulant effect of the two generations of β-glucan (0.1% β-G 1° and 0.1% β-G 2°) in commercial feed (28% CP) and its effects were compared with a β-glucan-free diet as positive control. After acclimatization, fish were fed with 1.5% of their body mass twice a day (11:00 – 16:00 h) during 15 days. We used 18 fish per treatment (Control, 0.1% β-G 1°and 0.1% β-G 2°). Another 10 fish were fed with the control feed to represent the negative control group that would be inoculated with PBS in the following bacterial inoculation. On the 16th day, fish were sampled to determine the prior infection condition. Following that, 12 fish per treatment, starved for 12 h, were anesthetized (benzocaine, 0.05 g L^{-1}) and inoculated in the mesenteric cavity with a sub lethal concentration of *A. hydrophila* (2.5µL g^{-1}), while the 10 fish from the negative control group were inoculated with the same quantity of phosphate buffer saline solution (PBS). At 6 and 24 hours post infection (hpi), anesthetized fish were bled in order to evaluate biochemical and cellular indicators of their immunological condition.

Experimental diets were prepared using an extruded commercial feed that was ground, to which 0.1% of two different glucans coded as β-G 1° and β-G 2° were incorporated and then, in order to be re-pelletized, the food was moistened with 40% water. The feed was then passed through a food processor, and finally dried in an oven with air extraction at 40 °C for 24 hours. The control feed was β-glucan-free. The two β-glucans were developed from *Saccharomyces cerevisiae*, being β-G1°

Macrogard 71% pure (batch number Q513187) and β-G 2° is a research and development substance for now will be called “R&D β-glucan” a new generation 62% pure (batch number T1411201). Both products contain β-glucans plus lipids, protein, ash and moisture, and no nucleotides. The batches were kindly provided by Biorigin, Brazil; http://www.biorigin.net/biorigin/macrogard/macrogard_en/index.html).

2.3. Acute bacterial challenge

The *A. hydrophila* strain was isolated from carp *Cyprinus carpio* (strain A135, LAPOA, UNESP) and identified by sequencing of the 16S rDNA (similarity of 97% with GenBank access: ATCC 7966). The strain was stocked in TSB (Tryptic Soy Broth, Media) medium with 30% glycerol (sterile), at -80° C. An aliquot of 20 µL (strain stock) was inoculated in 5 mL of autoclaved TSB medium and incubated in bacteriological incubator at 28 °C, for 24 h. Subsequently, 200 mL of autoclaved TSB medium was added and incubated again with the same procedure. The bacterial suspension was centrifuged at 8.000 xg for 10 min and supernatant was discarded. Then PBS buffer (0.01 M) was used to wash the pellets twice and the suspension was centrifuged at 8.000 xg for 10 min. The bacterial PBS suspension used was lower than lethal concentration CL-50 ($7.6 * 10^8$ CFU mL^{-1}) adjusted by UFC counting after bacterial culture and spectrophotometer reading (OD600=1.095), using PBS buffer (0.01 M). For stimulation of the fish immune response, the bacterial suspension was previously determined as a sub lethal dose (pre-experimental tests, data not shown). No mortality was recorded during the experiment period.

2.4. Sampling

At each sampling time, 6 fish per treatment (different fish at each sampling time were used) were anaesthetized and blood samples were drawn from the caudal vein using syringes without anticoagulant. Blood was dispensed in 2 ml microtubes with and without anticoagulant. Blood with the anticoagulant Glistab® was maintained under refrigeration for plasma glucose determination and NBT activity measure. The blood smears preparations were made with fresh blood. Blood in microtubes without anticoagulant was maintained at room temperature for 3 h and then centrifuged (3000

rpm for 5 min) for serum separation. Serum samples were stored at -20°C and further used to measure cortisol levels.

2.5. Serum cortisol and plasma glucose concentrations

Blood cortisol concentration was measured by enzyme-linked immunosorbent assay (ELISA) with a commercial kit (DRG® Cortisol ELISA, EIA-1887; DRG International, Inc., USA; http://www.drg-international.com). The plasma glucose concentrations were measured by enzymatic method (Labtest kit, Sao Paulo, Brazil, code 84) following the instructions of the manufacturer.

2.6. Leukocyte respiratory burst – NBT activity

The production of reactive oxygen species (ROS) was measured using NBT (Nitro tetrazolium blue chloride – Sigma Aldrich - N6876), following protocol by Anderson & Siwicki (1995), modified by Biller-Takahashi et al. (2013). Immediately after fish bleeding, 50 µL of heparinized blood was incubated with an equal volume of NBT buffer (0.2%) at room temperature for 30 min. Subsequently, 1 mL of DMF (Dimethylformamide, Sigma Aldrich – 227056) was added to the samples, and they were read using a spectrophotometer (Thermo Scientific; Genesys 10S), at room temperature, at 540 nm.

2.7. *Cellular counts*

The total count of red cells was performed in a Neubauer chamber, using whole blood diluted in formaldehyde citrate buffer 1:200. The total and differential count of leukocyte was performed using optical microscopy on blood smears stained with methanol blue eosin solution May-Grünwald-Giemsa-Wrigth (MGGW), according to the technique described (Tavares-Dias et al., 2008). The leukocytes were measured by the indirect method, which considers the amount of leukocytes found for every 2000 erythrocytes counted. To the leukocytes differentiation, 200 white blood cells were counted and the amount of each cell type was expressed as cells μl^{-1}.

2.8. Data analysis

To evaluate the immunostimulant effect of both β-glucans and to compare the control group prior to and after bacterial challenge all data was submitted to normality (Shapiro-Wilk) and homoscedasticity (Levene). A two-way-ANOVA was used with a factorial of 3x3, being 3 treatments (positive control, β-G 1° and β-G 2°) x 3 sampling times (prior to infection, 6 hpi, and 24 hpi). Duncan's post-hoc tests were made for comparison of means. Finally, a t-test was used to compare the means of positive and negative control groups 6 and 24 h after the respective inoculations. Values in the text and figures are represented by means ± standard error (SE) of the mean. P value < 0.05 was used to estimate the level of significance for statistical differences.

2.9. Ethical statement

All procedures that involved animal use in experimental practices were performed in accordance with ethical principles in animal experimentation, adopted by the Colégio Brasileiro de Experimentação (COBEA), Brasilia, Brazil, and approved by the Comissão de Ética no Uso de Animais (CEUA) protocol n° 014679/14 UNESP - Jaboticabal campus.

3. Results

To evaluate the immunostimulant effect of two generations of β-glucan (β-G 1° and β-G 2°) on the induction of fish immune response, matrinxã juveniles were fed for 15 days with or without the β-glucans before being inoculated with *A. hydrophila.* Biochemical and cellular indicators were evaluated just before inoculation and again 6 and 24 h after inoculation.

3.1. Blood cortisol and glucose concentrations

Prior to infection, fish fed with β-G 2° showed the highest levels of serum cortisol, followed by animals fed with β-G 1° and lastly by the positive control group. Fish from the positive control group presented similar serum cortisol levels 6 h post infection (hpi). However, cortisol levels were higher in fish fed with β-G 2° compared to fish fed with β-G 1° or the positive control diet. Only fish fed with the β-glucans reduced cortisol levels at 24 hpi, when compared to the levels measured prior to infection and at 6 hpi. Serum cortisol levels in fish of the negative control group were always lower than those of the positive control (Fig. 1A).

Prior to infection, plasma glucose concentrations did not differ among treatments. However, at 6 hpi all the challenged fish had a reduced plasma glucose concentration. At 24 hpi, the glucose levels of fish fed with β-G 1° were higher than those presented by fish from the positive control group; fish fed with β-G 2° revealed intermediate values. Negative control glucose values did not differ between 6 and 24 after PBS inoculation, being higher at 6 hpi compared to the positive control group (Fig. 1B).

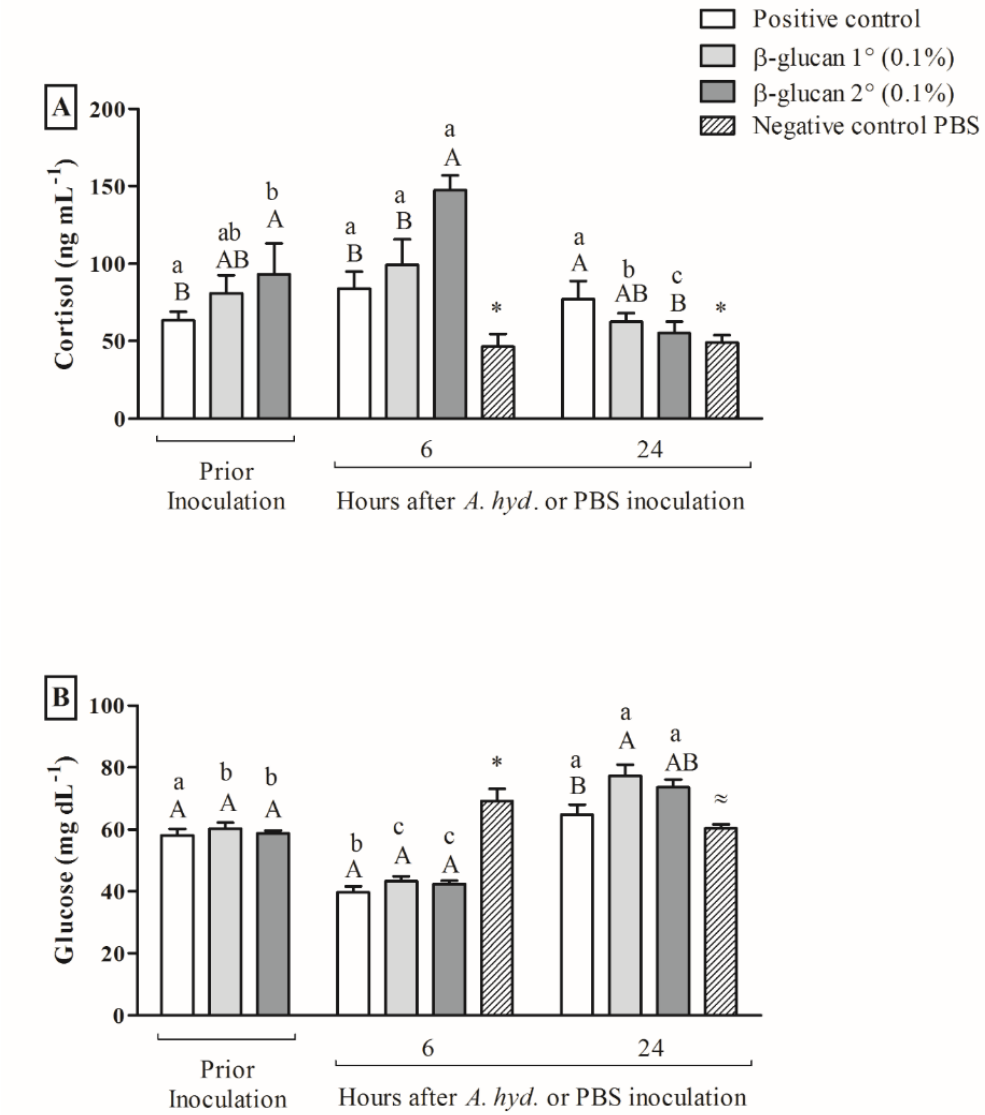

Figure 1. *Serum cortisol (A) and plasma glucose concentrations (B) in matrinxã*. Fish fed with β-G 1° and β-G 2°. The results are compared to fish from positive (bacterial challenge) and negative (PBS injected) control groups, prior to and after A. hydrophila inoculation. Different capital letters indicate differences between treatment groups in each isolated sampling time. Different lowercase letters indicate differences between the readings of the same treatment group, collected at all times shown. Symbols, ≈ indicates no difference and * indicates difference between positive and negative control groups 6 and 24 hpi. Columns represent the means ± SEM (n=6, P<0.05).

3.2. *Respiratory activity of leukocytes (RAL)*

Prior to infection, RAL did not differ among treatments. However, at 6 hpi, we observed increases in the reactive oxygen species (ROS) in all challenged fish. Furthermore, this increase was highest in fish fed with β-G 2° followed by fish from the positive control group and lastly by fish fed with β-G 1°. At this sampling time, RAL did not differ between fish from positive and negative control groups. Twenty-four hpi, RAL returned to the initial values in all treated fish, but remained higher in fish from the negative control group compared to the positive control group (Fig. 2).

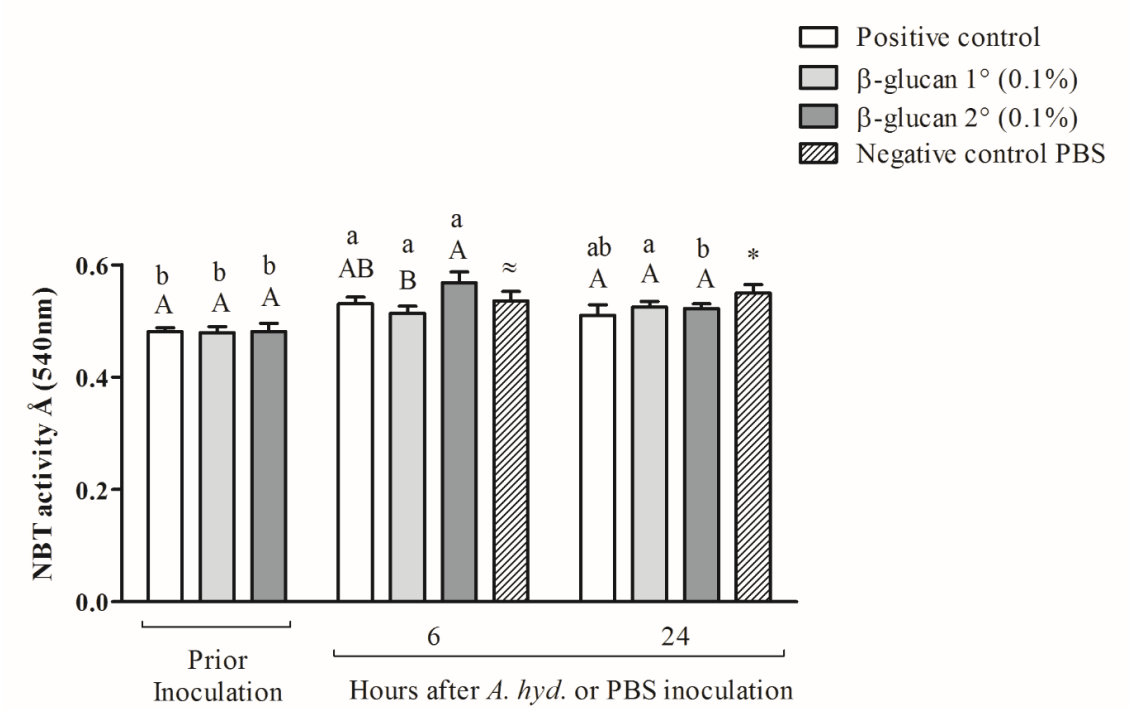

Figure 2. *Respiratory activity of leukocytes in matrinxã.* Fish fed with β-G 1° and β-G 2°. The production of reactive oxygen species was measured using nitro blue tetrazolium (NBT) (see Methods). The results are compared to fish from positive (bacterial challenge) and negative (PBS injected) control, prior to and after A. hydrophila inoculation. Different capital letters indicate differences between treatment groups in each isolated sampling time. Different lowercase letters indicate differences between the readings of the same treatment group, collected at all times shown. Symbols, ≈ indicates no difference and * indicates difference between positive and negative control groups 6 and 24 h post infection. Columns represented the means ± SEM (n=6, P<0.05).

