Fish & Shellfish Immunology 60 (2017) 197–204

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

β-Glucan-induced cortisol levels improve the early immune response in matrinxã (*Brycon amazonicus*)

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ARTICLE INFO

Article history:
Received 2 August 2016
Received in revised form
20 November 2016
Accepted 25 November 2016
Available online 27 November 2016

Keywords:
Acute stress
Handling
Capture
Early immune defense
Acute infection

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. For this, 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 β-glucan 0.1% (β-G) or β-glucan 0.1% + metyrapone 30 mg kg^{-1} fish (β-G + MTP). Dietary MTP was used to block cortisol production. After 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 exposure, and 24 h post infection (hpi). Herein we observed that dietary β-G modulated the cortisol profile prior to and after the stressor, increasing the number and activity of leukocytes. Moreover, cortisol showed to be an efficient modulator of both humoral and cellular innate immune system by increasing lysozyme and complement activity, as well as neutrophil and monocyte populations. Our results suggest that β-glucan-induced cortisol increase is one important mechanism to improve the innate immune response in matrinxã.

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 neuroendocrine systems from the brain to the chromaffin cells (catecholamine secretion) and interrenal cells (cortisol secretion), as well as 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, and its concentration increases 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, leukocyte mobilization, and lymphocyte response. On the other hand, chronic exposure to stressful stimuli can lead to immunosuppression, measured by a decrease of the innate immune response, as well as distribution and differentiation of leukocytes, making 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

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http://dx.doi.org/10.1016/j.fsi.2016.11.055
1050-4648/

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 mitochondrial inner membrane monooxygenase enzymes, such as the cholesterol side-chain cleavage enzyme (cytochrome P450scc, desmolase) and 11β-hydroxylase, which 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]. Metyrapone (2-methyl-1, 2-di-3-pyridyl-1-propanon) (MTP) is a competitive inhibitor of the conversion of 11-deoxycortisol to cortisol by 11-beta-hydroxylase during cortisol biosynthesis [14]. Dietary MTP in fish can also result in reduction of cortisol production under stress conditions [15,16]. 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 role of cortisol in immune response during acute stress in fish.

In an attempt to strengthen the immune system of farmed fish under stress, the use of immunostimulants has been exploited [20,21]. 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 [21–23]. The β-glucans are among the several immunostimulants used in aquaculture [21,24–27]. They represent part of a group of physiologically active compounds called “biological response modifiers” [28]. They are highly conserved and non-digestible carbohydrates that are structural components of cell walls of some plants, fungi, yeast, seaweed, and bacteria [23].

The β-glucans have been shown to be immunostimulatory and have beneficial properties for animals, including increased protection against infections [23,29,30], tumors development [31,32] and sepsis [33,34]. Their effect is attributed to the ability to bind to several types of leukocytes receptors, known as Toll-like receptors (TLR) and/or pattern recognition receptors (PRR) [25,35]. 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-glucans are an increase of bactericidal activity [29], modulation of cytokine production [36], increase of blood monocytes [33], and increase in number and phagocytic activity of leukocytes [24]. Additional effects include the increase of serum lysozyme concentration, hemolytic complement activity, as well as increase of antibody response and resistance to *Aeromonas hydrophila* [21,24,25,28,37].

The matrinxã *Brycon amazonicus* is a teleost fish, native of the Amazon River basin in Brazil, that has been considered a promising candidate for aquaculture throughout the country [38], especially in the Amazonian region. Due to its active and reactive temperament, matrinxã is an experimental model in studies on stress physiology [39–44]. In addition, matrinxã has been used in studies concerning aquaculture procedures, such as transport [45–48], capture [49,50], stocking densities [48,51] and behavioral responses [40,42–44,52]. In addition, it would be beneficial to assess the effects of the β-glucan on innate immune responses of this fish, bringing valuable knowledge for future application in its farming.

It is important to know the modulatory effects of cortisol on the immune response preceeding both immunostimulants and bacterial challenges in fish. To this end, 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 early immune response in matrinxã (*Brycon amazonicus*).

2. Material and methods

2.1. Experimental animals and lab conditions

This study utilized 64 (222.6 ± 26 g and 25 ± 1.0 cm) juvenile matrinxã that were kept individually in 64 40-L fiber tanks (1 fish per tank, density close to 5.5 g fish L^{-1}), during 10 days for acclimatization to laboratorial conditions, and were 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.

