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Immunomodulatory Effects of beta-Glucan on Neutrophil Function in Fathead Minnows (Pimephales Promelas Rafinesque, 1820)

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Keywords
beta-Glucan, Neutrophil function, Stress, Fish, Degranulation, Veterinary Microbiology & Preventive Medicine

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Immunomodulatory effects of β-glucan on neutrophil function in fathead minnows (*Pimephales promelas* Rafinesque, 1820)

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Abstract

Stimulatory effects of yeast β-1,3–1,6-glucans on neutrophils have long been recognized, but effects of glucans on degranulation of primary granules in fish neutrophils have not been previously reported. Neutrophil function was monitored during in vitro and in vivo application of glucans to non- (NS), acute- (AS) and chronically stressed (CS) fish. β-Glucan proved to be a strong and quick (80%, 2 min) stimulant of degranulation. Dietary glucan increased degranulation in NS fish, and prevented a decrease in AS fish. Degranulation in CS fish returned to NS levels 3 days after the glucan diet was fed. Fathead minnows appear to be a useful model to investigate neutrophil degranulation in fish exposed to different environmental conditions and immunomodulators. Use of β-glucans in fish diets prior to AS and during chronic stress can enhance neutrophil function, potentially increasing disease resistance and survival rates after transportation or exposure to poor water quality.

Keywords: β-Glucan; Neutrophil function; Stress; Fish; Degranulation

1. Introduction

β-1,3–1,6-Glucans are complex polysaccharide components of cell walls found in a large variety of organisms [1]. Stimulatory effects of β-1,3–1,6-glucans on neutrophils, as well as other components of the immune system, have long been recognized [2,3]. Glucan-specific receptors are present on phagocytic cell membranes of several species, including fish neutrophils [4,5], and potent activation of neutrophil function, including

Abbreviations: TAN, total ammonia nitrogen; TNN, total nitrite nitrogen; CaI, calcium ionophore; $HBSS^{CMF}$, Hank's balanced salt solution without Ca^{2+} and Mg^{2+}; PMA, phorbol myristate acetate; GB, β-glucan from barley; GY, β-glucan from yeast Sigma; MG, MacroGard Feed ingredient; soluble MG, MacroGard AquaSol; Z, zymosan; Cyt *C*, cytochrome *C*; MPO, myeloperoxidase; CTAB, cetyltrimethylammonium bromide; PMSF, phenylmethylsulfonyl fluoride; TMB, tetramethylbenzidine hydrochloride

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an increase in phagocytosis and killing, has been described in vitro [6,7]. The stimulatory effects of dietary β-glucan from baker's yeast (*Saccharomyces cerevisiae*) on neutrophil function, and increased disease resistance, have been recently demonstrated in several crustacean, fish and amphibian species [8–10].

Neutrophils are an important component of host defense against many bacterial, viral and fungal infections, and the evaluation of neutrophil function is valuable for assessment of the health status of individuals and animal populations [11]. Fish neutrophils have phagocytic, chemotactic and bactericidal functions, an intense respiratory burst, and peroxidase (myeloperoxidase, MPO) activity [12–14]. The process of degranulation is essential for the release of MPO and activation of the halide production pathway, as well as release of a diverse cocktail of antimicrobial enzymes. Changes in degranulation activity can influence the killing potential of the neutrophil, and potentially reduce the ability of the organism to defend against infection [15,16]. Measuring exocytosis of MPO from primary neutrophil granules in vitro is a direct, rapid and quantitative method to assess the degranulation process in fish neutrophils [16,17].

Severe or chronic stress is often associated with poor performance and has long been suspected to cause immunosuppression in cultured fish [18]. Effects of acute and chronic aquaculture stress on cortisol concentrations, innate and acquired immune function (cellular and humoral), and disease resistance have been reported [18]. Markedly elevated, as well as chronically increased concentrations of cortisol, act as inhibitors of neutrophil function [19], and a significant decrease in degranulation of fish neutrophil primary granules was observed after handling and crowding stress [20].

If fish are injured or exposed to other harmful conditions, there is a cause for concern not only in terms of responsible stewardship of fish populations, but also in terms of the welfare of individuals [21]. Decrease in neutrophil function can lead to increase in disease occurrence, and individuals with decreased or missing neutrophil degranulation had high incidence of bacterial and fungal infections often leading to terminal outcome [22]. Therefore, stress caused decrease in neutrophil degranulation could lead to reduced disease resistance and increased mortality rates, causing pain and distress in individual fish.