3.3. *Number of circulating leukocytes, lymphocytes, neutrophils and monocytes*

Prior to infection, the number of leukocytes did not differ among treatments. However, at 6 hpi, there was a drop of approximately 42% in the number of these circulating cells, being lower in fish fed with β-G 2° indicating increased cellular migration to the infected tissue. At 24 hpi, the number of leukocytes was around 38% lower than the initial values. Fish of the negative control group showed lower numbers of leukocytes at 6 and 24 hpi than the positive control group (Fig 3A).

Prior to infection, the number of lymphocytes did not differ among treatments. However, at 6 hpi, all fish from challenged groups showed a drop of approximately 83% in the number of circulating lymphocytes. Fish from the negative control group presented higher number of lymphocytes than those from the positive control group. At 24 hpi, all challenged fish revealed an increase in the number of lymphocytes. However, fish fed with both β-glucans (1° and 2°) showed higher circulating lymphocytes values compared to the positive and negative controls, indicating an immune modulation by β-glucan (Fig. 3B).

Prior to infection, the number of circulating neutrophils did not differ among treatment groups. However, at 6 hpi, these levels rose markedly in all fish groups, reaching a fivefold increase in fish from the positive control and the β-G 1° group. Fish fed with β-G2° also showed raised numbers of these cells, albeit lower than fish from the two groups aforementioned. At 24 hpi, the number of circulating neutrophils reduced in all challenged groups; the reduction was more evident in fish fed with β-G 2° than in those fed β-G 1°. Fish from the negative control group showed lower numbers of these cells than the positive control group in both samplings (Fig. 3C).

Prior to infection, fish fed with β-G 2° showed the highest number of circulating monocytes, followed by fish fed with β-G 1° and lastly fish from the positive control group. At 6 and 24 hpi, only fish fed with β-G 2° showed a drop of 83% and 60%, respectively. Fish from the negative control group showed lower numbers of these cells than the positive control group in both samplings (Fig. 3D).

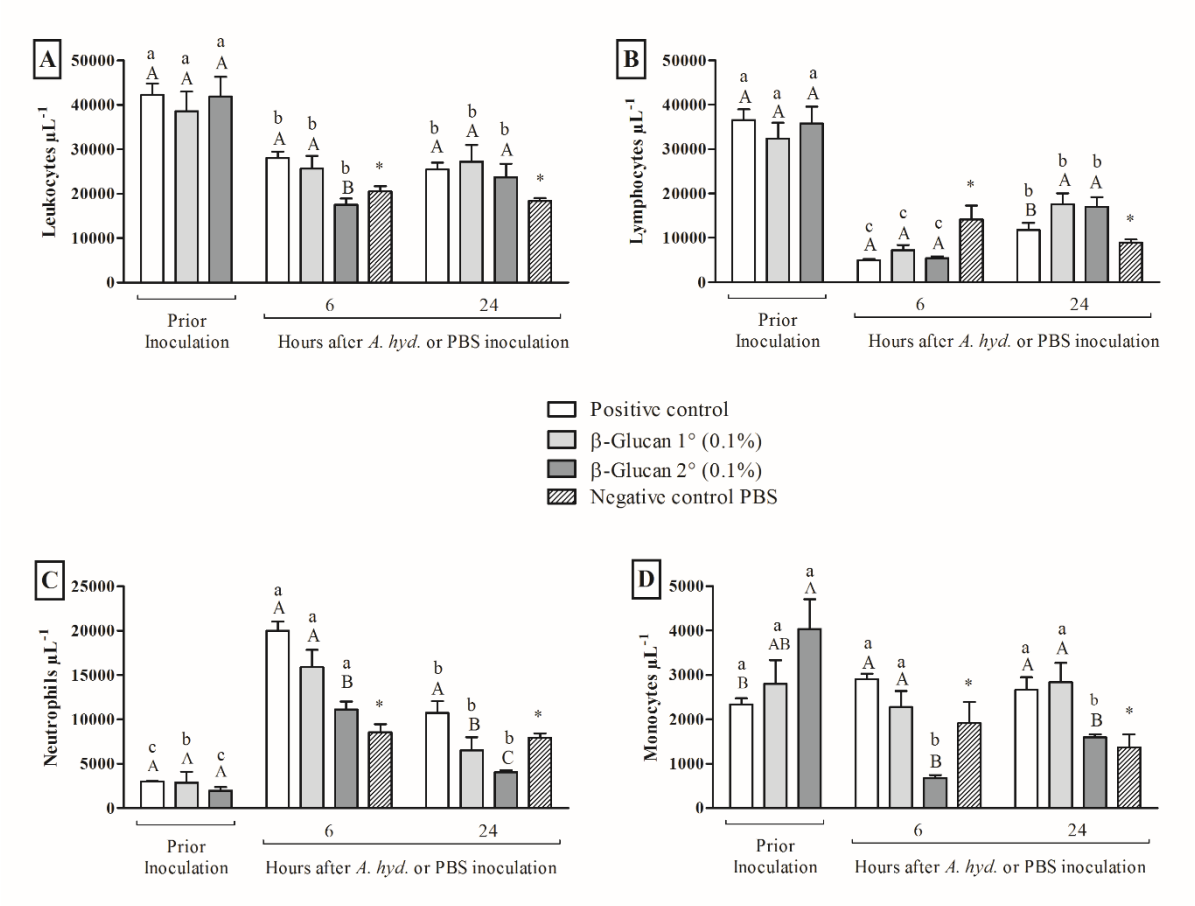

Figure 3. *Number of circulating leukocytes (A), lymphocytes (B), neutrophils (C) and monocytes (D) in matrinxã*. Fish fed with β-G 1° and β-G 2°. The results are compared to fish from positive (bacterial challenge) and negative (PBS injected) control groups, prior to and after A. hydrophila inoculation. Different capital letters indicate differences between treatment groups in each isolated sampling time. Different lowercase letters indicate differences between the readings of the same treatment group, collected at all times shown. Symbols, * indicates difference between positive and negative control groups at 6 and 24 h post infection. Columns represent the means ± SEM (n=6, P<0.05).

4. Discussion

Herein we tested the immunostimulant effect of two different β-glucan generations on the immune response of matrinxã. The results showed that β-G 2° was more efficient in elevating the cortisol serum levels and stimulating the innate immune response, prior to and after an acute experimental infection.

The β-glucan effect on the cortisol serum levels under resting conditions in fish is still minimal. In rainbow trout, physiological cortisol serum levels did not change in fish fed with 0.1% of β-glucan during four weeks [21]. Similarly, *Pangasianodon hypophthalmus* fed with different concentrations of β-glucan during 9 weeks also did not show changes in the levels of circulating cortisol [22]. We observed that in matrinxã fed with β-G 2° high levels of cortisol were maintained at 6 hpi. This profile may represent a mechanism to elicit an immune response in order to prepare fish to fight against the bacteria. At 24 hpi, serum cortisol levels decreased in fish fed with both β-glucans, especially in the β-G 2° group reinforcing the modulating effect of β-G 2° on cortisol serum levels. The separate effects of cortisol and β-glucans on the modulation of immune response are well characterized in fish [2,4,23,24].

Our results are the first evidence that β-glucan can modulate blood cortisol level in the resting condition, these can be explained because β-glucan has been recognized as a major fungal pathogen-associated molecular pattern (PAMP) [2,25]. For this reason, when β-glucan binds to receptors it can be similar to pathogen stimuli that lead to activation of monocyte / macrophages system which increases IL-6 expression and activates the hypothalamus / hypophysis / interrenal (HHI) axis and consequently cortisol secretion. This β-glucan effect and the cortisol responses vary between fish species. However, a direct association between bacterial infections or treatment with lipopolysaccharide (LPS; bacterial cell walls components of Gram-negative bacteria) with induction of cortisol levels has been described in fish. For instance, *Oreochromis mossambicus* injected with LPS from *Escherichia coli* showed the modulation of the HPI axis at the level of the cortisol producing tissue, as well as the corticotropic release hormone CRH production on the ventral telencephalon tissue [26].

Hypoglycemia was detected only after challenge with *A. hydrophila* (6 hpi) in all infected fish, suggesting there was an energetic cost to elicit the immunological response in matrinxã. A previous study showed that immune responses in pacu

Piaractus mesopotamicus has a high energetic cost reflected by decrease of the plasma glucose levels [27]. However, at 24 hpi, the glucose levels increased in all challenged fish, indicating a recovery of the energetic condition of fish after infection, regardless of dietary treatment.

β-glucan as a PAMP has strongly influenced the natural and adaptive host immune responses [2,25]. On the other hand, β-glucans are also known as leukocyte activators that promote immune protection in several animal disease models [13]. Herein we found that dietary β-G 2° modulated leukocyte response in all infected fish by increasing the number of the circulating leukocytes and by mobilizating them to the infection site.

The immune modulator effect of glucans occurs following their binding to specific receptors in monocytes/macrophages, neutrophils and natural killer cells [28]. Indeed, β-glucan binds to different types of receptors (βGR) in leukocytes, thereby triggering both innate and adaptive immune response [2]. Among these βGR is the scavenger receptor that binds to anionic β-glucans (sulphated β-glucans) (for a review see MEENA et al., 2013). The complement receptor 3 (CR3) is more expressed in neutrophils, monocytes, and NK cells compared to macrophages. Binding of β-glucan to CR3 increases leukocytes' phagocytosis and degranulation as well as their tumoricidal activity [29]. Lactosylceramide is a glycosphingolipid found on leucocytes and endothelial cells. It binds to β-glucan and is associated with production of reactive oxygen species (ROS) [4]. In addition, there are various types of Toll-like receptors (TLR2-6). These receptors are used by fungal, glucans and zymosan, all of which induce cytokines after binding to TLR2 and TLR4, thereby favoring Th1 cell differentiation [2]. Lastly, the dectin-1 βGR (dentritic cell-associated C-type lectin-1) is considered the main β-glucan receptor and it recognizes carbohydrates containing β-1,3 and/or β-1,6 glucan linkages, being expressed by monocyte/macrophages, neutrophils, dendrite cells, and T cells [25]. In carp, macrophages recognized β-glucans by multiple pattern recognition receptors that could include TLR but also non-TLR receptors and they were less, but not unresponsive, to selective dectin-1 agonists [30].

In our study, RAL increased in all challenged fish, particularly in those fed with β-G 2° at 6 hpi, which coincided with the reduction in plasma glucose levels, indicating that the fish mobilized energetic blood substrate to support higher immune cell

activity. RAL increases were also observed in carp intraperitoneally injected with β-glucan [8] and *in vitro*, in *Salmon salar* macrophages [31]. The phagocytic activity of fish leukocytes is one of the most important immune mechanisms for surviving to pathogen infection [24]. The RAL is an important indicator of the phagocytic activity. The increase in oxygen uptake at the initiation of the respiratory burst is followed by the production of reactive oxygen species which plays a role in the destruction of pathogens [18].

Total leukocyte counting reflected the counting of specific white cell types. It is well known that, during the acute stage of an infection or in conditions of immune exhaustion, there is a suppression of circulating lymphocytes [32]. Moreover, in teleost fish it was demonstrated that B cells have potent in vitro and in vivo phagocytic activities [33,34]. Accordingly, after bacterial inoculation, there was a strong lymphopenia caused by the acute infection that was reversed at 24 hpi, especially in fish fed with both β-glucans. Therefore, it is plausible to suggest there was a migration process stimulated by the immune activation and the reversal was due to immunostimulation by β-glucans that increased these cells' population. In contrast with our results, the effect of dietary Macrogard 0.1% during 60 days did not show a stimulation of lymphocytes populations in sea bass *Dicentrarchus labrax* [35].

Leukocyte profile was inversely associated to levels of serum cortisol, except in the case of lymphocytes. In fish, cortisol has been shown to affect the number of circulating leukocytes and their activity [24,36,37]. However, different cells of the immune systems show varied responses to cortisol. Previous studies have described cortisol induced lymphopenia and reduced lymphocyte proliferation as well as other effects, such as an increase of the number of circulating neutrophilic granulocytes [38,39]. According with studies in carp, when described, the cortisol induced apoptosis in activated B lymphocytes [40] whereas cortisol treatment caused an inhibition of neutrophil apoptosis [41].

In fish, as in mammals, neutrophils play a primary role in the inflammatory process and are the first phagocytic cells to arrive at a site of tissue injury or infection [33]. The neutrophils are the most abundant circulating leukocyte in humans and zebrafish and are typically the first responders [42,43]. Moreover, neutrophils can increase rapidly in circulation during acute stress when cortisol levels are high [44]. We observed that in matrinxã, under physiological conditions, lymphocytes are the most

abundant leukocytes. However, neutrophils observed in low number before bacterial inoculation become highly sensitive after acute infection, causing a rise in their numbers in the blood. In addition, at 6 hpi we observed an inverse association between the circulating neutrophils and the cortisol levels indicating the suppressor effect of glucocorticoids over the neutrophils populations. Fish of the β-G 2° treatment presenting the highest cortisol levels also had the lowest number of neutrophils. However, this association was not observed at 24 hpi. Cortisol-induced neutrophilia was observed also in channel catfish stressed by handling and transport [45].