2.2. Experimental design and diets

The present study evaluated the effect of dietary 0.1% β-glucan and 0.1% β-glucan + metyrapone (30 mg 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 and 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 the 16th day, fish were sampled to determine the condition prior to infection. Then, 12 fish per treatment group were subjected to acute stressor by air exposure during 3 min, aiming to increase 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* (2.5 μL g^{-1}) while 10 fish from the negative control group were inoculated with the same quantity of phosphate buffer saline solution (PBS). Lastly, fish were sampled 24 h post infection (hpi).

Experimental diets were prepared using an extruded commercial feed that was ground, then mixed to incorporate 0.1% of β-glucan and 0.1% of β-glucan + metyrapone (30 mg kg^{-1} fish). Following that, the feed was moistened with 40% water and passed through a food processor, and finally dried in an oven with air extraction at 40 °C for 24 h. The control feed was β-glucan-free. The immunostimulant used “R&D β-glucan”, derived from *Saccaromyces cerevisiae* (batch number T1411201), with 62% purity, was 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, Laboratório de Microbiologia e Parasitologia, LAPOA, Jaboticabal), and identified by sequencing of the 16S rDNA (similarity of 97% with GenBank access: ATCC 7966). The strain was stocked in TBS medium (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 at 28 °C, for 24 h. Subsequently, 200 mL of TSB medium was added and incubated again with the same procedure. The bacterial suspension was centrifuged at 8.000×*g* for 10 min and supernatant was discarded, then PBS buffer (0.01 M) was used twice to wash the pellets. The bacterial PBS suspension lower than lethal concentration CL-50 used (3.8×10^8 CFU mL^{-1}) was adjusted by UFC counting after bacterial culture and spectrophotometer reading (OD600 = 1.060). For stimulation of the fish immune response, the bacterial suspension was previously determined as sub-lethal dose (pre-experimental tests, data not shown).

2.4. Sampling

At each sampling time, 6 fish per treatment group were anaesthetized (benzocaine, 100 mg L^{-1}) and blood samples were drawn from the caudal vein and dispensed in 2 mL microtubes with and without anticoagulant Glistab® (http://www.labtest.com.br/reagentes#). 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.

2.5. Serum cortisol and plasma glucose concentrations

Blood cortisol concentration was determined 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 determined 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 [53], modified [54]. 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.

2.7. Complement system activity: alternative pathway (ACH50)

ACH50 was measured according to Refs. [55,56] 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 was adjusted to ratio 1:1.

2.8. Serum lysozyme concentrations

The serum lysozyme concentration was determined according to [57] with modifications by Ref. [58]. 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}.

2.9. 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 leukocytes was performed on blood smears stained with methanol blue eosin May-Grünwald-Giemsa-Wright (MGGW) solution, according to [59]. Calculation of leukocytes by the indirect method considered the number of leukocytes found at each 2000 erythrocytes. For leukocyte differentiation, 200 white blood cells were counted and the amount of each cell type was expressed as cells μL^{-1}.

2.10. 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 were submitted to normality (Shapiro-Wilk) and homoscedasticity (Levene). The experiment was set up in a completely randomized design with a factorial arrangement of 3 × 3, being 3 treatments (positive control, β-glucan 0.1%, and β-glucan 0.1% + metyrapone 30 mg kg^{-1} fish) × 3 sampling times (prior to infection, 30 min post stress, and 24 h 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.

2.11. 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, at 24 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 (Fig. 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 (Fig. 1B).

