Fish and Shellfish Immunology 93 (2019) 1076–1083

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

β-glucan modulates cortisol levels in stressed pacu (*Piaractus mesopotamicus*) inoculated with heat-killed *Aeromonas hydrophila*

Mariana Maluli Marinho de Mello[a], Camila de Fátima Pereira de Faria[a], Fábio Sabbadin Zanuzzo[a,1], Elisabeth Criscuolo Urbinati[a,b,*]

[a] Universidade Estadual Paulista UNESP – Centro de Aquicultura, Campus de Jaboticabal, Via de Acesso Prof. Paulo Donato Castelane, S/N - Vila Industrial, Jaboticabal, São Paulo, 14884-900, Brazil

[b] Universidade Estadual Paulista UNESP – Faculdade de Ciências Agrárias e Veterinárias, Campus de Jaboticabal, Via de Acesso Prof. Paulo Donato Castelane, S/N - Vila Industrial, Jaboticabal, São Paulo, 14884-900, Brazil

ARTICLE INFO

Keywords:
Transport
Stress
Immunostimulant
Immunomodulation

ABSTRACT

In this study, we show that β-glucan can modulate cortisol release in fish. We simulated a common situation in aquaculture: the transport of fish followed by contact with an opportunistic pathogen and observed what effect glucan had on the immune and stress response in these conditions. Pacu (*Piaractus mesopotamicus*) were fed with a diet containing β-glucan (0.1%) for 15 days prior to transport followed by an injection with heat-killed *Aeromonas hydrophila*. We sampled fish before transport, at arrival and at 3 and 24 h after bacterial injection. β-Glucans are used in aquaculture and have a known immunostimulatory effect, which was observed in this study. The results showed that β-glucan modulated the plasma cortisol levels differently by increasing these levels up to 24 h after transport and preventing the increase caused by bacterial inoculum injection. In addition, β-glucan enhanced the activity of the complement system at 24 h and reduced the monocytes and lymphocytes number in peripheral blood at 3 and 24 h after bacterial inoculation. Our results suggest that β-glucan modulated a bi-directional interaction between the stress and the immune responses. The modulation of cortisol levels and the immunostimulation by β-glucan at different moments in our study suggest the compound has a protective effect by avoiding higher levels of the hormone and improving resistance against bacterial infection in pacu. These results add evidence to support the use of β-glucan as an immunomodulator in the aquaculture industry.

1. Introduction

Intensive fish production exposes fish to several unavoidable procedures, such as transport, that may cause stress and immunosuppression [1,2]. Exposing fish to these stressful procedures triggers a cascade of adaptive reactions that allow the fish to cope with stressors in order to maintain their homeostatic state [1,3]. The responses affect mechanisms involving the mobilization of energy to meet the surplus of activity elicited by stressors [4,5] and, depending on the extension of the response, may consume energy destined to other mechanisms. This can adversely affect some responses as to mount an immune response and consequently resistance to pathogens [1]. Thus, stressed fish may experience immune suppression and low resistance to pathogens [1,6].

In fish, the modulation of immune mechanisms is regulated by hormones, mainly cortisol, secreted during the stress response [5]. The binding of cortisol to its receptor on immune cells can stimulate or inhibit the transcription of several cytokines [7,8]. Otherwise, cytokines and chemokines can modulate and act on hormonal secretion. During an immune response, cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6, and IL-12 result in the activation of the stress system [9]. In fish, the head kidney consists of cytokine-producing lymphoid cells from the immune system and also endocrine cells, which produce cortisol and catecholamines [10]. This unique organization of the fish immune and endocrine systems in one single organ makes bidirectional signaling between these systems closer and thus intriguing.

The use of immunostimulants in aquaculture is a strategy that is being increasingly employed to minimize the negative effects of stress on the immune system [11–13]. β-Glucans naturally occur in carbohydrates usually found in plants, fungi and bacterial cellular walls [14]

* Corresponding author. Via de Acesso Prof. Paulo Donato Castelane, S/N - Vila Industrial, Jaboticabal, São Paulo, 14884-900, Brazil.
E-mail addresses: marimaluli@gmail.com (M.M. Marinho de Mello), camilaf17@gmail.com (C. de Fátima Pereira de Faria), fabioszanuzzo@gmail.com (F.S. Zanuzzo), elisabeth.criscuolo-urbinati@unesp.br (E.C. Urbinati).
[1] Department of Ocean Sciences and Biology, Memorial University, St. John's, NL, Canada A1C5S7.

https://doi.org/10.1016/j.fsi.2019.07.068
Received 20 April 2019; Received in revised form 14 July 2019; Accepted 24 July 2019
Available online 25 July 2019
1050-4648/ Published by Elsevier Ltd.

and are well-known for their stimulating effects on the immune system. Macrophages and dendritic cells have typical cell surface receptors called pattern recognition receptors (PRRs) that innately detect non-self molecules including pathogen-associated molecular patterns (PAMPs) [15] and are considered the main target of β-glucans, although neutrophils, B cells, T cells, and natural killer cells are also activated by this immunostimulant [16]. It is established that β-glucans improve the activity of the serum lysozyme [17–19] and the hemolytic activity of the complement system [17,20], and the respiratory activity of leukocytes [17,19,21,22]. The immunostimulatory effect of β-glucans on immune effector cells results in the production of cytokines [23]. The treatment of tilapia with ß-glucan increased the plasma TNF-reactive protein, the levels of IL-1β, IL-10 and IL-12 antibody-reactive proteins, as well as the phagocytic indices of macrophages, suggesting that the effect was related to the production of cytokine-like proteins [24]. Both cortisol and β-glucan can regulate cytokines expression and the activation or inhibition of immune cells, so the interaction between them may provide interesting data on the bidirectional interaction between the immune and neuroendocrine systems.

In our study, we fed pacu (*Piaractus mesopotamicus*) for 15 days with diets containing β-glucan and then assessed the stress and immune responses to transport and inoculation with inactivated *Aeromonas hydrophila* (i.e., immunosuppressed). This is a gram-negative opportunistic bacterium that is commonly isolated from freshwater and takes advantage of opportunities not normally available, such as a host with a weakened immune system. Our fish model is a teleost species widely distributed in rivers of South America [25]. It has increasing commercial importance for aquaculture systems [26] and is vulnerable to diseases when stressed.

2. Material and methods

The experimental procedures were approved by the Comitê de Ética no Uso de Animais (CEUA) of the Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal campus (Protocol 23543/15).

2.1. Animals and experimental design

A total of 216 juvenile fish (104.4 ± 26.5 g and 16.0 ± 1.19 cm) supplied by the Centro de Aquicultura da Unesp (CAUNESP) were kept in 18 100-L tanks (12 fish per tank) with constant water renewal and aeration. Fish were fed with a commercial diet twice a day (3% of their body mass) and acclimated to the system during 7 days. They were distributed into two treatments: one fed with a control diet (β-glucan free) and the other with a diet supplemented with β-glucan at 0.1% for 15 days (9 tanks/108 fish per treatment). The β-glucan concentration was chosen according to a previous study [27]. On the 16th day, one fish from each tank (n = 9) in each treatment was sampled to assess the basal condition, and the remainders were packed in plastic bags (18 bags/11 fish per bag; density of 166 g L^{-1}), inflated with oxygen, and transported for 4 h. At the return to the lab, one fish from each bag (n = 9) was sampled. The remaining fish were anesthetized (benzocaine, 0.05 g L^{-1}), inoculated in the mesenteric cavity with 0.5 μL g^{-1} of heat-killed *Aeromonas hydrophila* and then returned to the tanks. The fish were then sampled 3 and 24 h after inoculation (n = 9). We evaluated the plasma concentrations of cortisol and glucose, the respiratory activity of leukocytes, the serum hemolytic activity of the complement system and serum lysozyme activity.