Lastly, dietary β-G 2° induced a marked increase in the circulating monocyte population before bacterial challenge. Beta-glucans are considered as strong mitogens inducing proliferation of peripheral blood mononuclear cells [13]. However, the monocytes decreased after inoculation with *A. hydrophyla*, indicating that β-G 2º induced cellular migration to the site of infection. A similar finding was described in *Pangasiodon hypoptalmus* supplemented with β-glucan during 4 weeks [11]. Moreover, the profile of cortisol before the inoculation and at 6 hpi is associated with the profile of the circulating monocytes. In the physiological conditions, cortisol would be stimulating proliferation of these cells and the lower number of monocytes at 6 hpi, coincident with the highest cortisol levels observed, suggests the involvement of cortisol in the cell migration process as suggested by Tort (2011). Monocytes/macrophages are part of the first line of defense cells from the innate immune system against infection and are also responsible for the regulation of inflammatory response [46,47]. Our results are in accordance to other studies describing the stimulator effect of β-glucan over monocyte functions [28].

The two different batches of Macrogard® we used promoted different results, the β-G 2° being the most efficient to stimulate cell-mediated immunity of matrinxã. This has occurred with the use of different β-glucans which vary in purity, solubility, primary structure, molecular weight, branching and polymer charge, which have been shown to influence their activity [48,49]. The binding to different cellular receptors has been implicated in these activities [50]. Although not yet fully understood, these attributes influence the way the carbohydrates interact with their receptors. Recently, and similarly to our experiment, a study tested the effects of adding two different glucans (B-G 1° 68.5% pure; B-G 2° 55.5% pure, both from *Saccharomyces cerevisiae*) into commercial feed of dogs to measure immune indicators. The study found that the two

glucans used had significant immunomodulating effects in dogs studied, but strongly suggested that B-G 1° activity was superior to that of B-G 2° [51]. According to the authors, the samples of glucans were developed using two different biotechnological processes. The same explanation can explain the differences we found in our study.

In summary, in matrinxã, β-G 2° is more efficient to stimulate the immune response cellular indicators and cortisol serum levels prior to and during an experimental acute infection. In addition, we observed an association of cortisol levels and immune response that indicate glucan-induced cortisol improves the start of the early immune response in matrinxã. Our results allow us to suggest that inclusion of β-G 2° in fish diet may help to prepare them to face stressful practices in fish farming.

5. Acknowledgement

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CHAPTER 4

Acute phase proteins in matrinxã (*Brycon amazonicus*): immunomodulation by β-glucan

(Research paper to be submitted to Fish & Shellfish Immunology)

Highlights

Matrinxã acute response is similar to that of other vertebrates.

Acute *A. hydrophila* infection alters serum protein profile in matrinxã.

Dietary β-glucan modulates the acute phase response in matrinxã.

Dietary β-glucan stimulates lysozyme and complement system activity in matrinxã.

Abstract

This study investigated the effects of two different generations of β-glucan on serum protein profile during bacterial challenge in matrinxã (*Brycon amazonicus*). In this sense, we evaluated the complement system activity and lysozyme serum concentration and the electrophoretic profile of serum proteins to evaluate the acute phase response of fish. These parameters were assessed prior to, 6, 24 and 72 hours post infection (hpi). During 15 days, fish were fed with 0.1% of two β-glucan generations (β-G 1° and β-G 2°) and then experimentally infected with *Aeromonas hydrophila* by intraperitoneal inoculation. Results from positive (bacterium challenged) and negative (PBS injected) control groups were compared to those of the fish fed with β-glucan-free diet. Our results showed that dietary supplementation with β-glucan can improve the lysozyme serum concentration and hemolytic activity of the complement system at 24 hpi. By electrophoresis SDS-PAGE, at 72 hpi, we observed proteins with molecular weight of ≈60 kDa and ≈75 kDa that behaved as negative acute phase proteins by reduction of its circulating concentrations. Moreover, ≈75 kDa-protein was stimulated by dietary β-glucan. Other proteins with molecular weight of ≈37 kDa and ≈27.5-kD behaved as positive acute phase proteins. The latter protein was remarkably increased by dietary β-glucan, mainly at 72 hpi. Concluding, dietary β-glucan acted as a immunostimulant by increasing the lysozyme concentration, stimulating the complement system activity, and modulating the serum proteins profile in matrinxã, prior to and after an acute response to bacterial challenge.

Key-words: Immunostimulant, humoral innate immunity, early defense, acute infection

1. Introduction

The first line of defense of vertebrates in immunological challenges is the innate immune system, or non-specific response, composed by several reactions, which precede the specific immune response [1]. One of these reactions is known as the acute phase response (APR), an early and synchronic response that leads to metabolic and physiologic changes in the host after local or systemic homeostatic disturbances, caused by tissue injury or infection [2–4]. The APR starts by the migration of tissue macrophages, blood monocytes, and dendritic cells to the site of tissue damage. These cells' activation leads to the production of cytokines IL-1, IL-6 and TNF-α, which not only initiate the APR, but also result in chemotactic recruitment of cells to the affected area to rapidly augment the response [5].

One clear indication of the acute phase response is the increase or decrease of the synthesis and secretion of several blood proteins, called Acute Phase Proteins (APPs) [6]. The APPs have been described from fish to mammals, but their type of and behavior during the acute phase response differ by species [5]. APPs synthesis takes place mainly in the liver, but also can occur in other cell types e.g., monocytes, endothelial cells, fibroblasts, and adipocytes [5,7]. APPs include blood proteins that undergo increases (positive APPs) or decreases (negative APPs) in concentration of >25% in response to pro-inflammatory cytokines stimulated during the disease process [7]. Negative APPs include transthyretin or thyroxine-binding prealbumin (TTR), retinol-binding protein (RBP), and cortisol-binding globulin, whose decrease indicates a temporal increase in availability of free hormones bound to those proteins [8]. The transferrin, iron transporter [9–11] and albumin, transporter protein of endogenous and exogenous ligands as nutrients or hormones, also represent negative APPs [3,7,12].

The positive APPs are mainly the C-reactive protein (CRP), which binds to microbes and enhances phagocytosis by macrophages, promoting complement system activation and opsonization. In addition, CRP modulates the cytokine production by monocytes and macrophages [2,13,14]. The serum amyloid A (SAA) helps the immune response by modulation of fever induction, platelet activation, neutrophil oxidative burst, and chemotaxis. The haptoglobin (Hp) binds to free hemoglobin and inhibits its oxidative activity [15,16]. Similar to mammals, in various fish species the β-chain of haptoglobin protein molecular mass is described as ≈35

kDa [15]. The ceruloplasmin (CP) is a serum ferroxidase that carries more than 90% of the copper in plasma and helps in iron homeostasis [17]. The CP molecular mass is ≈132 kDa and it participates in iron metabolism by oxidizing Fe^{2+} to Fe^{3+}, oxidizing biogenic amines and serving as an antioxidant [17,18]. The acute phase response is also accompanied by alteration in immunoglobulin concentrations that increase in response to antigen stimulation [19]. In mammals at least five different types of immunoglobulins have been described, but in teleost fish the most functional reported is the IgM with molecular weight of ≈72 kDa and ≈ 26.5 kDa, known as the heavy (H) and light (L) chains respectively [20].

Positive APPs also include the lysozymes and complement system protein complex [2,5,13,21]. Lysozymes are lytic enzymes that hydrolyze glycosidic bonds and are widely distributed in body fluids e.g., blood serum, mucus, and saliva [22,23]. In fish, lysozymes possess lytic activity against both Gram-positive and Gram-negative bacteria. They are also known to activate the complement system and promote phagocytosis processes [23–25].

Another important component of the innate immune response is the complement system, which is the major humoral system of innate immunity and is comprised of a complex of near 30 individual proteins [26,27]. The complement system activity is widely recognized as an indicator of innate immune response in fish [26–28]. Activation of the complement system takes place by different pathways: classical and lectin, alternative and cytolytic. These processes allow the activation of the protein complex that plays an important role in the killing and neutralization of microorganisms [26]. The complement system has also been described as mediator of phagocytosis, inflammatory reactions, immune complex clearance, and antibody production [27–29]. Both lysozyme and complement systems are currently considered APPs in fish because of their early response and increased blood concentrations during an acute phase response [2].

Currently, it is understood that the functions of both lysozyme and the complement system are related [30]. The fish complement system displays bactericidal activity against non-virulent Gram-negative bacteria, but not against Gram-positive bacteria or virulent Gram-negative bacteria [28]. Lipopolysaccharide in the cell walls of Gram-negative bacteria directly activates the alternative complement pathway that can lead to lysis of the bacterial cell. The bactericidal action of lysozymes consists in

hydrolyzing the β-(1,4) linkages between N-acetylmuramic acid and N-acetylglucosamine in the cell walls of Gram-positive bacteria, thus preventing them from invading. Gram-negative bacteria are not directly damaged by lysozyme. When the outer cell wall of Gram-negative bacteria is disrupted by the complement and other enzymes, exposing the inner peptidoglycan layer of bacteria, then lysozyme becomes effective [31].

Functions of APPs include defense activities such as the inactivation of proteases, limiting the dispersal of infection agents, repairing damaged tissue, killing of microbes or potential pathogens, and restoring health status [2]. The APPs can be used to assess the innate immune response to infection, inflammation or trauma [32,33]. In this sense, measure of APPs is currently used as an auxiliary diagnostic tool in human and veterinary medicine to diagnose primarily infectious diseases [7].

In the search for alternatives that strengthen the immune response of farmed fish, it is important to expand the existing knowledge on the effects of immunostimulant substances on said system. In this sense, we tested feed supplementation with dietary β-glucan as immunomodulatory substance, to activate the innate immune mechanisms after a bacterial challenge. Our aim was to evaluate the immunomodulatory effect of β-glucan over the acute phase response and APPs in matrinxã (*Brycon amazonicus*).

2. Material and methods

2.1. Experimental animals and lab condition

This study utilized 64 (250.9 ± 45.9 g and 25.7 ± 1.4 cm) juveniles of matrinxã that were kept individually in 64 40-liter fiber tanks (1 fish per tank, density near to 6.2 g fish L^{-1}), during 10 days for acclimatization to laboratorial conditions being fed with a commercial feed (28% crude protein CP). Temperature and oxygen levels were 30° ± 0.4° C and 5.7 ± 0.2 mg L^{-1}, respectively. Ammonia 71.1 ± 21.2 µg L^{-1} and photoperiod of 14 h light: 10 h dark, during summer season.

2.2. *Experimental design and diets*

The present study evaluated the individual immunostimulant effect of the two generations of β-glucan (0.1% β-G 1° and 0.1% β-G 2°) in commercial feed (28% CP) and its effects were compared with a β-glucan-free diet as positive control. After acclimatization, fish were fed with 1.5% of their body mass twice a day (11:00 – 16:00 h) during 15 days. We used 18 fish per treatment (Control, 0.1% β-G 1°and 0.1% β-G 2°). Another 10 fish were fed with the control feed to represent the negative control group that would be inoculated with phosphate buffer saline solution (PBS) in the following bacterial inoculation. On the 16th day, fish were sampled to determine the prior infection condition. Following that, 12 fish per treatment, starved for 12 h, were anesthetized (benzocaine, 0.05 g L^{-1}) and inoculated in the mesenteric cavity with a sub lethal concentration of *A. hydrophila* (2.5µL g^{-1}), while the 10 fish from the negative control group were inoculated with the same quantity of PBS. At 6, 24 and 72 hours post infection (hpi) anesthetized fish were bled in order to evaluate the humoral innate immune indicators.

Experimental diets were prepared using an extruded commercial feed that was ground, to which 0.1% of two different glucans coded as β-G 1° and β-G 2° were incorporated and then, in order to be re-pelletized, the food was moistened with 40% water. The ration was then passed through a food processor and finally dried in an oven with air extraction at 40 °C for 24 hours. The control feed was β-glucan-free. The two β-glucans were developed from *Saccharomyces cerevisiae*, being β-G1° Macrogard 71% pure (batch number Q513187) and β-G 2° is a research and development substance for now will be called “R&D β-glucan” a new generation 62% pure (batch number T1411201). Both products contain β-glucans plus lipids, protein, ash and moisture, and no nucleotides. The batches were provided by Biorigin, Brazil; http://www.biorigin.net/biorigin/macrogard/macrogarden/index.html).

2.3. Acute bacterial challenge

The *A. hydrophila* strain was isolated from carp, *Cyprinus carpio* (strain A135, LAPOA, Jaboticabal) and identified by sequencing of the 16S rDNA (similarity of 97% with GenBank access: ATCC 7966). The strain was stocked in TSB (Tryptic Soy Broth) medium with 30% glycerol (sterile), at -80° C. An aliquot of 20 µL (strain stock) was inoculated in 5 mL of autoclaved TSB medium and incubated in bacteriological

incubator at 28 °C, for 24 h. Subsequently, 200 mL of autoclaved TSB medium was added and incubated again with the same procedure. The bacterial suspension was centrifuged at 8.000 xg for 10 min and supernatant was discarded, then PBS buffer (0.01 M) was used to wash the pellets twice and centrifuged at 8.000 xg for 10 min. The bacterial PBS suspension lower than lethal concentration CL-50 used was (7.6 * 10^8 CFU mL^{-1}) adjusted by UFC counting after bacterial culture and spectrophotometer reading (OD600=1.095). For stimulation of the fish immune response, the bacterial suspension used was previously determined as sub lethal dose (pre-experimental tests, data not showed). No mortality was recorded during the experiment period.

2.4. Sampling

At each sampling time, six fish per treatment were anaesthetized and blood samples were drawn from the caudal vessel using syringes without anticoagulant. Blood was dispensed in 2ml microtubes that were maintained at room temperature for 3 hours and then centrifuged (3000 rpm for 5 min) for serum separation. Serum samples were stored at -20°C and further used to measure total serum protein and acute phase proteins.

2.5. Complement system activity: alternative pathway (ACH50)

ACH50 was measured according to Polhill et al. (1978) and Ferriani et al. (1990) and rabbit blood was collected and processed to isolate red blood cells (RaRBC) whose suspension was added to serum. Then, complement hemolytic activity was measured as time (in seconds) necessary to lyse 50% of RaRBC in kinetic assay at 700 nm. Some modifications were made to adjust the method to the matrinxã blood: the serum was fresh and the suspension of serum and rabbit erythrocytes was adjusted to ratio 1:1.

2.6. Serum lysozyme concentrations

The serum lysozyme concentration was determined according to Demers and Bayne (1997) with modifications by Zanuzzo et al. (2015). The assay is based on the lysis of *Micrococcus lysodeikticus* suspension (Sigma-Aldrich, São Paulo, Brazil; #M3770) using hen egg white lysozyme as standard (Sigma-Aldrich, São Paulo, SP, Brazil; #L6876). The assay was performed in 96-well plates in triplicate. The rate of decrease in absorbance for each sample (ΔOD) was then compared to the standard curve. Lysozyme concentration is expressed in ng μl^{-1}.