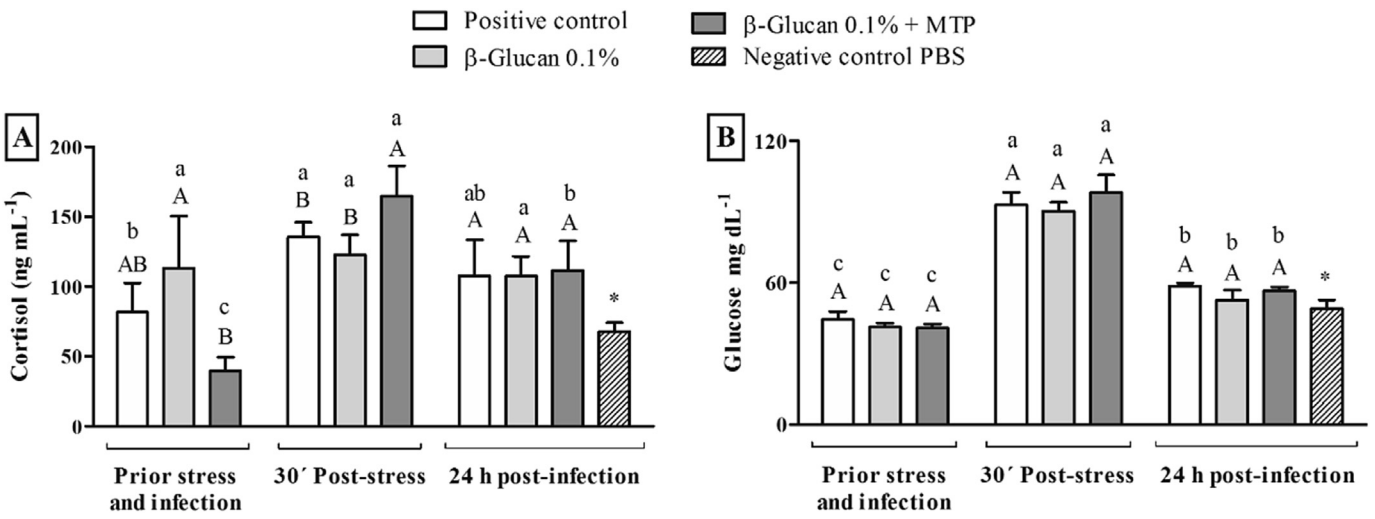

Fig. 1. *Cortisol serum (A) and plasma glucose (B) levels in matrinxã fed with β-glucan 0.1% or β-glucan 0.1% + metyrapone MTP 30 mg kg^{-1} fish.* The results are compared to fish from the positive (bacterial challenge) and the negative (PBS-injected) control groups, prior to and after both air exposure 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 for the same treatment group, collected at all times shown. The ≈ symbol 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 leukocyte 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 exposure, 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 from those of the positive control (Fig. 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 (Fig. 2B).

The hemolytic activity of the complement system did not differ among treatments prior to and after the stressor. However, 30 min after exposure 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 (Fig. 2C).

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, either prior to or 30 min after stressor exposure. However, at 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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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, at 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 (Fig. 3E).

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.