The fathead minnow (*Pimephales promelas*, Rafinesque, 1820) is a viable model to measure neutrophil function, such as degranulation and oxidative burst, during handling and crowding stress, and chemical compound (anesthetics) administration [20]. The widespread use of fathead minnows as laboratory models in toxicology research, their aquacultural and ecological relevance, and availability of functional assays, make them a species of choice for immunological research, including the effects of stress, and dietary immunomodulators on neutrophil function [20,23].

The effect of glucans on degranulation of neutrophil primary granules in fish, and the dietary application of β-glucans from baker's yeast in fathead minnows have not been reported. The purpose of this study was to determine the effects of glucans obtained from different commercial sources (Sigma; Biotec Pharmacon ASA, Norway) and species (baker's yeast, barley), when used as in vitro stimulants in fathead minnow neutrophil functional assays, and the optimal stimulant for dietary immunomodulation. In this study, commercially available particulate β-1,3–1,6-glucan from baker's yeast was found to be a potent stimulator of fish neutrophil degranulation and an optimal immunomodulator of fathead minnow neutrophil function during stress conditions.

2. Materials and methods

2.1. Fish

Adult fathead minnows with an average weight of 3 g were maintained in the Department of Natural Resource Ecology and Management, Iowa State University, Ames, IA, USA. Fish were held in 300–1000 L tank recirculation system supplied with dechlorinated tap water at 20 °C and fed daily with dried flake food (Aquatox®, Ziegler Bros Inc., PA, USA). Fish designated for experiments were fed 5% of the body weight, twice a day with a prepared basal diet at least 3 weeks prior to experiment. Water quality parameters were measured two times per week: water temperature was 20 ± 1 °C, pH was 8.0 ± 0.2, dissolved O_2 was $7 \pm 1\,mg\,L^{-1}$, total ammonia nitrogen (TAN) was $<0.5\,mg\,L^{-1}$ and total nitrite nitrogen (TNN) was below detection limit (HACH spectrophotometer 2000NR). Fathead minnows were cared for in accordance with approved Iowa State University animal care guidelines.

2.2. Reagents and diet preparation

Working solutions of reagents used in degranulation and oxidative burst assays were prepared as described before [17,20]. Stock solutions of zymosan (Z, Sigma; 10 mg mL^{-1}), particulate β-1,3-glucan from baker's yeast (MG, MacroGard Feed Ingredient®, Biotec Pharmacon ASA, Tromso, Norway; 10 mg mL^{-1}), particulate β-1,3-glucan from baker's yeast (GY, Sigma; 10 mg mL^{-1}), and particulate β-1,3-glucan from barley (GB, Sigma; 10 mg mL^{-1}) were prepared in Hank's balanced salt solution with Ca^{2+} and Mg^{2+} (HBSS, Mediatech-CellGro, AK, USA), sonicated for 2 min (Misonix XL2020; Misonix Inc., Farmingdale, NY) and stored at −70 °C. A stock solution of MacroGard AquaSol (soluble MG, 1% solution of water soluble β-1, 3-glucan from baker's yeast, courtesy of Biotec Pharmacon ASA, Tromso, Norway) was aliquoted and kept at 4 °C until use. Aliquots of the reagents were diluted in HBSS for each assay. Preliminary titrations were used to determine optimal reagent concentrations. The final concentrations of reagents used in the assay after addition of HBSS and resuspended neutrophils were: cytochalasin B (Cyto B, Sigma) 5 μg mL^{-1}; calcium ionophore (CaI) A23187 (Sigma) 5 μg mL^{-1}; phorbol myristate acetate (PMA, Sigma) 1 μg mL^{-1}; cytochrome *C* (Cyt *C* 490 μg mL^{-1}; soluble MG (4.125, 8.25, 16.5 and 33 μg mL^{-1}); Z, GB, GY and MG (25, 50, 100, 150, 200, 400 and 600 μg mL^{-1}).