2.7. Serum proteins levels by SDS-PAGE

Serum protein profiles were obtained by electrophoretic fractionation using 4%-10% gradient polyacrylamide gels containing sodium dodecyl sulfate (SDS-PAGE) according to Laemmli (1970) and modified for a unidimensional vertical electrophoresis system (BioRad Laboratories Inc.; http://www.bio-rad.com). Serum samples (10 µl) were prepared in 40 µl of Dulbecco phosphate-buffered saline solution and 10 µl of gel mixture (10% water, 2% SDS, 5% 2-mercaptoethanol, 10 mM ethylenediaminetetraacetic acid, 20 mM Tris phosphate [pH 7.4], 5% glycerol, and 0.001% bromophenol blue as the dye). The electric current for the 20 x 20.5 cm vertical gel electrophoresis system was programmed at 35 and 50 mA while samples were in the stacking and running gel, respectively. After fractionation, the gel was stained for 10 min in 0.2% Coomassie Brilliant Blue and then destained in a solution containing 250 mL methanol, 100 ml acetic acid and 650 ml water until protein fractions appeared clear. Concentrations of these protein fractions were determined according to Fagliari; McClenahan (1998) using a digital densitometer (9301PC Shimadzu, Tokyo, Japan). Proteins were identified using reference markers (http://www.sigmaaldrich.com/brazil) with molecular masses of 200, 116, 97, 66, 55, 45, 36, 29, 24 and 20 kDa. The electrophoresis was performed using six fish per treatment per sampling time to ensure constancy of protein pattern. The results were adjusted in relation to the total protein levels for each fish. The total serum protein concentration was measured with a commercial kit (Labtest® kit; http://www.labtest.com.br/reagentes).

2.8. Data analysis

All data was submitted to normality (Shapiro-Wilk) and homoscedasticity (Levene) to evaluate the immunomodulator effect of two generations of dietary β-glucan and compared to the control group prior to and after bacterial challenge. A two-way-ANOVA was used with a factorial of 3x3, being 3 treatments (Positive control, 1° β-glucan and 2°β-glucan) x 3 sampling times (prior to infection, 6, and 24 hours post infection). To compare data between 24 and 72 hours post infection (hpi), we used comparison by repeated measures because the same fish were sampled more than once. To compare the effects of treatments at 72 hpi we used a one-way-ANOVA. Finally, Duncan's post-hoc tests were made for comparison of means and a t-test was used to compare the means of positive and negative control groups 6 and 24 h after the respective inoculations. Values in the text and figures are represented by means ± standard error (SE) of the mean. P value < 0.05 was used to estimate the level of significance for statistical differences.

2.9. Ethical statement

All procedures that involved animal use on experimental practice were performed in accordance with ethical principles in animal experimentation, adopted by the Colégio Brasileiro de Experimentação (COBEA), Brasilia, Brazil, and approved by the Comissão de Ética no Uso de Animais (CEUA) protocol n° 014679/14 UNESP – Jaboticabal campus.

3. Results

We evaluated the effect of two generations of dietary β-glucan (β-G1° and β-G 2°) in the profile of some of the acute phase proteins of matrinxã prior to and after bacterial infection with *Aeromonas hydrophila*. In this sense, complement system activity and serum lysozyme concentration were assessed prior, 6, 24, and 72 hours post infection (hpi). The electrophoretic protein profile was measured in order to evaluate the dietary β-glucan effects over the acute phase response in matrinxã.

3.1. Serum lysozyme concentration and complement system activity

Lysozyme serum levels did not show differences prior to or 6 hpi, neither among treatments nor among sampling times. At 24 hpi, all infected fish showed increased lysozyme serum levels. Fish fed with β-G 1° showed the highest lysozyme levels, followed by fish from the positive control and lastly by animals fed with β-G 2°. At 72 hpi relative to 24 hpi, we observed an inverse situation: fish fed with β-G 2° maintained the highest levels of serum lysozyme, compared with fish from the positive control group and fish fed with β-G 1°. Finally, all fish from positive control groups after infection with *A. hydrophila* presented higher lysozyme levels compared to those of the negative control group; we observed an increase of lysozyme levels caused by the bacteria in all infected fish and stimulation of lysozyme by dietary β-glucans after 24 hpi (Figure 1A).

The hemolytic activity of the complement system (ACH50) did not differ among treatment groups neither prior to infection nor at 6 hpi. At 24 hpi, we observed that the ACH50 was higher in fish fed with β-G 2° compared to the other groups. At 72 hpi in relation to 24 hpi, the values did not show changes in any fish group. However, in fish fed with β-G 2° ACH50 was maintained moderately activated. On the other hand, positive and negative control groups did not differ throughout time (Figure 1B).

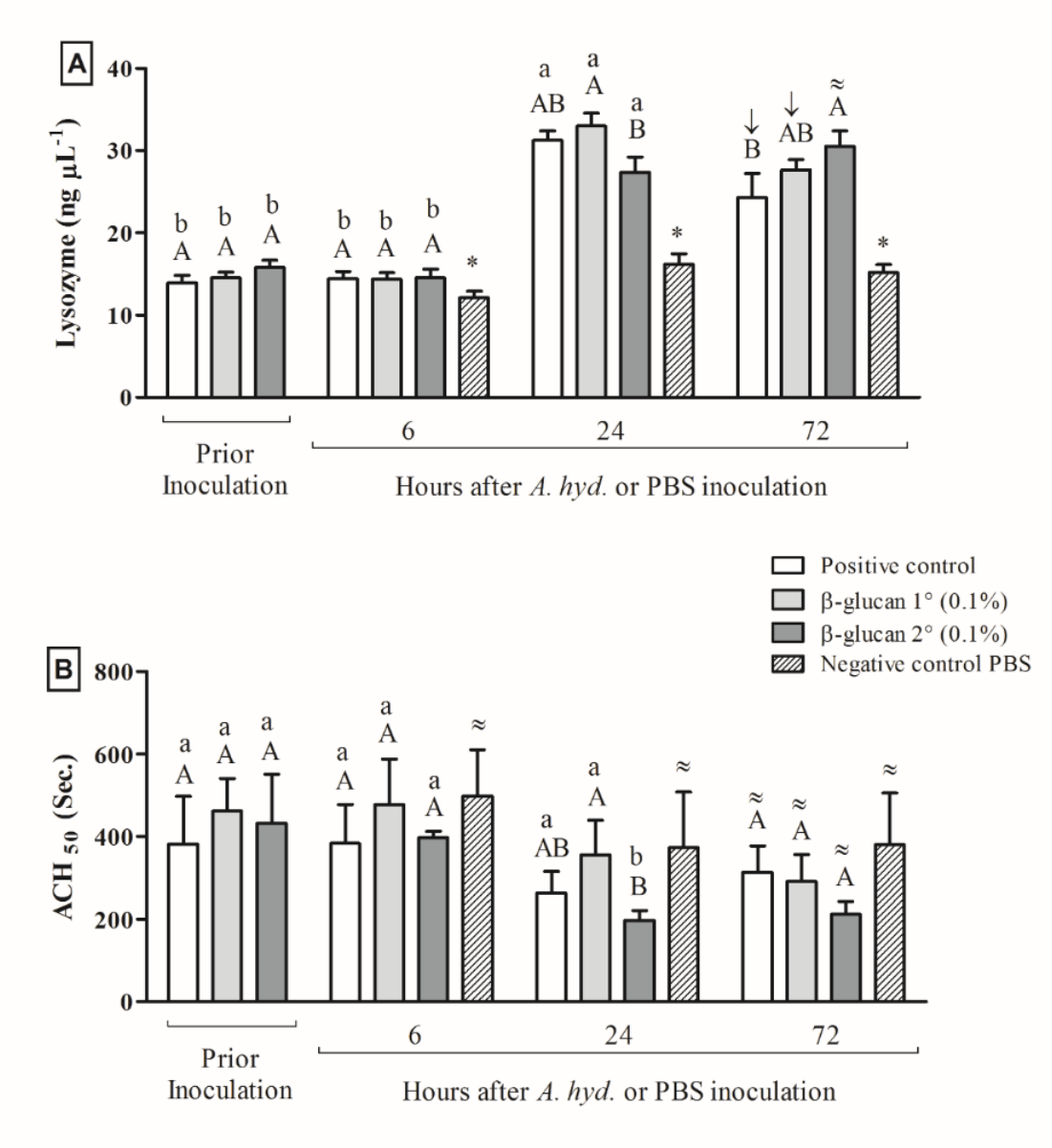

Figure 1. *Lysozyme serum concentrations (A) and complement system activity (B) in matrinxã*. Fish fed with 0.1% of two dietary β-glucan from 1° and 2°generations. Results were compared to fish from the positive control (bacterial challenge) and negative control (PBS injected), prior to and after intraperitoneal injection of *Aeromonas hydrophila*. Different capital letters indicate differences between treatment groups in each isolated sampling time. Different lowercase letters indicate differences between the readings of the same treatment group, collected at all times shown. Symbols over PBS bars, * indicates difference, ≈ indicates no difference between positive and negative control groups at 6, 24, and 72 h post infection. Symbols, ≈ indicates no difference, ↑ increase and ↓ decrease were used to compare only the effects of treatments between 24 and 72 hpi. Bars represent means ± SEM (n=6, P<0.05).

3.2. Serum protein profile in matrinxã by SDS-PAGE

To evaluate the serum profile protein in matrinxã, we used the SDS-PAGE method and described the proteins' behavior during acute infection with *A. hydrophila* (Figure 2). We observed alterations in proteins with molecular weight (mw) of ≈37 kDa and ≈27.5 kDa, as positive acute phase proteins. We also observed alterations in proteins with mw of ≈60 kDa and ≈75 kDa as negative acute phase proteins. Some of these proteins were modulated by dietary β-glucan.

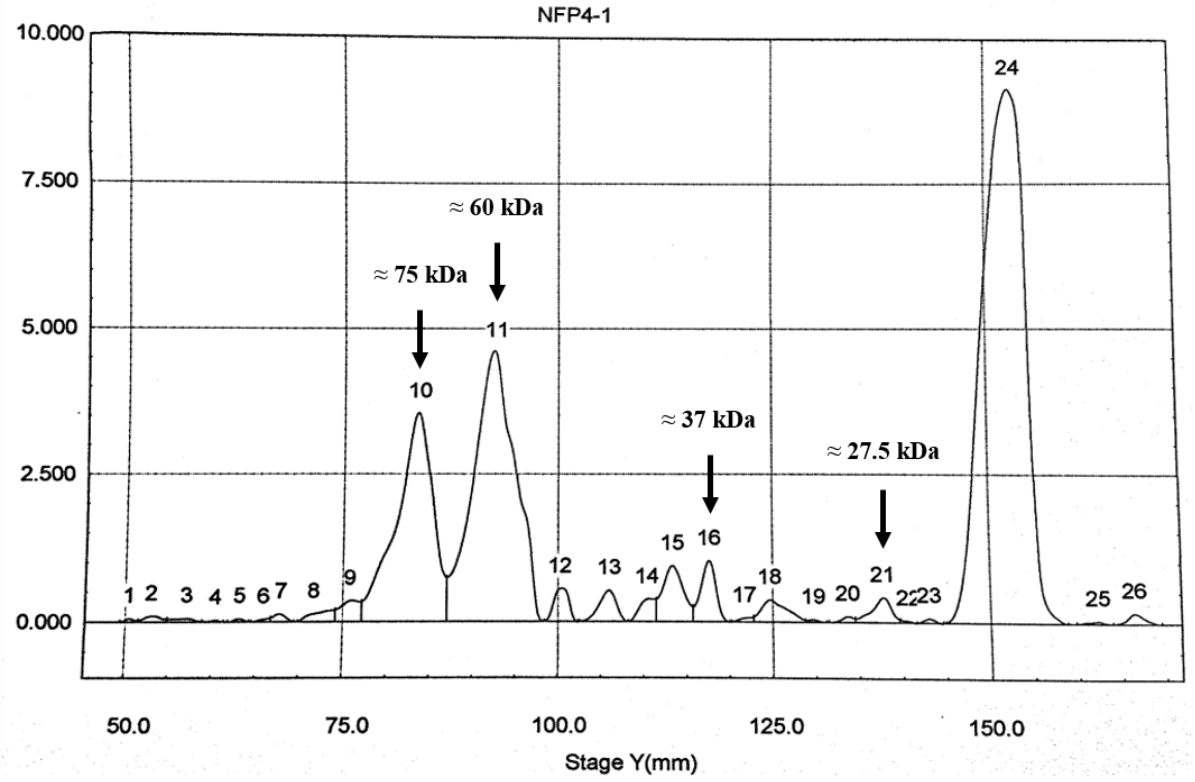

Figure 2. *Normal electrophoresis of serum protein in matrinxã.* Separation by molecular weight. The proteins were carried in SDS-PAGE polyacrylamide gel to 10%.

3.3. Positive acute phase proteins during infection in matrinxã

We observed protein band with similar pattern in all sampled fish, with molecular weight ≈37 kDa, and mean values of 10.61 ± 4.25 mg dL^{-1}. We described their behavior during the acute phase response. Prior to infection, they did not differ among treatment groups. Moreover, at 6 hpi, serum levels of ≈37 kDa-protein were higher in fish fed with β-G 1° followed by fish from the positive control group and lastly by fish fed with β-C 2°. At both 24 and 72 hpi these protein values more than doubled in all experimental groups without differences among treatments. Finally, we observed that, after infection, all negative control groups showed lower serum levels of ≈37 kDa-protein, compared to the positive control groups (Figure 3A).

In polyacrylamide gel SDS-PAGE, we observed in all samples a stable band with molecular weight of ≈27.5 kDa and basal levels near to 11.3 ± 11.4 mg dL^{-1}. Prior to infection, fish fed with β-G 2° showed lower levels of this protein. Between 6 and 24 hpi this protein did not differ between the treatment groups. However, at 24 hpi, this protein increased noticeably in all infected fish. At 72 hpi, we observed that all fish fed with β-G had high levels, indicating a modulation of ≈27.5 kDa-protein by both β-glucans generations. Finally, we observed that at 24 and 72 hpi, all negative control groups showed lower serum levels of ≈27.5 kDa-protein compared to positive control groups (Figure 3B).