Immediately before transport, the temperature was 27.9 ± 3.2 °C, the oxygen levels were 4.83 ± 0.1 mg L^{-1}, and total ammonia was 0.12 ± 0.01 mg L^{-1}. After transport, the values were 25.9 ± 3.2 °C, 12.36 ± 2.4 mg L^{-1} and 1.99 ± 0.08 mg L^{-1}, respectively, without differences between treatments (one-away ANOVA, with means compared by Tukey's test). The photoperiod was 12 h light: 12 h dark.

2.2. Experimental diet

The experimental diet was prepared using an extruded commercial feed (Fri-Aqua® Onivoros Engorda: 8–10 mm, 12% moisture, 28% crude protein and 3,600 kcal EB kg^{-1}), ground and mixed with 0.1% of β-glucan (Macrogard®, batch Q513187, 73% purity, Biorigin, Brazil). The diet was then moistened with 40% water, pelletized in a food processor and dried at 40 °C for 24 h. The control diet was processed following the same procedure without the inclusion of β-glucan.

2.3. Preparation of bacteria

The *A. hydrophila* strain, supplied by the Laboratório de Microbiologia e Parasitologia de Organismos Aquáticos (LAPOA/CAUNESP), was isolated from pacu and previously identified by gene sequencing (16S rDNA) (Genbank identification as KJ561014) [28]. The strain was stored in TSB culture medium (Tryptic Soy Broth, HMedia) at −80 °C, and, before inoculation, an aliquot of 20 μl was resuspended into 5 ml of TSB solution, autoclaved and incubated at 28 °C for 24 h. The pre-inoculum was added to 700 ml of TSB medium, autoclaved, and incubated again at 28 °C for an additional 24 h. The bacterial suspension was centrifuged at 8.000 × *g* for 30 min, and the supernatant was discarded. The pellets were then washed twice in PBS (0.01 M), and the suspension was centrifuged again. This operation was repeated twice. The bacteria were diluted at 10^8 CFU ml^{-1} for spectrophotometer reading (DO 625 = 0.08, corresponds to 1.5x10^8) and inactivated at 50 °C for 40 min.[2]

2.4. Samplings

At each sampling, fish were anaesthetized (benzocaine, 0.05 g L^{-1}), and the blood samples drawn from caudal vessels were dispensed in 2-ml microtubes with and without anticoagulant. Blood with Glistab® (EDTA 6 g dL^{-1} and KF 12 g dL^{-1}, Labtest, São Paulo, Brazil, code 29) was used to separate plasma for glucose and cortisol determination. Blood with heparin was utilized to measure the respiratory activity of leukocytes, and blood without anticoagulant was used to separate serum and stored at −20 °C for determination of lysozyme activity and at −80 °C for determination of the hemolytic activity of the complement system.

2.5. Stress indicators

2.5.1. Plasma cortisol and glucose concentrations

Cortisol concentrations were measured by enzyme-linked immunosorbent assay (ELISA, ThermoPlate Reader MN) with a commercial kit (DRG® Cortisol ELISA, EIA-1887). The intra-assay and inter-assay variations for cortisol were < 7% and < 10%, respectively. The glucose concentrations were measured by an enzymatic method with a commercial kit (Labtest, São Paulo, Brazil, code 133).

2.6. Immunological indicators

2.6.1. Respiratory activity of leukocyte (RAL)

The production of reactive oxygen species (ROS) was measured by the reduction of NBT (Nitroblue tetrazolium chloride - Sigma Aldrich, N6876), following the protocol described by Anderson and Siwicki [29] and modified for pacu by Biller-Takahashi et al. [30]. Heparinized blood was incubated with an equal volume (50 μL) of NBT solution (0.2%) at room temperature for 30 min. Thereafter, 1 mL of DMF (dimethylformamide, Sigma Aldrich, 227056) was added to the samples,

[2] Inactivation of bacteria was chosen to stimulate the pacu's immune system but avoid any significant mortality, since that transport could increase the susceptibility to the disease.

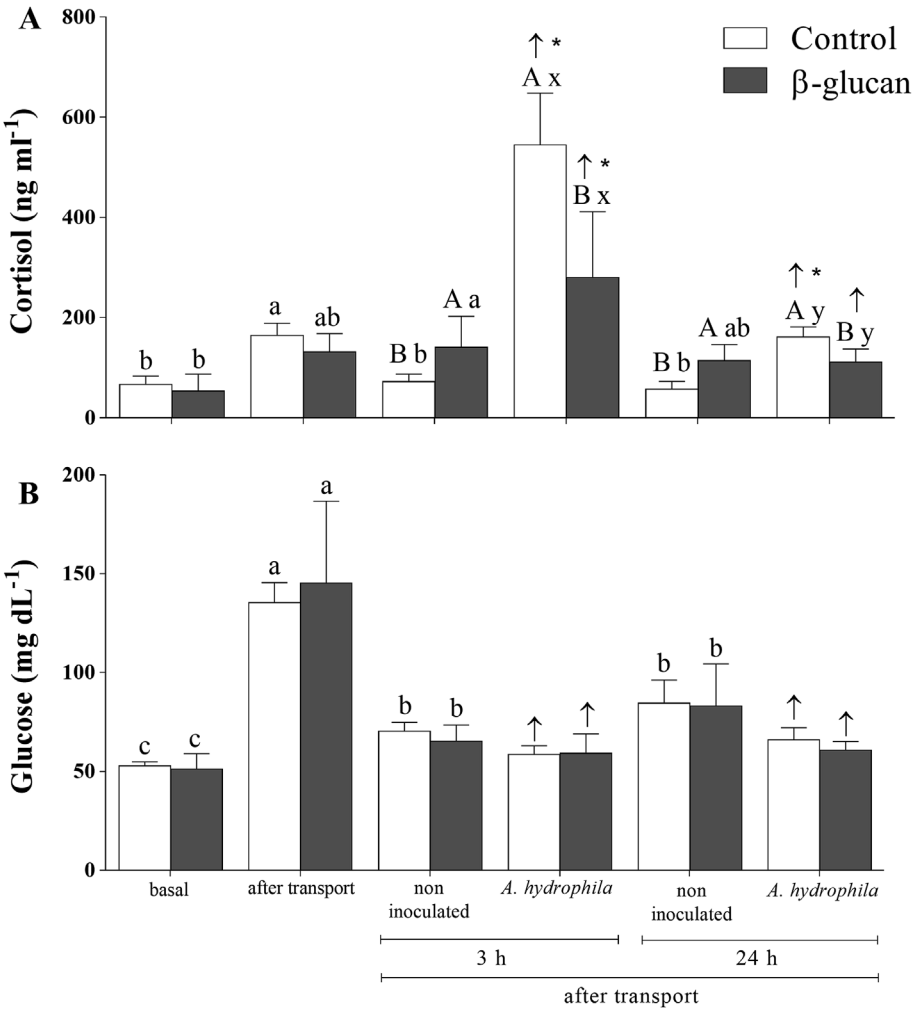

Fig. 1. Plasma cortisol (A) and glucose (B) levels in pacu fed with control diet and diet containing 0.1% β-glucan, before transport (basal), immediately after transport, 3 and 24 h after transport with none inoculation or intraperitoneal inoculation with heat-killed *Aeromonas hydrophila*. Different capital letters (A and B) indicate difference between treatments at particular injection in same sampling time. Letters a, b and c indicate differences between sampling times within the non-inoculated group. Letters x and y indicate difference between sampling times within the *A. hydrophila* group. Asterisk indicates difference between fish inoculated and non-inoculated in the same treatment and sampling time. Arrows indicate difference between basal sampling and each inoculated group. Symbols were omitted when there was no statistical difference. Values are means ± standard error (n = 9).

which were read in a spectrophotometer (Thermo Scientific®, Genesys 10S) at 540 nm.