The experimental fish diet was prepared by mixing pre-made powder (Gel Fish Food, Aquatic Eco-Systems, Apopka, FL, 50% protein, stable form of vit C) with warm (90 °C) water in ratio 1:1 (basal diet). Immediately after mixing, the recommended β-glucan dose of 10 g kg^{-1} (1% of glucan in feed) previously used in different fish species [24–26] was added to the diet and thoroughly mixed (treatment diet, 10 g kg^{-1} of MacroGard Feed Ingredient, MG). The diet was divided into daily rations and kept at 4 °C until use. Immediately before use, half of the daily ration was removed from the refrigerator, cut with a manual food processor to 0.1–0.5 mm pieces, and fed to the fish twice a day until apparent satiation.

2.3. Neutrophil separation and functional assays

Kidney tissue from six individual fish was aseptically collected, and neutrophils were separated using a previously described technique [17]. Briefly, kidneys were pooled in Hank's balanced salt solution without Ca^{2+} and Mg^{2+} ($HBSS^{CMF}$) and phenol red (Mediatech-CellGro, AK, USA), homogenized in a 15 mL tissue grinder (Wheaton, USA) and pelleted for 15 min at 250*g*. Cells were separated on a medium with a specific gravity of 1.078 g mL^{-1} (Lymphocyte separation medium 1078, Mediatech—CellGro, AK, USA), viability was determined, and cell suspensions were adjusted to a standard concentration of 2.5×10^7 cell mL^{-1}. The neutrophil to non-neutrophil ratio was determined by differential leukocyte counts on Hemacolor (Harleco, EM Science, NJ, USA) stained cytospin preparations of cell isolates [27,28]. The percent of neutrophils determined in cell suspensions from experimental groups were compared to control group using the following formula (N = neutrophil):

$$\%\ \text{of control group}\ N = \frac{N\ \text{ratio in experiment}}{N\ \text{ratio in control}} \times 100.$$

2.3.1. Total MPO content determination

The total MPO content was calculated from optical density (OD) of lysed neutrophils using a standard curve as previously described. In short, standard suspensions of bovine neutrophils (5×10^7 cell mL^{-1}) in phenylmethylsulfonyl fluoride (Sigma; 1 mM in absolute ethanol) were prepared and stored at −70 °C. Two-fold serial dilutions of a standard neutrophil suspension were lysed with cetyltrimethylammonium bromide (CTAB, Sigma; 0.02% in water). The OD due to the MPO content of these known concentrations of neutrophils was compared to OD values with serial dilutions of commercially available MPO (EC 1.11.1.7, Sigma), and a standard curve was calculated. Bovine neutrophil suspension with a total MPO content of 2.84 U/10^6 cell was used as an assay standard. Total MPO content for fathead minnow kidney neutrophil suspensions was normalized using the following correction formula:

$$\text{NU} = (\text{NCW} \times U)/[(\%N \times \text{NCW})/100],$$

where NU is the normalized MPO unit value, NCW the number of cells per well, U the measured MPO unit value and $\%N$ the percent of neutrophils in cell suspension.

The mean of total neutrophil MPO content in control group was used as 100%. Total MPO content from experiment groups were compared to control group activity using the following

formula:

$$\%\ \text{of control group total MPO content} = \frac{\text{total MPO content in experiment}}{\text{total MPO content of control}} \times 100.$$

2.3.2. Degranulation of neutrophil primary granules

The effects of different glucan concentrations on the stimulation of neutrophil degranulation were measured with a previously described assay with slight modifications [17]. In short, release of MPO from neutrophils was measured in response to different stimulants in a microtiter plate. Test wells received 75 μL of cyto B or HBSS and 50 μL of stimulant; control (background) wells received 125 μL of HBSS; and total MPO content (lysed cells) wells received 125 μL of CTAB. Twenty-five microliter of cell suspension (2.5×10^7 cell mL^{-1}) was added to each well and incubated at 30 °C for 20 min. To test the effect of time on MPO release in stimulated neutrophils, 200 μg mL^{-1} MG was used as described above, but with incubation times of 0, 30, 60 and 90 s, and 2, 3, 4 and 5 min. When CaI was used as the stimulant, incubation times were 0, 10, 20, 30, 40 and 60 min. To test if soluble β-glucan can prime neutrophils for degranulation, neutrophil suspensions were pre-treated with different concentrations of soluble MG (4.125, 8.25, 16.5 and 33 μg mL^{-1}) for 20 min and then primed cells were used as described above, stimulated with CaI (cells without pre-treatment and stimulated with CaI served as control). Change in degranulation due to pre-treatment was calculated using the following formula:

$$\%\ \text{of control neutrophil degranulation activity} = \frac{\%\ \text{MPO release of pre-treated neutrophils}}{\%\ \text{MPO release of control neutrophils}} \times 100.$$