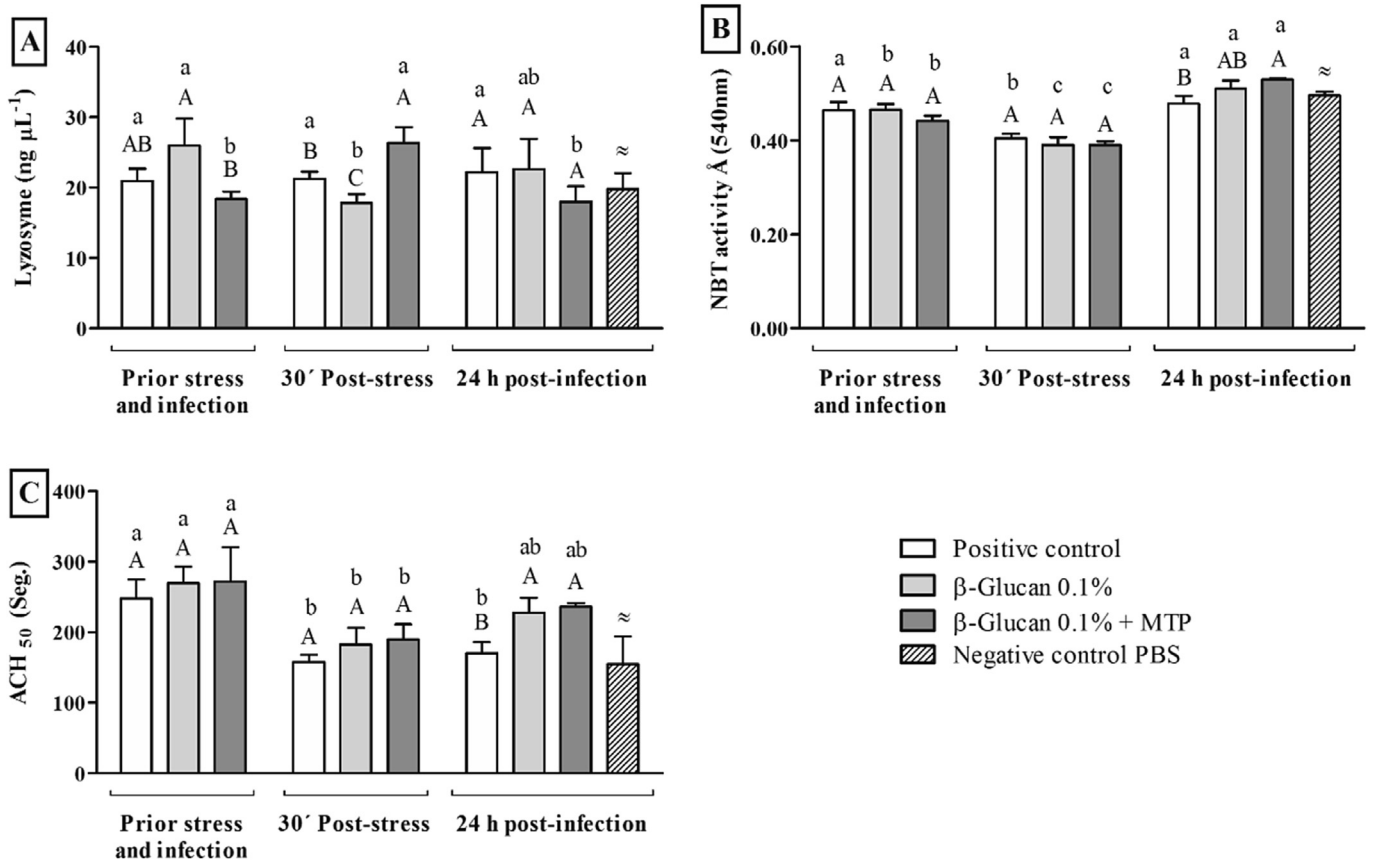

Fig. 2. *Lysozyme serum concentrations (A), respiratory activity of leukocytes (B), and complement system activity (C) in matrinxã.* Fish were fed with β-glucan 0.1% or β-glucan 0.1% + metyrapone MTP 30 mg kg^{-1} fish. Results are compared to fish from positive (bacterial challenge) and negative (PBS-injected) 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 for the same treatment group, collected at all times shown. The ≈ symbol 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).

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. Cortisol reduction by MTP was previously observed in fish under stress conditions [15,16]. However, dietary MTP did not prevent cortisol increase in matrinxã under stress as previously observed in pacu pre-treated with MTP [16]. 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.

β-glucans as a pathogen associated molecular pattern PAMP have strongly influenced the natural and adaptive host immune responses [25,60]. On the other hand, they are also known as leukocyte activators that promote immune protection in several animal disease models [61]. The immune modulator effect of glucans occurs following their binding to specific receptors in monocytes/macrophages, neutrophils and natural killer cells [62]. Indeed, β-glucan binds to different types of receptors (βGR) in leukocytes, thereby triggering both innate and adaptive immune response [25]. Among these βGR, the scavenger receptor 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. The binding of β-glucan to CR3 increases leukocytes' phagocytosis and degranulation as well as their tumoricidal activity [63]. Lactosylceramide is a glycosphingolipid found on leucocytes and endothelial cells that binds to β-glucan and is associated with production of reactive oxygen species (ROS) [21]. 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 production after binding to TLR2 and TLR4, thereby favoring Th1 cell differentiation [25]. Lastly, the dectin-1 βGR (dentritic cell-associated C-type lectin-1) is considered the main β-glucan receptor and recognizes carbohydrates containing β-1,3 and/or β-1,6 glucan linkages, being expressed by monocyte/macrophages, neutrophils, dendrite cells, and T cells [60]. 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 [64].

Moreover, we observed the effects of stress, and glucocorticoid levels on the early immune response in matrinxã and we inferred the influence of β-glucan supplementation in cortisol serum levels. The stress condition was confirmed by the two-fold increase of plasma glucose concentration, that normalized at 24 hpi, as previously described in matrinxã subjected to transport stress [51] and the stress response by capture [49]. 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