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

The hemolytic activity of the alternative pathway of serum complement was determined according to Zanuzzo et al. [2]. A kinetic assay determined the time (in seconds) necessary for 75 µl of a serum sample to lyse 50% of 400 µl of a rabbit erythrocyte suspension in Alséver solution (anticoagulant, pH 6.1), measured in a spectrophotometer (Thermo Scientific®, Genesys 10S), with readings every 30 s for 20 min at 700 nm. The HA-AP in each sample was measured as the time (seconds) required for the initial optical density to be reduced by one-half and obtained through the equation of a straight line representing the decay of the absorbance by time.

2.6.3. Lysozyme activity

The lysozyme concentrations were determined according to Demers and Bayne [31] with modifications by Zanuzzo et al. [32], based on lysis of the gram-positive bacterium *Micrococcus lysodeikticus* suspension (Sigma Aldrich, M3770) using hen egg white lysozyme as a standard (Sigma Aldrich, L6876). The absorbance of the solution was measured at 450 nm over 10 min in a microplate reader (ThermoPlate Reader MN) at room temperature. The rate of decrease in absorbance for each sample (ΔOD) was then compared to the standard curve. The concentration was used to calculate the enzymatic activity using the formula: UA = ([lysozyme]/0.001)/10.

2.7. Cellular counts

Blood smears were stained with eosin [33] and observed under optical microscopy for total and differential counts of leukocytes. The total count of red cells was performed in a Neubauer chamber, using whole blood diluted in formaldehyde citrate buffer 1:200. The leukocytes calculated by indirect method was the number of leukocytes found at each 2000 erythrocytes counted. For the leukocyte differentiation, 200 white cells were counted and the amount of each cell type was expressed as cell μ^{-1}.

2.8. Statistical analysis

All data were submitted to normality (Shapiro-Wilk) and homoscedasticity (Brown-Forsythe). To adjust cortisol and glucose concentrations, the number of circulating monocytes, serum hemolytic activity of the complement system and lysozyme activity data for normality, data were transformed using log10. Oxidative burst and the number of circulating leukocytes, lymphocytes and neutrophils were not transformed. Before the inoculation with heat-killed *A. hydrophila*, we used a completely randomized design with a factorial arrangement of two treatments (control and β-glucan 0.1%) vs. time point (basal and after transport). After the inoculation, we analyzed the data in an arrangement of two treatments (control and β-glucan 0.1%) vs. two inoculation groups (non-inoculated and inoculated with *A. hydrophila*) and two sampling times (3 and 24 h) after inoculation. Means were compared by Duncan's post-hoc tests. Finally, a *t*-test was used to compare the means of the inoculation groups, treatments and sampling

time after inoculation with basal and after transport bars. P value < 0.05 was used to estimate the level of significance for statistical differences. N = 9.

3. Results

To evaluate the effect of dietary β-glucan on the stress and innate immune responses of pacu, we assessed the profile of cortisol and glucose in plasma, the respiratory activity of leukocytes, the hemolytic activity of the complement system and lysozyme activity in serum and total and differential counts of leukocytes after the transport and after bacterin inoculum. There was no mortality during the experiment..

3.1. Plasma cortisol and glucose concentrations (Fig. 1 A and B)

The plasma cortisol concentrations increased (p = 0.004, a; letter in figure) immediately after transport in the control group, returning to the basal levels 3 h after (p = 0.006, b) and did not change in the β-glucan group (p = 0.2797). At 3 h, among non-inoculated fish, the cortisol levels were higher in fish fed β-glucan than in the control (p = 0.0451, A), and among inoculated fish, the levels were higher in the control fish compared to those fed β-glucan (p = 0.0280, A). At this sampling time, the control fish that were inoculated with *A. hydrophila* showed a higher increase (p < 0.0001, *) in cortisol concentrations compared to the non-inoculated fish, and we observed the same profile among fish fed with β-glucan, i.e. the cortisol levels were higher in fish inoculated (p = 0.0200, *) compared to those non-inoculated. At 24 h, we observed a significant reduction in cortisol levels in both inoculated fish (control – p = 0.0252, β-glucan – p = 0.0128; y), but the cortisol levels were still significantly higher in control fish (p = 0.0197, A). At this sampling time, cortisol levels remained higher in fish fed with β-glucan among those non-inoculated (p = 0.0174, A) and lower in fish fed with β-glucan among those inoculated.

In all fish, the plasma glucose concentrations increased (p < 0.0001, a) after transport, and reduced (control – p < 0.0001, β-glucan – p = 0.0052, b) at 3–24 h, but the values were still significantly higher at 24 h compared to the basal levels. There was no difference between control fish or fish fed with β-glucan or between non-inoculated vs. inoculated fish in plasma glucose concentration..

3.2. Respiratory activity of leukocytes (RAL), serum hemolytic activity of the alternative pathway of the complement system (HA-AP) and lysozyme activity (Fig. 2 A, B and C)

The respiratory activity of leukocytes (RAL) in control fish increased (p < 0.0001, a) immediately after transport and reduced (non-inoculated – p < 0.001, c; *A. hydrophila* – p < 0.0001, y) 3 h later, with recovery (non-inoculated – p = 00.50, b; *A. hydrophila* – p < 0.0001, x) of the basal activity at 24 h in both inoculated and non-inoculated fish. Fish fed with β-glucan did not present increased RAL after transport (p = 0.4793) but reduced (non-inoculated – p < 0.001, c; *A. hydrophila* – p < 0.001, y) 3 h later, and increased (non-inoculated – p = 0.0004 b; *A. hydrophila* – p < 0.001, x) at 24 h, without differing (p = 0.4674) from the basal activity.

The HA-AP decreased in fish of both treatments at the end of transport (control – p = 0.0257, β-glucan – p = 0.0053, a) and remained lower until 24 h in fish fed with β-glucan and non-inoculated (p < 0.0001, ↑) and in inoculated control fish (p = 0.0276, ↑). At 24 h, among inoculated fish, the fish fed with β-glucan showed higher HA-AP than the control group (B, p = 0.0402).

The lysozyme activity increased at 24 h in fish fed with β-glucan (non-inoculated – p = 0.0011, a; *A. hydrophila* – p = 0.0126, ↑) independent of the inoculum. In control fish, the activity also increased at 24 h when compared with 3 h (p = 0.0006, a) but without a difference with basal activities (p = 0.2406)..