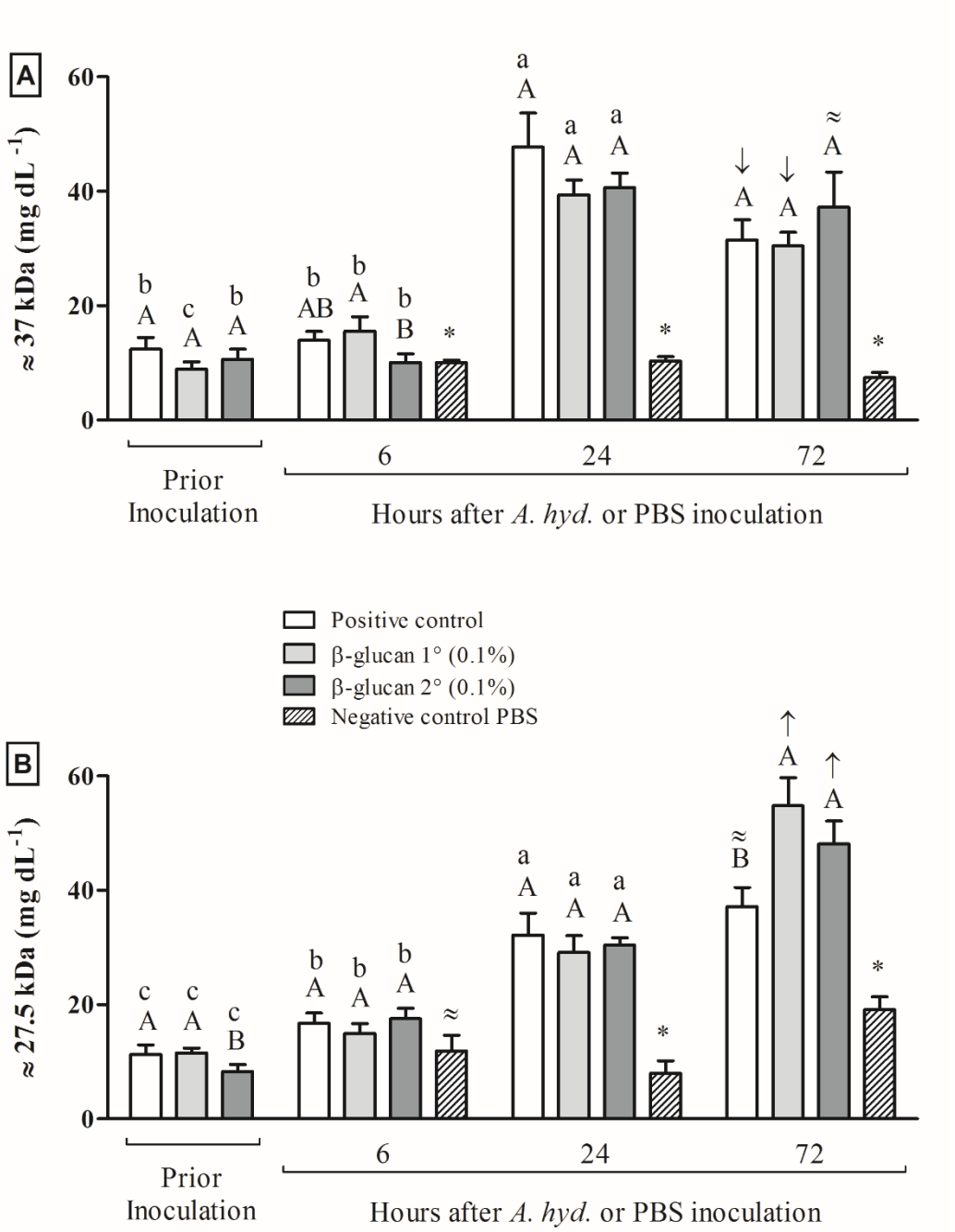

Figure 3. *Serum proteins levels of ≈37 kDa-protein (A) and ≈ 27.5 kDa-protein (B) in matrinxã.* Fish fed with 0.1% of two dietary β-glucan from 1° and 2°generations. Results were compared to fish from of positive control (bacterial challenge) and negative control (PBS injected), prior to and after intraperitoneal injection of *Aeromonas hydrophila*. Different capital letters indicate differences between treatment groups in each isolated sampling time. Different lowercase letters indicate differences between the readings of the same treatment group, collected at all times shown. Symbols over PBS bars, * indicates difference, ≈ indicates no difference between positive and negative control groups at 6, 24 and 72 h post infection. Symbols, ≈ indicates no difference, ↑ increase and ↓ decrease were used to compare only the effects of treatments between 24 and 72 hpi. Bars represent means ± SEM (n=6, P<0.05).

3.4. Negative acute phase proteins during infection in matrinxã

Prior to infection, we observed ≈60 kDa-protein levels higher in fish fed with β-G 2°, followed by fish from the positive control group and lastly by animals fed with β-G 1°. At 6 hpi, these protein levels did not differ among treatments, but fish fed with β-G 2° showed a decrease compared to initial values. At 24 hpi, ≈60 kDa-protein serum levels were similar to those observed at 6 hpi and fish fed with β-G 2° showed values still lower compared to other groups. At 72 hpi compared to 24 hpi, all experimental groups showed a decrease in these protein levels. In addition, at 72 hpi, fish from the negative control group showed higher ≈60 kDa-protein values compared to infected fish independent of the diet (Figure 4A).

Prior to infection, ≈75 kDa-protein levels were higher in fish fed with β-G 2° followed by fish fed with β-G 1° and lastly by animals from the positive control group. At 6 hpi, this protein was increased in fish from the positive control group respect to initial values, and levels from fish fed with both β-G generations were similar to the initial sampling time. At 24 hpi, these proteins levels did not differ among treatments. At 72 hpi, all infected fish showed a drop in ≈75 kDa-protein levels compared to 24 hpi. However, this drop was more abrupt in fish from the positive control group compared to fish fed with β-Gs. Therefore, we observed a modulation of ≈75 kDa-protein serum levels by β-glucans diet (Figure 4B).

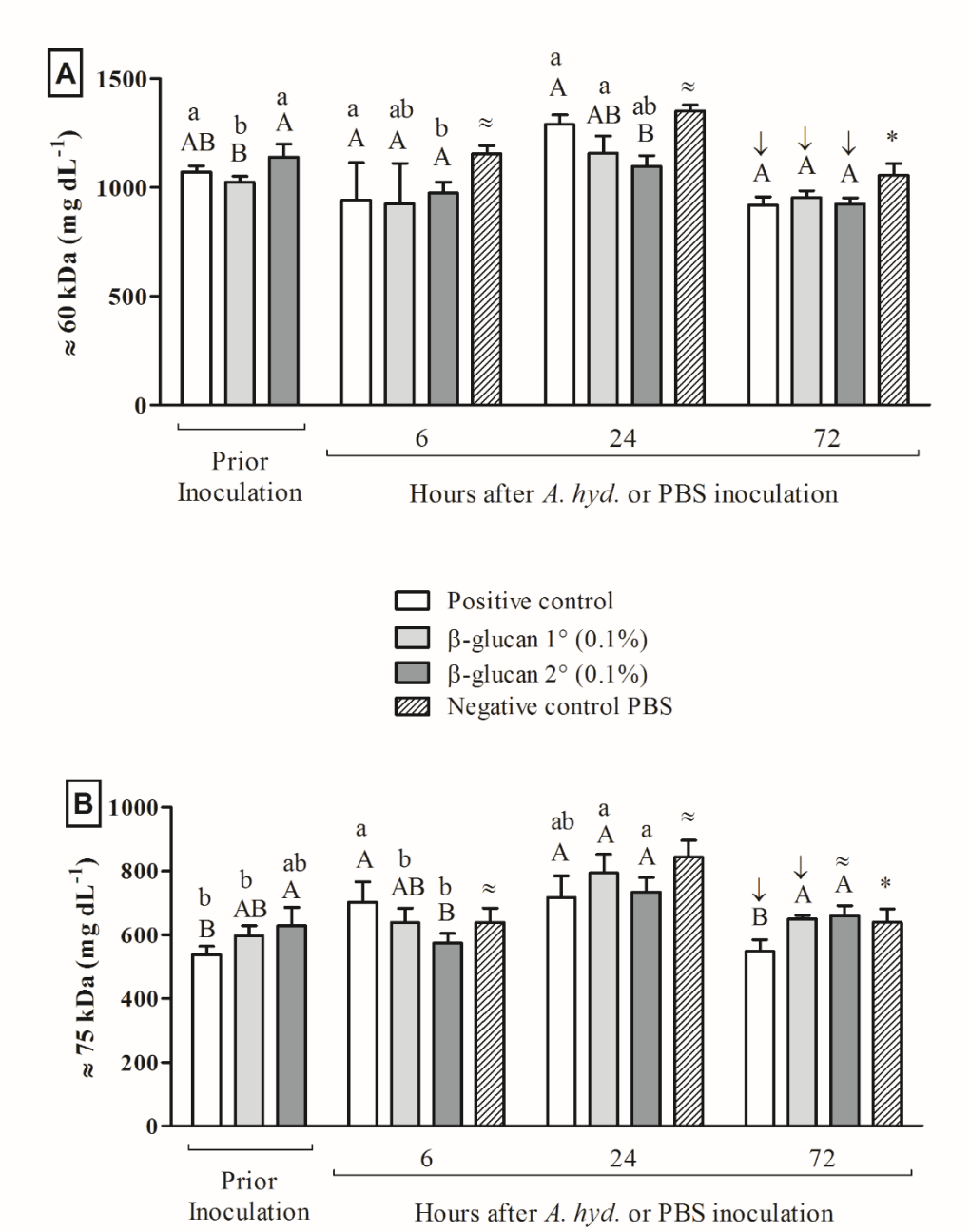

Figure 4. *Serum proteins levels of ≈60 kDa-protein (A) and ≈75 kDa-protein (B) in matrinxã.* Fish fed with 0.1% of two dietary β-glucan from 1° and 2° generations. Results were compared to fish from the positive control (bacterial challenge) and negative control (PBS injected), prior to and after intraperitoneal injection of *Aeromonas hydrophila*. Different capital letters indicate differences between treatment groups in each isolated sampling time. Different lowercase letters indicate differences between the readings of the same treatment group, collected at all times shown. Symbols over PBS bars, * indicates difference, ≈ indicates no difference between positive and negative control groups at 6, 24, and 72 h post infection. Symbols, ≈ indicates no difference, ↑ increase and ↓ decrease were used to compare only the effects of treatments between 24 and 72 hpi. Bars represent means ± SEM (n=6, P<0.05).

4. Discussion

In order to evaluate the effect of two generations of dietary β-glucan (β-G1° and β-G 2°) in the profile of some of the acute phase proteins of matrinxã prior to and after bacterial infection with *Aeromonas hydrophila*, we assessed the serum lysozyme concentration, the hemolytic activity of the complement system, and the electrophoretic serum protein profile in matrinxã prior to, 6, 24, and 72 hours post infection (hpi).

The innate or non-specific immune system of fish is the first line of defense against a wide variety of pathogens [40,41]. Lysozyme level or activity is an important index of innate immunity of fish. Lysozymes possess lytic activity against both Gram-positive and Gram-negative bacteria, in addition to being opsonic in nature and activating the complement system and phagocytes [23,42]. We observed the immunostimulant effect of dietary β-glucan through the increase of lysozyme serum concentrations that improved the innate immune response in matrinxã. Our results are in accordance to studies that described the β-glucan's ability to enhance innate humoral immunity such as increased levels of lysozyme in different fish species [43–46]

The complement system is the major humoral system of innate immunity and is comprised of a complex of near 30 individual proteins [26,27]. The complement system activity is widely recognized as indicator of innate immune response in fish [26–28]. The complement system has also been described as mediator of phagocytosis, inflammatory reactions, immune complex clearance, and antibody production [27–29]. We observed the immunostimulant effect of dietary β-glucan through a time response reduction of hemolytic activity of the complement system that led to an improvement in the innate immune response in matrinxã, as similarly described in other studies in fish supplemented with β-glucan [44,47,48].

In fish, studies have been fruitful and clearly established that homologs of at least some known APPs are present in both elasmobranchs and teleost, and that in teleost some of these plasma proteins increase in concentration in response to inflammatory stimuli [2,49]. In this sense, APPs are currently studied in commercial fish: *Salmo salar* and *Salmo gairdneri* [13]; *Pargus auratus* [50]; *Oreochromis mossambicus, Channa punctatus, Cyprinus carpio* and *Cirrhina mrigala* [51], and *Oreochromis niloticus* [52]. These studies described alterations observed in some APPs blood

concentrations, mainly during initial bacterial or parasites experimental infections, which indicate the early innate immune system response.

We observed one protein band with similar pattern in all sampled fish, with molecular weight ≈37 kDa, and mean values of 10.61 ± 4.25 mg dL^{-1}. By its molecular weight and behavior during the acute phase response, we speculated that this protein band could represent the haptoglobin (Hp). The Hp binds to free hemoglobin and inhibits its oxidative activity [15,16]. Similar to mammals, in various fish species the β-chain of haptoglobin protein molecular mass is described as ≈35 kDa [15]. We did not observe effects or responses of dietary β-glucan over this protein in matrinxã.

We observed in all samples a stable band with molecular weight of ≈27.5 kDa and basal levels near to 11.3 ± 11.4 mg dL^{-1}. By its molecular weight, serum concentration and acute response we associated this protein band in matrinxã with the IgM light (L) chain. The acute phase response is accompanied by alteration on immunoglobulin concentrations that increase in response to antigen stimulation [19]. In mammals, at least five different types of immunoglobulins are described, but in teleost fish the reportedly most functional is the IgM with molecular weight of ≈72 kDa and ≈26.5 kDa known as the heavy (H) and light (L) chains respectively [20]. In addition, we observed the immunostimulant effect of dietary β-glucan on ≈27.5 kDa. It increased at 24 hpi, and at 72 hpi was noticeably stimulated in all fish fed with β-glucan. Previous studies report the immunostimulant effect of β-glucan in antibody response after pathogen attack [53,54,55].

Finally, we described two big band proteins observed by electrophoresis with mw ≈60 kDa and ≈75 kDa, which were shown to be negative acute phase proteins (see Fig. 2 and 4). By its mw and serum levels we associated these bands with the albumin and transferrin proteins respectively. The dietary β-glucan did not affect the ≈60-kDa albumin protein levels. However, we observed that ≈75 kDa-protein was stimulated by dietary β-glucan at 72 hpi. Transferrin is a glycoprotein of approximately 70–80 kDa and is responsible for the transport and delivery of iron to cells. Transferrin is abundant in nature and has been identified in a wide range of organisms e.g., insects, crustaceans, fish, and mammals. Binding of iron to transferrin creates a bacteriostatic environment by limiting the availability of iron to replicating pathogens [10,11]. Transferrin may exhibit behavior as a positive as well as a negative APP, this alteration depending on the species, however transferrin can increase and decrease

along the acute phase response with the aim to limit the viability of iron to pathogens [2,10,11]. To our knowledge, there are no reports about the effects of dietary β-glucan over the circulation of transferrin levels.