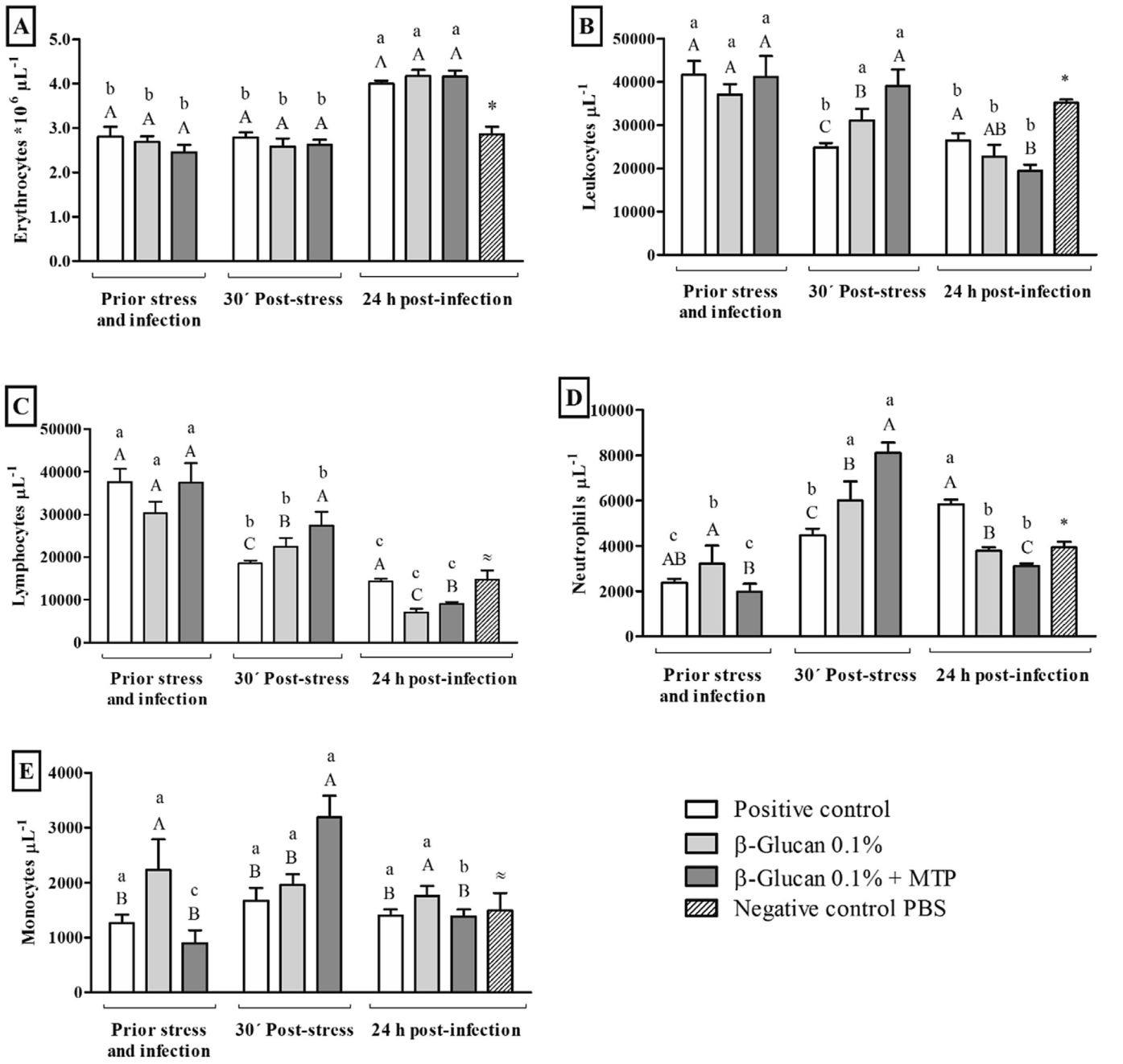

Fig. 3. *Number of circulating erythrocytes (A), leukocytes (B), lymphocytes (C), neutrophils (D), and monocytes (E) in matrinxã.* Fish were fed with β-glucan 0.1% or β-glucan 0.1% + metyrapone MTP 30 mg kg^{-1} fish. Results are compared to fish from positive (bacterial challenge) and negative (PBS 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 for the same treatment group, collected at all times shown. The ≈ symbol 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).

with cortisol levels. Fish with 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 stimulation of 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 [12,65].

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, cortisol, is by contrast inhibitory or suppressive [12]. We observed a decrease of RAL after stress and an increase after acute infection; fish treated with MTP showed the highest RAL. The RAL is considered an important indicator of 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 [54]. Previous studies described that after acute stress, there was a strong reduction of phagocytic activity in trout and sea

bream [66,67].

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 4 h transport [39] or after 2 min air exposure and sampled after 5 min [68]. The different results can be explained by different stressor intensity, duration, and sampling time.

The total leukocyte count 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 [69]. 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 [12,70,71]. Previous studies have described a short-term cortisol secretion increase in the number of circulating neutrophilic granulocytes and reduced lymphocyte proliferation and functions [72,73].

Neutrophils are highly motile phagocytic cells that play a critical role in the immune response to infection [74]. Neutrophils increase rapidly in circulation during acute stress when cortisol levels are high [12,75]. 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 [76].

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 Ref. [12]. 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 [77,78]. 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 a 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 [62].

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 is a fundamental mediator of the immunostimulant effect of β-glucan. The association of cortisol levels and immune response indicates that glucan-induced cortisol enhances the start of the early innate immune response in matrinxã.

Acknowledgement

The authors would like to thank the Centro de Aquicultura (CAUNESP) for supplying the fish for the research and the Laboratório de Microbiologia e Parasitologia (LAPOA – CAUNESP), for supplying the bacteria. The first author was granted a PhD scholarship from the Asociación Universitaria Iberoamericana de Posgrado-AUIP, through the program PAEDEX from Universidad Estadual Paulista UNESP (nº 151185 - 1).

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