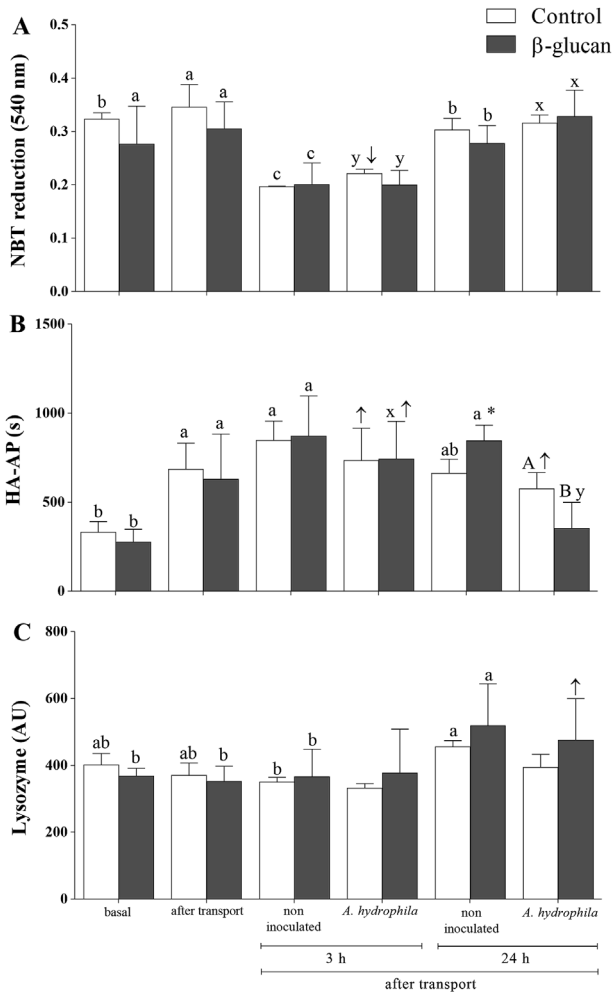

Fig. 2. Respiratory activity of leukocytes (A), serum hemolytic activity of alternative pathway of complement system (B) and lysozyme activity (C) in pacu fed with control diet and diet containing 0.1% β-glucan, before transport (basal), immediately after transport, 3 and 24 h after transport with none inoculation or intraperitoneal inoculation with heat-killed *Aeromonas hydrophila*. Different capital letters (A and B) indicate difference between treatments at particular injection in same sampling time. Letters a, b and c indicate differences between sampling times within the non-inoculated group. Letters x and y indicate difference between sampling times within the *A. hydrophila* group. Asterisk indicates difference between fish inoculated and non-inoculated in the same treatment and sampling time. Arrows indicate difference between basal sampling and each inoculated group. Symbols were omitted when there was no statistical difference. Values are means ± standard error (n = 9).

3.3. Count of circulating leukocytes, lymphocytes, neutrophils and monocytes (Fig. 3 A, B, C and D)

The number of leukocytes reduced after transport in both fish groups (control – p = 0.0019, β-glucan = 0.0003, b) and remained lower until 3 h independent of the inoculum and treatment (control non-inoculated – p < 0.0001, b; control inoculated – p = 0.0006, ↓; β-glucan non-inoculated – p = 0.0359, b; β-glucan inoculated – p < 0.0001, ↓). At 24 h, non-inoculated fish recovered the basal number (control – p = 0.0462, β-glucan – p = 0.0003, a), but this did not occur in the inoculated group (control – p = 0.1037, β-glucan – p = 0.2170, ↓), although in inoculated fish, the control group had a higher number of leukocytes compared to fish fed with β-glucan (p = 0.0019, A). In fish fed with β-glucan and non-inoculated, the number of leukocytes was higher at 3 h (p = 0.0005, *) and 24 h (p = 0.0005, *) samplings.

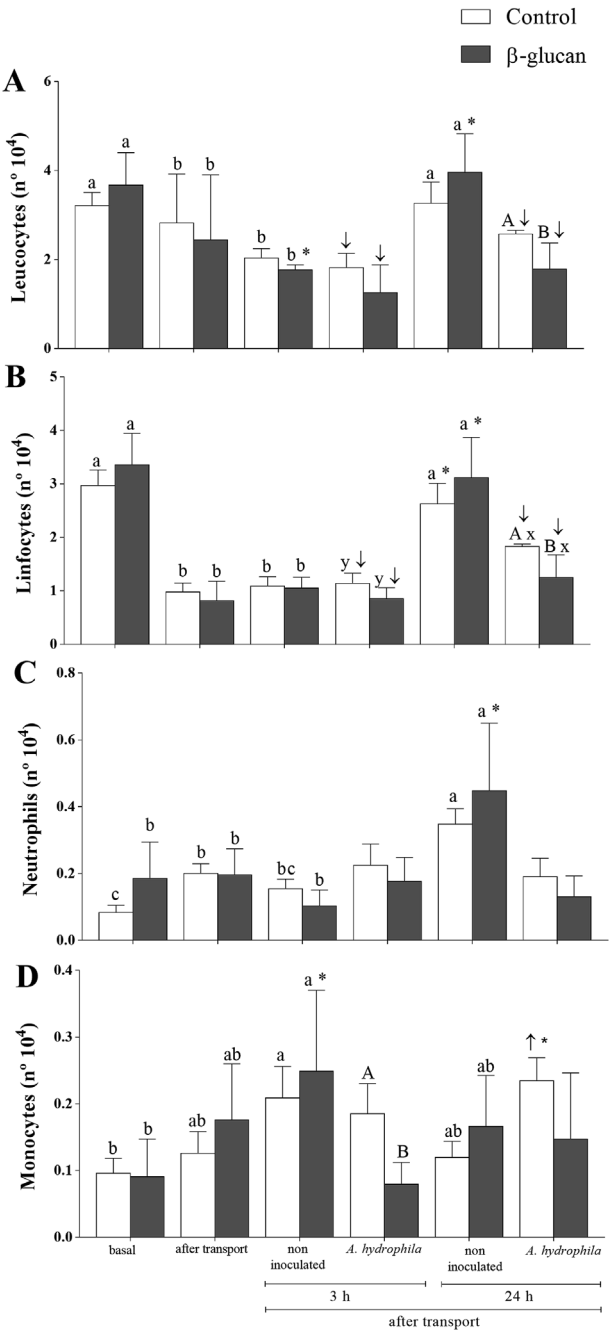

Fig. 3. Number of circulating leucocytes (A), lymphocytes (B), neutrophils (C) and monocytes (D) in pacu fed with control diet and diet containing 0.1% β-glucan, before transport (basal), immediately after transport, 3 and 24 h after transport with none inoculation or intraperitoneal inoculation with heat-killed *Aeromonas hydrophila*. Different capital letters (A and B) indicate difference between treatments at particular injection in same sampling time. Letters a, b and c indicate differences between sampling times within the non-inoculated group. Letters x and y indicate difference between sampling times within the *A. hydrophila* group. Asterisk indicates difference between fish inoculated and non-inoculated in the same treatment and sampling time. Arrows indicate difference between basal sampling and each inoculated group. Symbols were omitted when there was no statistical difference. Values are means ± standard error (n = 9).

After transport, the number of lymphocytes decreased (control – $p = 0.0002$; β-glucan – $p = 0.0004$, b) and remained lower until 24 h among inoculated fish (control – $p = 0.0439$; β-glucan – $p = 0.0439$, ↓) and at 3 h among non-inoculated fish (control – $p = 0.0076$, β-glucan – $p < 0.001$, b). In inoculated fish, at 24 h, those fed with β-glucan showed a lower number of lymphocytes compared to the control group ($p = 0.0002$, B).

The number of neutrophils did not vary over time in inoculated fish in both treatments (control – $p = 0.7149$; β-glucan – $p = 0.03441$). Non-inoculated fish had more neutrophils immediately after transport ($p = 0.0095$, b) and 24 h later ($p = 0.0087$, a) in the control group and only at 24 h after inoculation ($p = 0.0068$, a) in the β-glucan group. At 24 h, in fish fed β-glucan, the number of neutrophils was higher in the non-inoculated group than in inoculated ones ($p = 0.0039$, *).