In summary, we observed the immunostimulant effect of dietary β-glucan mainly in fish fed β-G 2°, on the humoral innate immune factors as lysozyme serum levels and complement system activity as well as some proteins of the acute phase response. These effects can improve the early innate immune response in matrinxã. In addition, the proteins observed by electrophoresis can be interesting tools in the diagnosis of fish disease and future studies should focus on elucidating the identity and functions of these proteins.

5. Acknowledgement

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CHAPTER 5

β-glucan-induced cortisol improves the early immune response in matrinxã (*Brycon amazonicus*)

(Research paper to be submitted to Fish & Shellfish Immunology)

Highlights

β-glucan increased cortisol serum levels in matrinxã.

β-glucan improved early immune defense in matrinxã.

Cortisol serum levels increased the lysozyme serum levels.

Cortisol serum levels modulated circulating leukocytes populations.

Acute stress induced the hemolytic activity of the complement system.

Abstract

This study investigated the role of endogenous cortisol on the innate immune response in matrinxã (*Brycon amazonicus*) fed with β-glucan prior to and after stressor exposure and bacterial challenge. In this sense, we evaluated the serum cortisol and plasma glucose levels, the serum lysozyme levels, the hemolytic activity of the complement system and the respiratory activity of leukocytes, as well as the number of circulating erythrocytes and leukocytes of fish fed during 15 days with diets containing the immunostimulant β-glucan 0.1% (β-G) or β-glucan 0.1% + metyrapone 30mg kg^{-1} fish (β-G+MTP). Dietary MTP was used to block the cortisol production. After experimental feeding, fish were air exposed during 3 min, to endogenously increase the cortisol levels. Following that, they were challenged with intraperitoneal injection of *Aeromonas hydrophila*. Results were compared with a positive control group fed with a β-glucan-free diet. A negative control group, also fed with β-glucan-free diet but inoculated with PBS, was established to evaluate the effect of the handling during injection. Fish were sampled prior to the stressor exposure, 30 min after and 24 hours post infection (hpi). Herein we observed that the dietary β-G modulated the cortisol profile prior to and after the stressor, increasing the number and activity of leukocytes. Moreover, the cortisol showed to be a strong modulator of both humoral and cellular innate immune system by increasing lysozyme and complement activity, as well as neutrophils and monocytes populations. Our results suggest that β-glucan-induced cortisol increase is one important mechanism to improve the innate immune response in matrinxã.

Key-words: acute stress, handling, capture, early immune defense, acute infection

1. Introduction

Stress is defined as a condition in which the homeostasis of the animal body is threatened or disturbed by intrinsic or extrinsic stimuli, commonly defined as stressors [1]. In teleost fish, stress response shows many similarities to that of terrestrial vertebrates, in both neuroendocrine systems, from the brain to the chromaffin cells (catecholamine secretion), and to the interrenal cells (cortisol secretion), as well as, to the metabolic and ionic adjustments [2–5]. The stressors display coordinated compensatory or adaptive physiological and behavioral responses, allowing the animal to overcome the threat [5,6]. Cortisol is the main circulating glucocorticoid in teleost fish, whose concentration increase dramatically during the stress response [7]. As in mammals, cortisol has metabolic effects by increasing plasma glucose levels to supply the homeostatic mechanisms activated during exposure to stressors [8,9]. Besides that, cortisol effects also include osmotic and ionic regulation, growth, reproduction, immune responses, and behavior [3,4,10,11].

The fish's exposure to acute stressors activates the immune system by increasing the innate response, the leukocytes mobilization and the lymphocytes response. On the other hand, chronic exposure to stressful stimuli can lead to immunosuppression, measured by the decrease of the innate immune response, distribution and differentiation of leukocytes, which make the fish more vulnerable to pathogens [12]. Under unstressed conditions, cortisol plays metabolic maintenance or "housekeeping" roles; however its functions tend to be appreciated when its concentration and actions go well beyond housekeeping range [10]. The cortisol biosynthesis in fish is similar to that in mammals and occurs in the interrenal cells distributed in the head-kidney region [7,10,11]. The biosynthesis involves microsomal enzymatic pathways, including 21-hydroxylation (P450c21), 17α-hydroxylation (P450c17), and 3β-hydroxy steroid dehydrogenation (3β-HSD). In addition, fish possess the mitochondrial inner membrane monooxygenase enzymes, such as the cholesterol side-chain cleavage enzyme (cytochrome P450scc, desmolase) and the 11β-hydroxylase that catalyze the 11β-hydroxylation of deoxycortisol/deoxy-corticosterone (cytochrome P450c11) [10].

To better understand of the role of cortisol in regulating physiological events, different experimental protocols have been used and include stimulus or inhibition of

its biosynthesis. Several substances used to inhibit production of cortisol are utilized therapeutically, such as metyrapone, ketoconazole, and fluconazole which act by inhibiting the steroidogenesis [13]. The metyrapone (2-methyl-1, 2-di-3-pyridyl-1-propanon) (MTP) is a competitive inhibitor of the conversion of 11-deoxycortisol to cortisol by the 11-beta-hydroxylase during the cortisol biosynthesis [14]. Dietary MTP in fish can also result in reduction of cortisol production under stress conditions [15,16]. The MTP has been used in different experimental designs in fish to evaluate the role of cortisol in metabolism, as a regulator of the muscle glycogen production after exercise in rainbow trout *Oncorhynchus mykiss* [17], and also to assess the effect of cortisol on the metabolism of catfish, *Clarias batrachus* [18]. In addition, MTP was used to determine the role of cortisol in the regulation of estrogen biosynthesis and masculinization of Japanese flounder [19]. However, there are no reports of the use of MTP to evaluate the cortisol role on the immune response during the acute stress in fish, especially in fish β-glucan immunostimulated.

It is important to know the modulatory effects of cortisol on the immune response preceding both immunostimulants and bacterial challenges in fish. In this sense, we tested a feed supplemented with β-glucan, and evaluated the role of cortisol during the acute immune response through the blocking of its production by dietary metyrapone or by its endogenous increase in stressed fish, prior to and after bacterial challenge. Our aim was to evaluate the effect of both cortisol serum levels and a protocol with dietary β-glucan to stimulate the early immune response in matrinxã.

2. Material and methods

2.2. Experimental animals and lab condition

This study utilized 64 (222.6 ± 26 g and 25 ± 1.0 cm) juvenile matrinxã that were kept individually in 64 40-liter fiber tanks (1 fish per tank, density near to 5.5 g fish L^{-1}), during 10 days for acclimatization to laboratorial conditions being fed with a commercial feed (28% crude protein CP). Temperature was 30.4° ± 0.4 °C, oxygen 5.66 ± 0.3 mg L^{-1}, and ammonia 36.5 ± 14.5 µg L^{-1}. The photoperiod was 14 h light: 10 h dark, during summer season.

2.3. Experimental design and diets

The present study evaluated the effect of dietary 0.1% β-glucan and 0.1% β-glucan + metyrapone (30mg kg^{-1} fish) in commercial feed (28% CP). Their effects were compared with a β-glucan-free diet as the positive control. After acclimatization, fish were fed with 3.0 % of their body mass twice a day (11:00 – 16:00 h) during 15 days. We used 18 fish per treatment group (Control, β-G, and β-G + MTP). Ten fish were fed with control feed to represent the negative control group. On 16th day, fish were sampled to determine the prior infection condition. Then, 12 fish per treatment group were subject to acute stressor by air exposure during 3 min, aimed to increase the endogenous cortisol secretion, and sampled 30 min after acute stress. Following that, 6 fish per treatment group were inoculated in the mesenteric cavity with a sub lethal concentration of *A. hydrophila* (2.5µL g^{-1}) while 10 fish from the negative control group were inoculated with the same quantity of phosphate buffer saline solution (PBS). Lastly, 24 hours post infection (hpi) fish were sampled.

Experimental diets were prepared using an extruded commercial feed that was ground, mixed to incorporate the 0.1% of β-glucan and 0.1% of β-glucan + Metyrapone (30mg kg^{-1} fish). Following that, the feed was moistened with 40% water and passed through food processor, and finally dried in an oven with air extraction at 40 °C for 24 hours. The control feed was β-glucan-free. The immunostimulant additive used is a research and development substance called “R&D β-glucan” derived from *Saccaromyces cerevisiae* (batch number T1411201) with 62% of purity according to the manufacturer. The product contained β-glucans plus lipids, protein, ash and moisture, and no nucleotides. The batch was kindly provided by Biorigin, Brazil; http://www.biorigin.net/biorigin/macrogard/macrogard_en/index.html).

2.4. Acute bacterial challenge

The *A. hydrophila* strain was isolated from carp, *Cyprinus carpio* (strain A135, LAPOA, Jaboticabal), and identified by sequencing of the 16S rDNA (similarity of 97% with GenBank access: ATCC 7966). The strain was stocked in medium TSB (Tryptic Soy Broth, Media) with 30% glycerol (sterile), at -80° C. An aliquot of 20 µL (strain stock) was inoculated in 5 mL of autoclaved TSB medium and incubated in bacteriological incubator at 28 °C, for 24 h. Subsequently, 200 mL of autoclaved TSB

medium was added and incubated again with the same procedure. The bacterial suspension was centrifuged at 8.000 xg for 10 min and supernatant was discarded, then PBS buffer (0.01 M) was used twice to wash the pellets and centrifuged at 8.000 xg for 10 min. The bacterial PBS suspension lower than lethal concentration CL-50 used was ($3.8 * 10^8$ CFU mL^{-1}) adjusted by UFC counting after bacterial culture and spectrophotometer reading (OD600=1.060). For stimulation of the fish immune response, the bacterial suspension used was previously determined as sub lethal dose (pre-experimental tests, data not shown).

2.5. Sampling

At each sampling time, 6 fish per treatment group were anaesthetized (benzocaine, concentração) and blood samples were drawn from the caudal vein and dispensed in 2 ml microtubes with and without anticoagulant Glistab®. Blood with the anticoagulant was maintained under refrigeration for plasma glucose determination and NBT activity measure. Blood smears were made with fresh blood. Blood without anticoagulant was maintained at room temperature for 3 h and then centrifuged (3000 rpm for 5 min) for serum separation. Complement system activity was measured with fresh serum. The remaining serum samples were stored at -20°C and further used to measure serum cortisol levels.

2.6. Serum cortisol and plasma glucose concentrations

Blood cortisol concentration was measured by enzyme-linked immunosorbent assay (ELISA) with a commercial kit (DRG® Cortisol ELISA, EIA-1887; DRG International, Inc., USA; http://www.drg-international.com). The plasma glucose concentrations were measured by enzymatic method (Labtest kit, Sao Paulo, Brazil, code 84) following the instructions of the manufacturer.

2.7. Leukocyte respiratory burst – NBT activity

The production of reactive oxygen species (ROS) was measured using NBT (Nitro tetrazolium blue chloride – Sigma Aldrich - N6876), following protocol by Anderson and Siwicki (1995), modified by Biller-Takahashi et al. (2013). Immediately after fish bleeding, 50 µL of heparinized blood were incubated with an equal volume of NBT buffer (0.2%) at room temperature for 30 min. Subsequently, 1 mL of DMF (Dimethylformamide, Sigma Aldrich – 227056) was added to the samples, and they were read in a spectrophotometer (Thermo Scientific®; Genesys 10S), at room temperature and 540 nm.

2.8. Complement system activity: alternative pathway (ACH50)

ACH50 was measured according to Polhill et al. (1978) and Ferriani et al. (1990) and rabbit blood was collected and processed to isolate red blood cells (RaRBC) whose suspension was added to serum. Then, complement hemolytic activity was measured as time (in seconds) necessary to lyse 50% of RaRBC in kinetic assay at 700 nm. Some modifications were made to adjust the method to the matrinxã blood i.e. the serum was fresh and the suspension of serum and rabbit erythrocytes adjusted to ratio 1:1.

2.9. Serum lysozyme concentrations

The serum lysozyme concentration was determined according to Demers and Bayne (1997) with modifications by Zanuzzo et al. (2015). The assay is based on the lysis of *Micrococcus lysodeikticus* suspension (Sigma-Aldrich, São Paulo, Brazil; #M3770) using hen egg white lysozyme as standard (Sigma-Aldrich, São Paulo, SP, Brazil; #L6876). The assay was performed in 96-well plates in triplicate. The rate of decrease in absorbance for each sample (∆OD) was then compared to the standard curve. Lysozyme concentration is expressed in ng μl^{-1}.

2.10. *Cellular counts*

The total count of red cells was performed in a Neubauer chamber, using whole blood diluted in formaldehyde citrate buffer 1:200. The total and differential count of leukocyte was performed on blood smears stained with methanol blue eosin solution May-Grünwald-Giemsa-Wrigth (MGGW), according to Tavares-Dias et al. (2008). The leukocytes calculated by the indirect method was the amount of leukocytes found at each 2000 erythrocytes counted. To the leukocytes differentiation, 200 white blood cells were counted and the amount of each cell type was expressed as cells μl^{-1}.

2.11. Data analysis

To evaluate the immunostimulant effect of 0.1% of dietary β-glucan and the role of cortisol on this response we compared the treatments to the positive control group prior to and after bacterial challenge. All data was submitted to normality (Shapiro-Wilk) and homoscedasticity (Levene). The experiment was set up in a completely randomized design with a factorial arrangement of 3x3, being 3 treatments (positive control, β-glucan 0.1% and β-glucan 0.1% + metyrapone 30 mg kg^{-1} fish) x 3 sampling times (prior to infection, 30 min post stress, and 24 hours post infection). Means were compared by Duncan's post-hoc tests. Finally, a t-test was used to compare the means of positive and negative control groups 30 min and 24 h after the respective inoculations. Values in the text and figures are represented by means ± standard error of the mean (SEM). n=6 and P value < 0.05 was used to estimate the level of significance for statistical differences.

2.12. Ethical statement

All procedures that involved animal use in this study were performed in accordance with ethical principles in animal experimentation, adopted by the Colégio Brasileiro de Experimentação (COBEA), Brasilia, Brazil, and approved by the Comissão de Ética no Uso de Animais (CEUA) protocol n° 014679/14 UNESP - Jaboticabal, Brazil.