At 3 h, the number of monocytes increased among non-inoculated fish (control – $p = 0.0031$; β-glucan – $p = 0.0304$, a). At this time, in inoculated groups, the number decreased ($p = 0.0269$, B) in fish fed β-glucan compared to the controls. Still, at 3 h, fish fed β-glucan and non-inoculated had higher ($p = 0.0452$, *) circulating monocytes than those that were inoculated. At 24 h, control fish that were inoculated showed a higher number of monocytes than the control non-inoculated fish ($p = 0.0304$, *) and compared to the basal sampling ($p = 0.0468$, ↑).

4. Discussion

The strengthening of the fish immune system through dietary additives, such as β-glucans, has been an area of great interest [11–13]. Despite a large number of studies, few reports have explored the anti-stress effect of β-glucan in fish. Here, we fed fish for 15 days with β-glucan and assessed their stress and innate immune responses after transport followed by the inoculation of heat-killed bacteria. We observed that β-glucan modulated the stress responses, mainly the profile of circulating cortisol, by preventing the recovery of basal levels 3 h after transport as observed in control fish and contrarily preventing the additional increase after bacterial injection. In addition, β-glucan enhanced the activation of the complement system and the mobilization of monocytes and lymphocytes 3 and 24 h after bacterin inoculation, respectively. However, β-glucan did not modulate lysozyme levels or the respiratory activity of leukocytes. Our results provide evidence that β-glucan can modulate the stress response in addition to its role as an immunostimulant.

Interestingly, β-glucan sustained higher levels of cortisol after transport, while in the control fish, those levels already had returned to the basal levels. The increase in cortisol levels after transport is a known response in fish, as previously described for Atlantic salmon (*Salmo salar*) [34] red drum (*Sciaenops ocellatus*) [35], matrinxã (*Brycon cephalus*) [36], rainbow trout (*Oncorhynchus mykiss*) [37], and pacu (*Piaractus mesopotamicus*) [2]. However, to the authors' knowledge, no other study showed similar effects of β-glucan. Additionally, the higher levels of cortisol in pacu fed with β-glucan 3 h after transport in non-inoculated fish and lower cortisol levels after the bacterin inoculation have not yet been described to the best of our knowledge. Bacterial infection is also known to promote an increase in circulating cortisol levels, as observed in channel catfish (*Ictalurus punctatus*) after infection with *Edwardsiella ictaluri* [38], in rainbow trout (*Oncorhynchus mykiss*) following *Vibrio anguillarium* infection [39] and in blunt snout (*Megalobrama amblycephala*) after inoculation with *Aeromonas hydrophila* [40]. Similar to our findings, although using another immunostimulant product, Zanuzzo et al. [2] fed pacu with *Aloe vera* and observed that the plant extract increased cortisol levels 24 h after transport and prevented high cortisol levels after inoculation in treated fish, but the authors did not explain the results. We believe that β-glucan, as well as *Aloe vera*, potentiated the stress response to the transport and prevented an overshooting of the hormone after the inoculum as a protection response. Cortisol is important to ensuring fish survival, as it prepares the immune system for possible challenge, but high levels or prolonged periods of exposure can force fish beyond their normal biological limits, weaken them and decrease their capacity to respond to an unfavorable situation.

Reactive oxygen species plays an essential role in the control of host

immune responses and resistance to pathogens [41]. In our study, the bacterin inoculum decreased respiratory activity of leucocytes in fish from both treatments. This reduction can reflect the migration of leukocytes to infection tissue, since the numbers of circulating leucocytes also decreased. Fish fed with glucan diet and inoculated recovered their basal ROS production 3 h after injection, while control fish showed this increase at 24 h. This effect can be attributed to the ability of β-glucan to bind to several types of leukocytes receptors [11], activating directly those cells and stimulating their cytotoxic, antimicrobial and phagocytic activities [21], including the production of reactive oxygen and nitrogen intermediates [15].

The complement system is an essential component of innate immunity [42]. It has been used as an indicator of fish immune competence and plays an essential role in detecting the presence of pathogens [43,44] in addition to mediating the opsonization of pathogens and facilitating the recognition of microorganisms by phagocytes [43]. Additionally, these proteins break the bacteria membrane, making possible the action of antimicrobial enzymes such as lysozyme [45], induce inflammation and stimulate respiratory burst in leukocytes [46]. In our study, the transport impaired the complement activity (HA-AP), as has been described in other studies that showed increased susceptibility of fish to disease after stress [47,48]; however, the β-glucan enhanced the HA-AP 24 h after the inoculum. The bacterin inoculation after the transport was not able to modify the HA-AP in pacu, even though, as in mammals, some complement components have been recognized as acute-phase reactant also in teleost, suggesting their important role in first-line defense against pathogen infection [42]. In a previous study, transport also caused a reduction of the HA-AP in pacu, which was reversed by the use of dietary *Aloe vera* [2]. Short-term or long-term crowding also depressed the HA-AP in gilthead seabream (*Sparus aurata*) [49,50]. Several studies have confirmed the immunostimulatory properties of β-glucans in several fish [12,13,51]. Silver catfish (*Rhamdia quelen*) fed with a β-glucan-supplemented diet showed higher hemolytic activity of complement system compared to control fish [52]. Similarly, olive flounder (*Paralichthys olivaceus*) fed with diets containing β-1,3-glucan presented elevated HA-AP [53]. Otherwise, in carp (*Ciprinus carpio*), classical and alternative complement pathways were not affected by β-glucan through different routes [21]. Also, in Asian catfish (*Clarias batrachus*), the HA-AP was not affected by the dietary supplementation of yeast glucan [54].

Lysozyme is an enzyme found in serum and leucocytes-rich tissues [55], which mediates protection against microbial invasion by digesting cell wall components causing bacteria lysis [45,55]. In addition, it activates leucocytes, promotes phagocytosis and has an opsonic effect [45]. Lysozyme activity is dependent on intensity, duration and type of stressors [56]. Studies have shown reduction [2,57–60], or increase [31,61,62] in lysozyme activity after stressful situations. In our study, the transport or bacterin inoculation was not able to change lysozyme activity. However, β-glucan increased the lysozyme activity in inoculated and non-inoculated fish 24 h after the injection when compared to basal activity. Although β-glucan has modulated RAL, HA-PA and lysozyme differently, the improvement in those immune indicators after transport and bacterin inoculation in pacu fed with β-glucan indicates the effect of β-glucan in the immune defense of stressed fish.

Regarding the leukocytes profile, we observed lymphopenia acutely, after stress, with or without the bacterin inoculum, and recovery of the number of lymphocytes within 24 h in non-inoculated fish without the effect of β-glucan. In the inoculated group, we observed lymphopenia to be more intense in fish fed with β-glucan. The effects of β-glucans on immune cells are well established, as discussed before [15,16]. Otherwise, the presence of cytokine-producing lymphoid cells and endocrine cells in the head kidney of fish [10] favors a bidirectional signaling between endocrine and immune systems. Considering that lymphocytes in fish have potent phagocytic and microbicidal abilities [63], their reduction in blood can represent the migration of cells to the inflammation site, which is intensified by β-glucan at 24 h after bacterial injection. In response to glucocorticoids, circulating lymphocytes adhere to the endothelial cells that line the walls of blood vessels, and subsequently undergo migration from circulation into other tissues [64]. These cells are key regulators in microbial infections, and after the recognition of microbial structures, they can release several cytokines [65] to maintain communication with the immune and endocrine system during the response. Neutrophilia was observed in both fish groups regardless of the β-glucan, 24 h after bacterin injection, suggesting a later effect of cortisol secreted during transport and additionally to the stimulatory effect of β-glucan. Monocytes increased in both fish groups after transport, decreasing in fish fed β-glucan 3 h after bacterin inoculum, suggesting activation caused by transport and migration to the inflammatory site after inoculum. These cells are highly phagocytic cells and are critical components of teleost innate immune defenses [66]. According to Davis et al. [67], glucocorticoids act to increase the number and percentage of neutrophils while decreasing the number and percentage of lymphocytes. This phenomenon is seen in all five vertebrate taxa in response to either natural stressors or the exogenous administration of stress hormones. The profile of leukocytes in pacu was modified by β-glucan, which increased the cell mobilization caused by stress.

As discussed above, the immune system is regulated by a complex bidirectional pathway mediated by cytokines [7]. All our results together suggest that β-glucan modulated a bidirectional interaction between the neuroendocrine and immune systems. According to Hida et al. [23], the immunomodulating activity of β-glucans is mainly related to their effects on immune effector cells, such as macrophages, mononuclear cells, and neutrophils involved in innate immunity, resulting in the production of cytokines. In tilapia orally treated with ß-glucan, the plasma TNF, a reactive protein, was stimulated, and the levels of IL-1ß, IL-10 and IL-12 antibody-reactive proteins as well as the phagocytic indices of macrophages also increased, suggesting that the immunostimulation was related to the production of cytokine-like proteins [24]. In turn, cytokines and chemokines can modulate brain function and hormonal secretion by the stress system. During an immune reaction, the cytokines secretion results in the activation of the stress system [9], and the β-glucan binding with PRRs receptors also activate this axis. A basic relationship exists between the immune and endocrine system to modulate an adequate response to stressors, and due to recognition of the immune cells, β-glucan acts as an immune and stress modulator.

5. Conclusion

The results of the present study provide additional evidence that β-glucan modulated not only the immune system, but also the release of cortisol. The β-glucan modulated cortisol levels differently after transport and after inoculation of pacu with *Aeromonas hydrophila*. Up to 24 h after transport, β-glucan increased the levels of cortisol, while in fish that were additionally inoculated with the bacterium, the elevation of the hormone levels was prevented. In inoculated fish, with reduced levels of cortisol due to the effect of β-glucan, we observed a reduction of monocytes (3 h after inoculation) and a reduction of lymphocytes as well as enhanced complement system activity (24 h). In conclusion, our results reinforce the benefits of the adoption of feeding β-glucan as a dietary supplement prior to handling to modulate stress response in a protective way.

Acknowledgements

We thank the Centro de Aquicultura da UNESP (CAUNESP) for supplying the fish for the research and the Laboratório de Microbiologia e Parasitologia de Organismos Aquáticos for supplying the bacteria inoculum. The first author was granted a scholarship from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) [process number: 138990/2014-0].

References

[1] L. Tort, Stress and immune modulation in fish, Dev. Comp. Immunol. 35 (2011) 1366–1375, https://doi.org/10.1016/j.dci.2011.07.002.

[2] F.S. Zanuzzo, R.E. Sabioni, L.N.F. Montoya, G. Favero, E.C. Urbinati, Aloe vera enhances the innate immune response of pacu (*Piaractus mesopotamicus*) after transport stress and combined heat killed *Aeromonas hydrophila* infection, Fish Shellfish Immunol. 65 (2017) 198–205, https://doi.org/10.1016/j.fsi.2017.04.013.

[3] B.A. Barton, Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroid, Integr. Comp. Biol. 42 (2002) 517–525, https://doi.org/10.1093/icb/42.3.517.

[4] S.E. Wendelaar Bonga, Hormone response to stress, in: A.P. Farrel, J.J. Cech, J.G. Richards, E.D. Stevens (Eds.), Encyclopedia of Fish Physiology: from Genome to Environment, Elsevier Academic Press Inc, UK, 2011, pp. 1515–1523.

[5] T. Yada, L. Tort, Stress and disease resistance: immune system and immunoendocrine interactions, in: C.B. Schreck, L. Tort, A.P. Farrell, C.J. Brauner (Eds.), Fish Physiology - Biology of Stress in Fish, vol. 35, Academic Press, San Diego, 2016, pp. 365–403.

[6] G. Nardocci, C. Navarro, P.P. Cortés, M. Imarai, M. Montoya, P. Jara, C. Acuña-Castillo, R. Fernández, Neuroendocrine mechanisms for immune system regulation during stress in fish, Fish Shellfish Immunol. 40 (2014) 531–538, https://doi.org/10.1016/j.fsi.2014.08.001.

[7] M.Y. Engelsma, M.O. Huising, W.B.V. Muiswinkel, G. Flik, J. Kwang, H.F.J. Savelkoul, B.M.L.V.V. Kemenade, Neuroendocrine-immune interactions in fish: a role for interleukin-1, Vet. Immunol. Immunopathol. 87 (2002) 467–479, https://doi.org/10.1016/S0165-2427(02)00077-6.

[8] L. Pijanowski, P. Jurecka, I. Irnazarow, M. Kepka, E. Szwejser, B.M.L.V.V. Kemenade, M. Chadzinska, M, Activity of the hypothalamus-pituitary-interrenal axis (HPI axis) and immune response in carp lines with different susceptibility to disease, Fish Physiol. Biochem. 41 (2015) 261–278, https://doi.org/10.1007/s10695-015-0084-3.

[9] E. Calgnani, I. Elenkov, Stress system activity, innate and T helper cytokines, and susceptibility to immune-related diseases, Ann. N. Y. Acad. Sci. 1069 (2006) 62–76, https://doi.org/10.1196/annals.1351.006.

[10] E.J.W. Geven, P.H.M. Klaren, The teleost head kidney: integrating thyroid and immune signaling, Dev. Comp. Immunol. 66 (2017) 73–83, https://doi.org/10.1016/j.dci.2016.06.025.

[11] R.A. Dalmo, J. Bøgwald, β-Glucans as conductors of immune symphonies, Fish Shellfish Immunol. 25 (2008) 384–396, https://doi.org/10.1016/j.fsi.2008.04.008.

[12] E. Vallejos-Vidal, F. Reyes-Lopez, M. Teles, S. Mackenzie, The response of fish to immunostimulant diets, Fish Shellfish Immunol. 56 (2016) 34–69, https://doi.org/10.1016/j.fsi.2016.06.028.

[13] D.K. Meena, P. Das, S. Kumar, S.C. Mandal, A.K. Prusty, S.K. Singh, M.S. Akhtar, B.K. Behera, K. Kumar, A.K. Pal, S.C. Mukherjee, Beta-glucan: an ideal immunostimulant in aquaculture (a review), Fish Physiol. Biochem. 3 (2013) 431–457, https://doi.org/10.1007/s10695-012-9710-5.

[14] A. Synytsya, M. Novak, Structural analysis of glucans, Ann. Transl. Med. 2 (2) (2014) 17, https://doi.org/10.3978/j.issn.2305-5839.2014.02.07.

[15] G.D. Brown, S. Gordon, Immune recognition of fungal beta-glucan, Cell Microbiol. 7 (2005) 471–479, https://doi.org/10.1111/j.1462-5822.2005.00505.x.

[16] H. Kim, J.T. Hong, Y. Kim, S.-B. Han, Stimulatory effect of β-glucans on immune cells, Immune Network 4 (2011) 191–195, https://doi.org/10.4110/in.2011.11.4.191.

[17] C. Misra, B. Das, S. Mukherjee, P. Pattnaik, Effect of long term administration of dietary beta-glucan on immunity, growth and survival of *Labeo rohita* fingerlings, Aquaculture 255 (2006) 82–94, https://doi.org/10.1016/j.aquaculture.2005.12.009.