3. Results

3.1. Serum cortisol and plasma glucose concentrations

Prior to the stressor exposure, fish fed with β-glucan 0.1% (β-G) showed the highest levels of serum cortisol, followed by fish from the positive control group and lastly by fish fed with β-glucan 0.1% + metyrapone (β-G + MTP). However, 30 min after stressor exposure, fish fed with β-G + MTP showed the highest levels of serum cortisol. Finally, 24 hours post infection (hpi), cortisol serum levels in all challenged fish showed similar values compared to positive control group at the initial sampling time. Serum cortisol levels in fish from the negative control group were lower than those of the positive control (Figure 1A). Plasma glucose concentrations did not differ among treatment groups at any sampling time. However, 30 min after, all fish showed increased plasma glucose concentrations. At 24 hpi, the glucose levels in fish from all groups reduced but to values lower than those of their initial condition. Negative control glucose values were lower compared to the positive control group (Figure 1B).

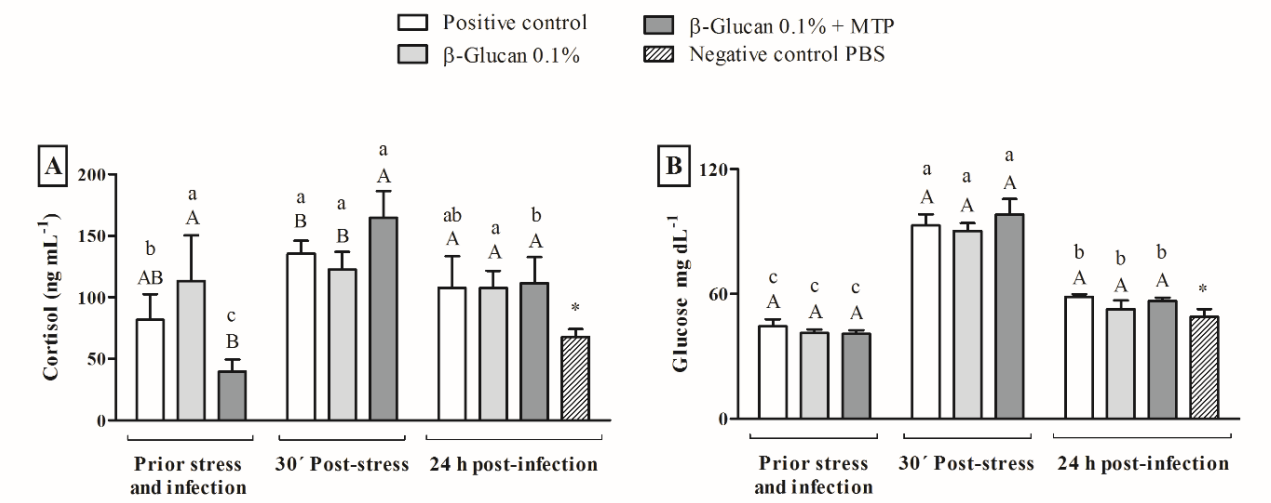

Figure 1. *Cortisol serum (A) and plasma glucose (B) levels in matrinxã fed with β-glucan 0.1% or β-glucan 0.1% + metyrapone MTP 30mg kg^{-1} fish.* The results are compared to fish from the positive (bacterial challenge) and the negative (PBS injected) control groups, prior to and after both air exposure and *A. hydrophila* inoculation. Different capital letters indicate differences between treatment groups in each isolated sampling time. Different lowercase letters indicate differences between the readings of the same treatment group, collected at all times shown. Symbols, ≈ indicates no difference and * indicates difference between positive and negative control groups 30 min after stressor and 24 h post infection. Bars represent means ± SEM (n=6, P<0.05).

3.2. Lysozyme concentration, respiratory burst activity of leukocytes RAL and complement system activity

Prior to the stressor exposure, fish fed with β-glucan 0.1% (β-G) showed the highest levels of serum lysozyme, followed by fish from the positive control group, and lastly by fish fed with β-G + MTP. However, 30 min after, fish fed with β-G + MTP showed the highest levels of serum lysozyme, followed by those from the positive control group. Fish fed with β-G showed the lowest values. Finally, 24 hpi lysozyme serum levels did not differ among treatments but decreased in fish fed with β-G + MTP. Serum lysozyme levels in the negative control group did not differ to those of the positive control (Figure 2A).

Prior to and after the stressor exposure, RAL did not differ among treatments. However, 30 min after, we observed a reduction of the reactive oxygen species (ROS) production in fish from all treatments. Twenty-four hpi, RAL increased in all challenged fish with the highest values in fish fed with β-G + MTP, followed by fish fed with β-G, and lastly by fish from the positive control group. At this sampling time, RAL did not differ between fish from positive and negative control groups (Figure 2B).

The hemolytic activity of the complement system did not differ among treatments prior to and after the stressor. However, 30 min after we observed reduction of the hemolytic activity in fish from all treatments. At 24 hpi, both groups of fish pre-treated with β-G showed delayed responses compared to those of positive and negative control groups (Figure 2C).

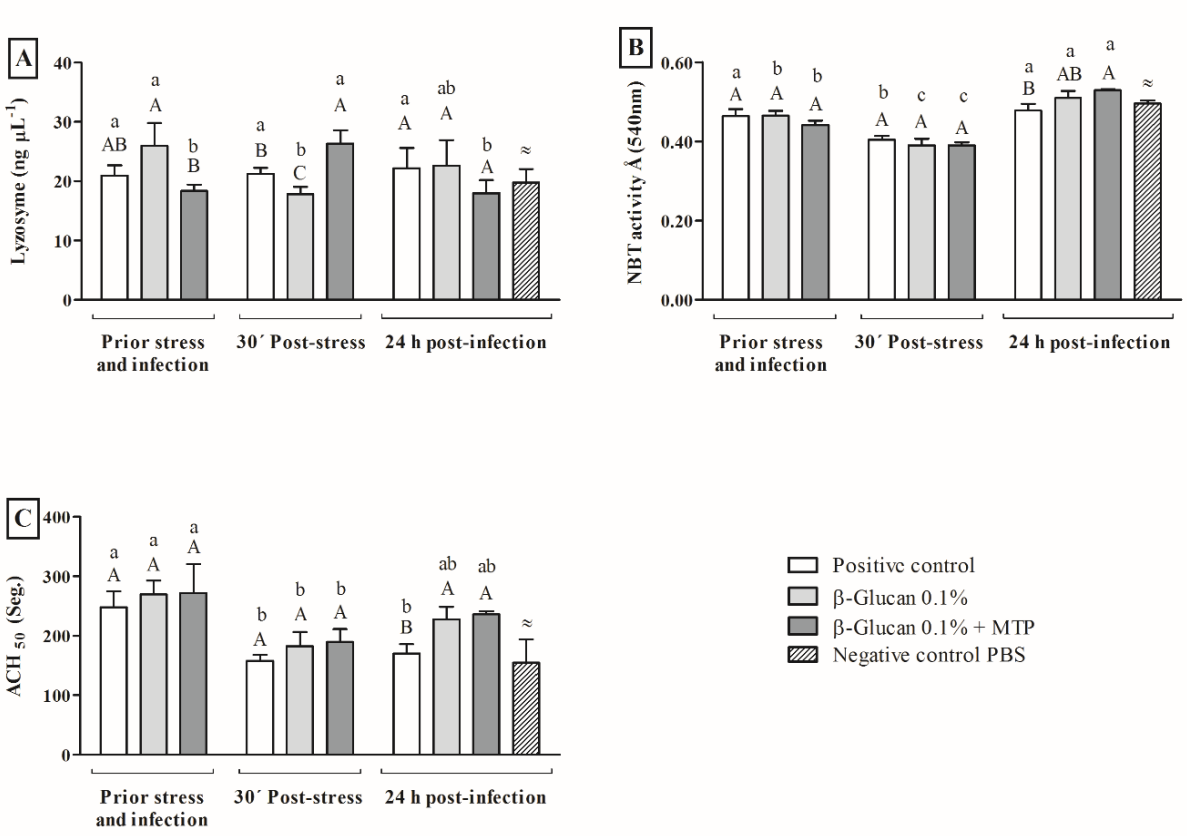

Figure 2. *Lysozyme serum concentrations (A), respiratory activity of leukocytes (B) and complement system activity (C) in matrinxã*. Fish fed with β-glucan 0.1% or β-glucan 0.1% + metyrapone MTP 30mg kg^{-1} fish. Results are compared to fish from positive (bacterial challenge) and negative (PBS injected) control groups, prior to and after both air exposure and *A. hydrophila* inoculation. Different capital letters indicate differences between treatment groups in each isolated sampling time. Different lowercase letters indicate differences between the readings of the same treatment group, collected at all times shown. Symbols, ≈ indicates no difference and * indicates difference between positive and negative control groups 30 min after stressor and 24 h post infection. Bars represent means ± SEM (n=6, P<0.05).

3.3. Number of circulating erythrocytes, leukocytes, lymphocytes, neutrophils and monocytes

The number of circulating erythrocytes did not differ among treatments at any sampling time, not prior to nor 30 minutes after stressor exposure. However, 24 hpi all challenged fish showed a marked increase of circulating erythrocytes. Negative control group values were lower than those of the positive control group (Figure 3A).

Prior to the stressor exposure, the number of leukocytes did not differ among treatment groups. However, 30 min after, fish fed with β-G + MTP showed the highest number of circulating leukocytes, followed by those fed with β-G and lastly by fish from the positive control group. At 24 hpi we observed an inverse situation: the highest values in the positive control group and the lowest values in fish fed with β-G + MTP. The negative control group showed more circulating leukocytes than the positive control (Figure 3B).

Prior to the stressor exposure, the number of lymphocytes did not differ among treatments. However, 30 min after, fish fed with β-G + MTP showed the highest number of circulating lymphocytes, followed by those fed with β-G, and lastly by fish from the positive control group. At 24 hpi, all challenged fish revealed a decrease in the number of lymphocytes. However, fish from both β-G treatments showed lower circulating lymphocytes values compared to the positive and negative control groups (Figure 3C).

Prior to the stressor exposure, fish fed with β-G showed the highest number of circulating neutrophils, followed by those from the positive control group and lastly by fish fed with β-G + MTP. However, 30 min after, fish fed with β-G + MTP showed the highest number of neutrophils, followed by fish fed with β-G, and lastly by those from the positive control group. At 24 hpi we observed an inverse situation: the highest values in the positive control group and the lowest values in fish fed with β-G + MTP. The negative control group showed a lower number of circulating neutrophils compared to the positive control group (Figure 3D).

Prior to the stressor exposure, fish fed with β-G showed a higher number of circulating monocytes compared to fish from the positive control and fish fed with β-G + MTP. However, 30 min after, fish fed with β-G + MTP showed a clear increase in the number of circulating monocytes compared to the other fish groups. Finally, 24 hpi the values tended to return to the initial condition, the highest number being in fish fed with β-G. Circulating monocytes from the positive and negative control fish groups did not differ (Figure 3E).

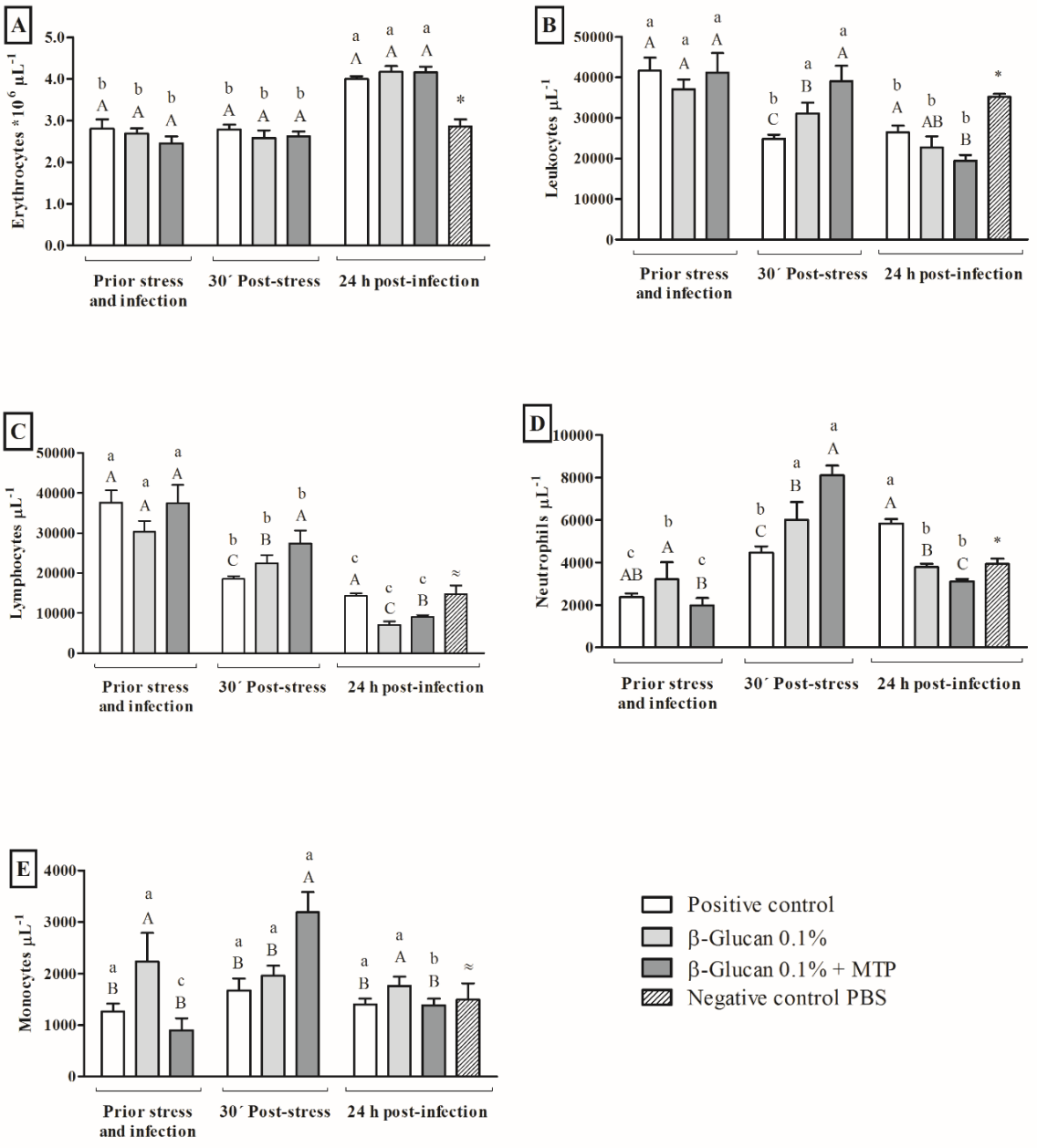

Figure 3. *Number of circulating erythrocytes (A), leukocytes (B), lymphocytes (C), neutrophils (D) and monocytes (E) in matrinxã.* Fish fed with β-glucan 0.1% or β-glucan 0.1% + metyrapone MTP 30mg kg^{-1} fish. Results are compared to fish from positive (bacterial challenge) and negative (PBS injected) control groups, prior to and after air exposure and *A. hydrophila* inoculation. Different capital letters indicate differences between treatment groups in each isolated sampling time. Different lowercase letters indicate differences between the readings of the same treatment group, collected at all times shown. Symbols, ≈ indicates no difference and * indicates difference between positive and negative control groups 30 min after stressor and 24 h post infection. Bars represent the means ± SEM (n=6, P<0.05).