[18] S.K. Gupta, A.K. Pal, N.P. Sahu, R. Dalvi, V. Kumar, S.C. Mukherjee, Microbial levan in the diet of *Labeo rohita* Hamilton juveniles: effect on non-specific immunity and histopathological changes after challenge with *Aeromonas hydrophila*, J. Fish Dis. 31 (2008) 649–657, https://doi.org/10.1111/j.1365-2761.2008.00939.x.

[19] A. Rodríguez, A. Cuesta, J. Ortuño, M. Esteban, J. Meseguer, Immunostimulant properties of a cell wall-modified whole *Saccharomyces cerevisiae* strain administered by diet to seabream (*Sparus aurata* L.), Vet. Immunol. Immunopathol. 96 (2003) 183–192, https://doi.org/10.1016/j.vetimm.2003.07.001.

[20] M.A. Esteban, A. Cuesta, J. Ortuño, J. Meseguer, Immunomodulatory effects of dietary intake of chitin on gilthead seabream (*Sparus aurata* L.) innate immune system, Fish, Shellfish Immunol 11 (2001) 303–315, https://doi.org/10.1006/fsim.2000.0315.

[21] V. Selvaraj, K. Sampath, V. Sekar, Administration of yeast glucan enhances survival and some non-specific and specific immune parameters in carp (*Cyprinus carpio*) infected with *Aeromonas hydrophila*, Fish Shellfish Immunol. 19 (2005) 293–306, https://doi.org/10.1016/j.fsi.2005.01.001.

[22] F. Pilarski, C.A. Ferreira de Oliveira, F.P.B. Darpossolo de Souza, F.S. Zanuzzo, Different β-glucans improve the growth performance and bacterial resistance in Nile tilapia, Fish Shellfish Immunol. 70 (2017) 25–29, https://doi.org/10.1016/j.fsi.2017.06.059.

[23] T. Hida, K. Ishibashi, N. Miura, Y. Adachi, Y. Shirasu, N. Ohno, Cytokine induction by a linear 1,3-glucan, curdlan-oligo, in mouse leukocytes, Inflamm. Res. 58 (2009) 9–14, https://doi.org/10.1007/s00011-008-8141-3.

[24] N. Chansue, M. Endo, T. Kono, M. Sakai, The stimulation of cytokine-like proteins in Tilapia (*Oreochromis niloticus*) orally treated with beta-1, 3-glucan, Asian Fish Sci. 13 (2000) 271–278.

[25] J.R.M. Petrere JR., River fisheries in Brazil: a review, Regul. Rivers Res. Manag. 4 (1989) 1–16, https://doi.org/10.1002/rrr.3450040102.

[26] G.M.R. Valladão, S.U. Gallani, F. Pilarski, South American fish for continental aquaculture, Rev. Aquac. 10 (2016) 351–369, https://doi.org/10.1111/raq.12164.

[27] L.N. Franco Montoya, T.P. Martins, R.Y. Gimbo, F.S. Zanuzzo, E.C. Urbinati, β-Glucan-induced cortisol levels improve the early immune response in matrinxã (*Brycon amazonicus*), Fish Shellfish Immunol. 60 (2017) 197–204.

[28] F.A. Sebastião, L.R. Furlan, D.T. Hashimoto, F. Pilarski, Identification of bacterial fish pathogens in Brazil by direct colony PCR and 16S rRNA gene sequencing, Adv. Microbiol. 5 (2015) 409–424.

[29] D.P. Anderson, A.K. Siwicki, Basic haematology and serology for fish health programs, in: M. Shariff, J.R. Arthur, R.P. Subasinghe (Eds.), Diseases in Asian Aquaculture II, Manila: Fish Health Section, Asian Fisheries Society, 1995, pp. 185–202.

[30] J.D. Biller-Takahashi, L.S. Takahashi, M.V. Saita, R.Y. Gimbo, E.C. Urbinati, Leucocytes respiratory burst activity as indicator of innate immunity of pacu *Piaractus mesopotamicus*, Braz. J. Biol. 73 (2013) 425–429.

[31] N.E. Demers, C.J. Bayne, The immediate effects of stress on hormones and plasma lysozyme in rainbow trout, Dev. Comp. Immunol. 21 (1997) 363–373.

[32] F.S. Zanuzzo, E.C. Urbinati, M.L. Rise, J.R. Hall, G.W. Nash, A.K. Gamperl, *Aeromonas salmonicida* induced immune gene expression in Aloe vera fed steelhead trout *Oncorhynchus mykiss* (Walbaum), Aquaculture 435 (2015) 1–9, https://doi.org/10.1016/j.aquaculture.2014.09.010.

[33] G. Rosenfeld, Corante pancrômico para hematologia e citologia clínica. Nova combinação dos componentes do May-Grüwald e do Giemsa num só corante de emprego rápido, Mem. Inst. Butantan (Sao Paulo) 20 (1947) 329–334.

[34] M. Iversen, B. Finstad, K.J. Nilssen, Recovery from loading and transport stress in Atlantic salmon (*Salmo salar* L.) smolts, Aquaculture 168 (1998) 387–394, https://doi.org/10.1016/S0044-8486(98)00364-0.

[35] L. Robertson, P. Thomas, C.R. Arnold, Plasma cortisol and secondary stress responses of cultured red drum (Sciaenops ocellatus) to several transportation procedures, Aquaculture 68 (2) (1988) 115–130.

[36] E.C. Urbinati, J.S. Abreu, A.C.S. Camargo, M.A.L. Parra, Loading and transport stress in juvenile martinxã (*Brycon cephalus*) at various densities, Aquaculture 229 (2004) 389–400.

[37] L. Tacchi, L. Lowrey, R. Musharrafieh, K. Crossey, E.T. Larragoite, I. Salinas, Effects of transportation stress and addition of salt to transport water on the skin mucosal homeostasis of raibow trout (*Oncorhynchus mykiss*), Aquaculture 435 (2015) 120–127, https://doi.org/10.1016/j.aquaculture.2014.09.027.

[38] L.A. Bilodeau, G.C. Waldbieser, J.S. Terhune, D.J. Wise, W.R. Wolters, A real-time polymerase chain reaction assay of the bacterium *Edwardsiella ictaluri* in channel catfish, J. Aquat. Anim. Health 15 (2003) 80–86, https://doi.org/10.1577/1548-8667.

[39] P.A. Ackerman, G.K. Iwama, Physiological and cellular stress responses of juvenile rainbow trout to vibriosis, J. Aquat. Anim. Health 13 (2001) 173–180, https://doi.org/10.1577/1548-8667.

[40] B. Liu, X.P. Ge, J. Xie, P. Xu, Y.T. Cui, J.H. Ming, Q. Zhou, L. Pan, Effects of anthraquinone extract from Rheum officinale Bail on the physiological responses and HSP70 gene expression of *Megalobrama amblycephala* under *Aeromonas hydrophila* infection, Fish Shellfish Immunol. 32 (2012) 1–7, https://doi.org/10.1016/j.fsi.2011.02.015.

[41] B.M. Babior, R.S. Kipnes, J.T. Curnutte, Biological defense mechanisms – production by leukocytes of superoxide a potential bactericidal agent, J. Clin. Investig. 52 (3) (1973) 741–744, https://doi.org/10.1172/JCI107236.

[42] M. Nakao, M. Tsujikura, S. Ichiki, T.K. Vo, T. Somamoto, The complement system in teleost fish: progress of post-homolog-hunting researches, Dev. Comp. Immunol. 35 (2011) 1296–1308, https://doi.org/10.1016/j.dci.2011.03.003.

[43] H. Boshra, J. Li, J.O. Sunyer, Recent advances on the complement system of teleost fish, Fish Shellfish Immunol. 20 (2006) 239–262, https://doi.org/10.1016/j.fsi.2005.04.004.

[44] S.C. Zhang, P.F. Cui, Complement system in zebrafish, Dev. Comp. Immunol. 46 (2014) 3–10, https://doi.org/10.1016/j.dci.2014.01.010.

[45] S. Saurabh, P.K. Sahoo, Lysozyme: an important defence molecule of fish innate immune system, Aquacult. Res. 39 (3) (2008) 223–239, https://doi.org/10.1111/j.1365-2109.2007.01883.x.

[46] J. Rotllant, D. Parra, R. Peters, H. Boshra, J.O. Sunyer, Generation, purification and functional characterization of three C3a anaphylatoxins in rainbow trout: role in leukocyte chemotaxis and respiratory burst, Dev. Comp. Immunol. 28 (7–8) (2004) 815–828, https://doi.org/10.1016/j.dci.2003.11.001.

[47] L. Tort, E. Gomez, D. Montero, J.O. Sunyer, Serum haemolytic and agglutinating activity as indicators of fish immunocompetence: their suitability in stress and dietary studies, Aquacult. Int. 4 (1996) 31–41, https://doi.org/10.1007/BF00175219.

[48] Z. Yin, T.J. Lam, Y.M. Sin, The effects of crowding stress on the nonspecific immune-response in fancy carp (*Cyprinus carpio* L.), Fish Shellfish Immunol. 5 (7) (1995) 519–529, https://doi.org/10.1016/S1050-4648(95)80052-2.

[49] D. Montero, M.S. Izquierdo, L. Tort, L. Rabaina, J.M. Vergana, High stocking density produces crowding stress altering some physiological and biochemical parameters in gilthead seabream, *Sparus aurata*, juveniles, Fish Physiol. Biochem. 20 (1999) 53–60, https://doi.org/10.1023/A:1007719928905.

[50] J. Ortuño, M.A. Esteban, J. Meseguer, Effects of short-term crowding stress on the gilthead seabream (*Sparus aurata* L.) innate immune response, Fish Shellfish Immunol. 11 (2001) 187–197, https://doi.org/10.1006/fsim.2000.0304.

[51] V. Vetvicka, L. Vannucci, P. Sima, The effects of β-glucan on fish immunity, N. Am. J. Med. Sci. 5 (10) (2013) 580–588, https://doi.org/10.4103/1947-2714.120792.

[52] J.D. Domenico, R. Canova, L.F. Soveral, C.O. Nied, M.M. Costa, R. Frandoloso, L.C. Kreutz, Immunomodulatory effects of dietary β-glucan in silver catfish (*Rhamdia quelen*), Pesqui. Vet. Bras. 37 (2017) 73–78, https://doi.org/10.1590/

s0100-736x2017000100012.
[53] J.H. Lee, J.W. Kim, Y.J. Kang, D.W. Ko, J.M. Kim, S.H. Choi, K.H. Park, Effects of β-1,3-glucan on innate immunity responses and mortality induced by *Vibrio harveyi*, hemorrhagic septicemia virus, or Miamiensis avidus in the olive flounder *Paralichthys olivaceus*, Aquacult. Int. 26 (2018) 743–756, https://doi.org/10.1007/s10499-018-0248-0.
[54] J. Kumari, P.K. Sahoo, Dietary β-1,3 glucan potentiates innate immunity and disease resistance of Asian catfish, *Clarias batrachus* (L.), J. Fish Dis. 29 (2006) 95–101, https://doi.org/10.1111/j.1365-2761.2006.00691.x.
[55] A.E. Ellis, Lysozyme assays, in: J.S. Stolen, T.C. Fletcher, D.P. Anderson, B.S. Roberson, W.B. Van Muiswinkel (Eds.), Techniques in Fish Immunology Fair Haven, SOS Publications, Fair Haven, 1990, pp. 101–103.
[56] H.Y. Yildiz, Plasma lysozyme levels and secondary stress response in rainbow trout, *Oncorhynchus mykiss* (Walbaum) after exposure to Leteux-Meyer mixture, Turkey J Anim Sci 30 (2006) 265–269.
[57] A. Mock, G. Peters, Lysozyme activity in rainbow trout, *Oncorhynchus mykiss* (Walbaum) stressed by handling, transport, and water pollution, J. Fish Biol. 37 (1990) 873–885, https://doi.org/10.1111/j.1095-8649.1990.tb03591.x.
[58] D. Caruso, J. Lazard, Subordination stress in Nile tilapia and its effect on plasma lysozyme activity, J. Fish Biol. 55 (1999) 451–454, https://doi.org/10.1111/j.1095-8649.1999.tb00690.x.
[59] B. Liu, R. Jia, C. Han, B. Huang, J.L. Lei, Effects of stocking density on antioxidant status, metabolism and immune response in juvenile turbot (Scophthalmus maximus), Comp. Biochem. Physiol. C Toxicol. Pharmacol. 190 (2016) 1–8, https://doi.org/10.1016/j.cbpc.2016.07.007.
[60] P. Yarahmadi, H.K. Miandare, S.H. Hoseinifar, N. Gheysvandi, A. Akbarzadeh, The effects of stocking density on hemato-immunological and serum biochemical parameters of rainbow trout (*Oncorhynchus mykiss*), Aquacult. Int. 23 (1) (2015) 55–63.
[61] M.P. Soares, F.C. Oliveira, E.C. Urbinati, C. Meldau de Campos, H. Hisano, Glucan-MOS® improved growth and innate immunity in pacu stressed and experimentally infected with *Aeromonas hydrophila*, Fish Shellfish Immunol. 73 (2018) 73–133, https://doi.org/10.1016/j.fsi.2017.11.046.
[62] B.K. Velmurugan, C.R. Chan, C.F. Weng, Innate-immune responses of tilapia (*Oreochromis mossambicus*) exposure to acute cold stress, J. Cell. Physiol. (2019) 1–11, https://doi.org/10.1002/jcp.28270.
[63] J. Li, D.R. Barreda, Y.A. Zhang, H. Boshra, A.E. Gelman, S. LaPatra, L. Tort, J.O. Sunyer, B lymphocytes from early vertebrates have potent phagocytic and microbicidal abilities, Nat. Immun. 7 (2006) 1116–1124, https://doi.org/10.1038/ni1389.
[64] F.S. Dhabhar, A hassle a day may keep the doctor away: stress and the augmentation of immune function, Integr. Comp. Biol. 42 (2002) 556–564, https://doi.org/10.1093/icb/42.3.556.
[65] K.D. Cain, L. Grabowski, J. Reilly, M. Lytwyn, Immunomodulatory effects of a bacterial-derived β-1,3 glucan administered to tilapia (*Oreochromis niloticus* L.) in a Spirulina-based diet, short communication, Aquacult. Res. 34 (2003) 1241–1244, https://doi.org/10.1046/j.1365-2109.2003.00934.x.
[66] A.M. Rieger, D.R. Barreda, Antimicrobial mechanisms of fish leukocytes, Dev. Comp. Immunol. 35 (2011) 1238–1245, https://doi.org/10.1016/j.dci.2011.03.009.
[67] A.K. Davis, D.L. Maney, J.C. Maerz, The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists, Funct. Ecol. 22 (2008) 760–772, https://doi.org/10.1111/j.1365-2435.2008.01467.x.