4. Discussion

Herein we studied the immunostimulant effect of dietary β-glucan 0.1%, and the role of cortisol levels over the innate immune response in matrinxã. Our results showed that cortisol levels can modulate both humoral and cellular components of the early immune response in matrinxã, also we observed that immunostimulation by β-glucan also has a relationship with cortisol secretion.

Both strategies we used to manipulate the levels of cortisol (dietary metyrapone and stressor exposure) were efficient to alter the physiological cortisol levels. Dietary MTP reduced the cortisol levels prior to stressor exposure, a condition that was determinant to the understanding of the relation between cortisol and innate immune response, in both humoral and cellular baseline parameters. The cortisol reduction by MTP was previously observed in fish under stress conditions [15,16]. However, the dietary MTP did not prevent the cortisol increase in matrinxã under stress as previously observed by Zanuzzo and Urbinati (2016). Contrarily, the increase of cortisol was more accentuated in fish in control groups and those fed with β-glucan. Matrinxã with reduced levels of cortisol were more sensitive and responsive to the effect of the stressor.

The stress condition was confirmed by the two fold increase of plasma glucose concentration, that normalized at 24 hpi, as previously described in matrinxã subject to transport stress [28] and capture stress [29]. It was also confirmed by the increase in circulating cortisol levels in the positive control fish and those that received MTP, differently from fish fed with β-glucan whose cortisol levels were already elevated before the stressor exposure.

The lysozyme serum concentrations showed a clear relation with the cortisol levels. Fish with the higher cortisol levels were also those that showed higher levels of serum lysozyme. The results suggest that high cortisol production induced by dietary β-glucan or by acute stress is responsible for the stimulation of the lysozyme concentration. In addition, we observed that the hemolytic activity of the complement system was also induced by stress in matrinxã, without modulation by dietary β-glucan. A variety of immune changes have been described after acute stress in fish. Immediate responses during the activation phase enhance innate humoral immunity such as increased levels of lysozyme and C3 complement component in different fish species [12,24,30].

Both the activated and suppressed respiratory activity of leukocytes (RAL) have been described following stress episodes. The initial secretion of ACTH can stimulate, whereas the final product, the cortisol, is rather inhibitory or suppressive [12]. We observed a decrease of the RAL after stress and an increase after the acute infection; fish treated with MTP showed the highest RAL. The RAL is considered an important indicator of the phagocytic activity. The increase in oxygen uptake at the beginning of the respiratory burst is followed by the production of reactive oxygen species which attack the pathogens [21]. Previous studies described that after acute stress, there was a strong reduction of the phagocytic activity in trout and sea breams [31,32].

The number of circulating erythrocytes increased only after acute infection, suggesting that the response of matrinxã to stress by air exposure did not affect this parameter and it was not modulated by dietary β-glucan. In contrast, previous reports indicated that stress increased the number of circulating erythrocytes in matrinxã after 4h transport [33], or after 2 min air exposure and sampled after 5 min [34]. The different results can be explained by different stressor intensities, duration, and sampling time.

The total leukocyte counting reflected the number of different white cell types. It is well known that, during the acute stage of an infection or in conditions of immune exhaustion, there is a suppression of circulating lymphocytes [35]. We observed that the leukocyte profile was markedly associated to levels of serum cortisol. In fish, cortisol has been shown to affect the number of circulating leukocytes and their activity [12,36,37]. Previous studies have described a short-term cortisol secretion increase in the number of circulating neutrophilic granulocytes and reduced lymphocyte proliferation and functions [38,39].

Neutrophils increase rapidly in circulation during acute stress when cortisol levels are high [12,40]. We observed that, in matrinxã, the physiological number of neutrophils was low before stress; but after acute stress the number increased, with a clear association with cortisol levels. Fish with the highest cortisol presented the highest neutrophilia. However, this association was not observed at 24 hpi. Cortisol-induced neutrophilia was observed also in channel catfish stressed by handling and transport [41].

In this study, the profile of cortisol was clearly associated to the profile of circulating monocytes. Under physiological conditions, cortisol would be stimulating

proliferation of these cells both prior to and after stress conditions. The highest number of monocytes coincident with the highest cortisol levels suggests the involvement of cortisol in this cell migration and function as described by Tort (2011). Monocytes/macrophages are part of the first line of cell defense against infection in the innate immune system and are also responsible for the regulation of inflammatory response [42,43]. We observed at 24 hpi that the circulating monocytes decreased probably by migration to infection tissue. At this sampling, fish fed with β-G showed high number of monocytes in their blood. Our results are in accordance with another study describing the stimulating effect of β-glucan over monocyte functions [44].

In summary, the cortisol secreted as response to an acute stressor and/or bacterial challenge is able to modulate both humoral and cellular innate immune response in matrinxã, and it is a fundamental mediator of the immunostimulant effect of β-glucan. The association of the cortisol levels and the immune response indicate that glucan-induced cortisol enhance the start of the early innate immune response in matrinxã.

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CHAPTER 6

Final considerations

The intensive aquaculture system affects fish health because of high stocking densities and handling, promoting a potentially stressful environment as well as rapid proliferation of pathogens. Stressful conditions in fish farming lead to alterations in the neuroendocrine hypothalamus-pituitary-interrenal tissue axis and cortisol production. Depending of the stressor type, intensity and duration, the stress response has acute or chronic characteristics, affecting the physiological homeostasis of fish, which includes the impairment of the immune system response. To control infectious diseases in fish, caused by chronic stress-induced immunosuppression, risky measures have been taken such as the uncontrolled use of antibiotics, which has contributed to the emergence of several resistant pathogenic organisms. Thus, it is necessary to develop strategies for pathogen control and immuno-prophylactic measures aiming to strengthen fish immune response to face challenges imposed by the production system.

Currently, the use of natural immunostimulant products is becoming a healthy alternative to counteract the negative effects of stress situations in many livestock species, including fish. Addition of immunostimulants to the diet, known as “functional food”, has been shown to enhance fish’s innate humoral and cellular immune responses against infectious diseases. Dietary β-glucan derived from the cell wall of baker’s yeast *(Saccharomyces cerevisiae)* effectively promoted the activation of early immune response in juvenile matrinxã when used during 15 days in concentration of 0.1% of diet offered to fish at 1.5-3.0% of biomass. The immunostimulation of dietary β-glucan affects both humoral and cellular innate immunity. Herein, based on our results, we propose that the immunostimulant mechanism of β-glucan in matrinxã involve the acute increase of cortisol secretion during the initial response, which leads to monocytes-macrophages and neutrophils activation and proliferation. In addition, it is known that the binding of β-glucan, as pathogen-associated molecular pattern “PAMP”, to specific receptor in monocytes/macrophages is an activating stimulus to these cells. Thus, the β-glucan can improve the leukocytes activity and proliferation by both pathways: direct (through receptor-binding) and indirect (cortisol-induced).

The dietary use of β-glucan in matrinxã improved the lymphocytes proliferation and probably the production of immunoglobulins (≈26 - 27.5 kDa-proteins). The acute secretion of cortisol during the protocol of immunostimulation with dietary β-glucan also induced the increase of lysozyme serum concentration, an important indicator of innate immune response, as well as a moderate increase of the hemolytic activity of the complement system.

It is worth noting that the new generation of β-glucan, from Biorigin, "R&D β-glucan" tested in this study, was more efficient than the previous generation to stimulate the humoral immune response e.g., lysozyme serum levels and complement activity, as well as in the cellular indicators: neutrophils, monocytes, and lymphocytes. In addition, it increased the cortisol serum levels prior to and during an experimental acute infection, which may help to prepare the fish to face stressful practices and bacterial challenges during fish farming.

We also highlight that it is the first description of the electrophoretic profile of serum proteins in matrinxã, as well as the characterization of the proteins during the acute phase response as negative (≈60 and ≈75 kDa) or positive (≈26-27.5 kDa and ≈36-37 kDa) APPs. This protein profile has important implications for aquaculture and medical fields, since it represents a potential starting point for future development of diagnostic tools for human and veterinarian medicine. The identification of positive and negative APPs, and the elucidation of β-glucan-mediated defense mechanisms in matrinxã and other fish should be subject to study in future research to obtain a broader understanding of these mechanisms and to provide tools for the improvement of protocols of immunostimulation.

Finally, based on our results, this study encourages the use of functional food in matrinxã aquaculture, with an addition of 0.1% of β-glucan derived from the cell wall of baker's yeast *(Saccharomyces cerevisiae)* as an immunostimulant. This functional food may be offered for 15 days in proportion of 1.5% of biomass in fish >200g or 3.0% of biomass in fish <200g, before expected management practices e.g., capture, transport, or artificial reproduction. This protocol aims to strengthen fish defense mechanisms, reduce disease outbreak, and enhance fish resistance to generate nutritional products with high quality and low environmental cost.

Considerações finais

O sistema de aquicultura intensiva afeta a saúde dos peixes em virtude das altas densidades de estocagem e manejo, promovendo um ambiente potencialmente estressante, bem como a rápida proliferação de patógenos. Condições estressantes na piscicultura levam a alterações no eixo neuroendócrino hipotálamo-hipófise-interrenal e produção de cortisol. Dependendo do tipo de estressor, intensidade e duração, a resposta de estresse tem caráter agudo ou crônico, afetando a homeostase fisiológica dos peixes, que inclui prejuízos ao sistema imunitário. Na luta contra as doenças infecciosas nos peixes causadas por imunossupressão induzida pelo estresse crônico, tem-se adotado medidas de risco, como o uso descontrolado de antibióticos, que contribui para o surgimento de patógenos altamente resistentes. Portanto, é necessário desenvolver estratégias para controle de patógenos e medidas imuno-profiláticas que visem reforçar a resposta imune dos animais para enfrentar os desafios impostos pelo sistema de produção.

Atualmente, a utilização de produtos naturais imunoestimulantes torna-se numa alternativa saudável para combater os efeitos negativos de situações de estresse em muitas espécies animais incluindo peixes. A adição na ração de imunestimulantes é conhecida como "alimentos funcionais" e já foi mostrado que melhora as respostas imunes inatas humorais e celulares contra doenças infecciosas em peixes. O β-glucano dietético derivado da parede celular de levedura de padeiro (*Saccharomyces cerevisiae*) promoveu eficazmente a ativação da resposta imunitária inicial em juvenil de matrinxã, quando usado durante 15 dias, na concentração de 0,1% da dieta, oferecido na proporção de 1,5 - 3,0% da biomassa. A imunoestimulação mediada por β-glucano na dieta envolve tanto a imunidade inata humoral e celular. No presente estudo, com base em nossos resultados, propomos que o mecanismo imunoestimulante de β-glucano em matrinxã envolve o aumento agudo da secreção de cortisol, o qual, durante a resposta inicial, estimula tanto a ativação quanto a proliferação dos monócitos-macrófagos e neutrófilos. No entanto, a literatura sugere que a ligação direta de β-glucano, como padrão molecular associado a patógenos "PAMP", ao receptor específico nos monócitos / macrófagos é um estímulo ativador destas células. Assim, o β-glucano pode melhorar a resposta imune inata mediada por células por ambas as vias: direta (de ligação ao receptor das células) e indireta (induzida pelo cortisol).

O uso de β-glucano na dieta melhorou a proliferação de linfócitos e, provavelmente, a produção de imunoglobulinas (≈26 - ≈27,5 kDa-proteínas). Além disso, a secreção aguda de cortisol durante o protocolo de imunoestimulação com dieta suplementada com β-glucano induziu o aumento da concentração sérica de lisozima, importante indicador da resposta imune inata, bem como aumentou moderadamente a atividade hemolítica do sistema do complemento no matrinxã.

É importante notar que a nova geração de β-glucano, da Biorigin, "R&D β-glucano" testado neste estudo foi mais eficiente em estimular a resposta imune humoral, por exemplo, níveis séricos de lisozima e atividade hemolítica do sistema complemento, assim como os indicadores celulares (neutrófilos, monócitos e linfócitos). Além disso, aumentou os níveis séricos de cortisol antes e durante uma infecção aguda experimental, o que pode ajudar a preparar os peixes para enfrentar desafios bacterianos e/ou práticas de manejo estressantes durante o ciclo produtivo.

Ressaltamos, ainda, que o perfil eletroforético de proteínas séricas em matrinxã foi apresentado pela primeira vez, bem como a caracterização das proteínas durante a resposta de fase aguda como proteínas de fase aguda negativas (≈60 ≈75 e kDa) ou positivas (≈26-27.5 kDa e ≈36-37 kDa). Este perfil proteína tem implicações importantes para a aquicultura, uma vez que representam potencial ponto de partida para o futuro desenvolvimento de ferramentas de diagnóstico como utilizadas atualmente na medicina humana e veterinária. A identificação destas proteínas e suas funções, e a elucidação dos mecanismos de defesa mediados pelo β-glucano em matrinxã e outros peixes, deve ser objeto de novos estudos para um entendimento mais amplo destes mecanismos visando fornecer ferramentas para aperfeiçoamento de protocolos de imunestimulação.

Finalmente, com base em nossos achados, este estudo incentiva o uso de alimentos funcionais durante a criação de matrinxã, com adição na ração de 0.1% de β-glucano derivado da parede celular de levedura de panificação (*Saccharomyces cerevisiae*) como inmunoestimulante. Na proporção de 1,5% de biomassa em peixes > 200g ou 3,0% da biomassa peixes < 200g, que pode ser oferecido durante 15 dias antes de práticas de manejo previstas como captura, transporte ou reprodução artificial. Este protocolo visa reforçar os mecanismos de defesa do peixe, além de reduzir surtos de doenças e aumentar a resistência dos peixes, como objetivo de gerar produtos nutricionais de alta qualidade com baixo custo ambiental.