After incubation, 50 μL of 3,3′,5,5′-tetramethylbenzidine hydrochloride (Sigma; 2.5 mM in water) was added, followed immediately with 50 μL of hydrogen peroxide (H_2O_2, 5 mM in water). The color change reaction was allowed to proceed for 2 min, and 50 μL of sulfuric acid (2 M) was added to stop the reaction. Test plates were centrifuged at 600*g* for 15 min, 200 μL of supernatant from each well was transferred to another plate and OD in each well was determined at 405 nM using a microtiter plate spectrophotometer (V-Max, Molecular Devices, USA) with SOFTmax PRO 4.0 software. The percent release of MPO was calculated using the following formula:

$$\%\ \text{release} = \frac{\text{OD}_{\text{stimulated}} - \text{OD}_{\text{background}}}{\text{OD}_{\text{lysed}} - \text{OD}_{\text{background}}} \times 100.$$

The degranulation assay was used to determine effects of stress on neutrophil function as previously described. The mean % of neutrophil MPO release from primary granules of control group was used as 100% of neutrophil degranulation activity for each day. Neutrophil degranulation activity from experimental groups was compared to control group activity using the following formula:

$$\%\ \text{of control group neutrophil degranulation activity} = \frac{\%\ \text{MPO release of experiment}}{\%\ \text{MPO release of control}} \times 100.$$

2.3.3. Neutrophil oxidative burst

Neutrophil oxidative burst was assayed using modifications of a previously described method [29]. Detection of extracellular superoxide was based on reduction of Cyt *C* (Sigma), when PMA, GB and MG were used as stimulants in microtiter plates. All samples were tested in duplicate wells. Briefly, 50 μL of HBSS and stimulant was added to each test well, followed by 150 μL of Cyt *C*. Non-stimulated test wells received 100 μL of HBSS and 150 μL of Cyt *C*. Background wells received all reagents, but no cells. All test wells received 50 μL of cell suspension containing 2.5×10^7 cell mL^{-1}. To test if soluble β-glucan can prime neutrophils for oxidative burst, neutrophil suspensions were pre-treated with different concentrations of soluble MG (4.125, 8.25, 16.5 and 33 μg mL^{-1}) for 20 min and then used primed cells, as described, stimulated with PMA (cells without pre-treatment and stimulated with PMA served as control).

After addition of cells, plates were immediately placed in a microtiter plate spectrophotometer at room temperature, OD in each well was determined every minute for 40 min using two wavelengths (OD = V1−V2; V1 = 550 and V2 = 650 nM), and background values were subtracted from the plate. Total superoxide release was measured as nmol O_2^- produced per 10^6 neutrophils using the following correction formula:

$$O_2^- = [(\text{OD}_{\text{av}}/2) \times 100/\%N] \times 15.87/1.25,$$

O_2^- is the nmol of superoxide produced per 10^6 neutrophils, OD_{av} the average measured OD, $\%N$ the percent of neutrophils in cell suspension, 15.87

the correction factor for transformation of OD value to nmol of O_2^- [30] and 1.25 the neutrophil dilution factor.

The oxidative burst assay was used to determine effects of stress on neutrophil function [20]. The mean neutrophil superoxide release from the control group at 20 min was used as 100% of neutrophil burst activity for each day. The 20 min time was used as the average time when O_2^- production reached 95% of the maximal recorded value. Experimental group neutrophil burst activity was compared to control group activity using the following formula:

$$\%\ \text{of control group neutrophil burst activity at 20 min} = \frac{O_2^-\ \text{release of experiment at 20 min}}{O_2^-\ \text{release of control at 20 min}} \times 100.$$

2.4. Effects of β-glucan on neutrophil function

To determine effects of aquaculture stress on neutrophil function, fish were exposed to acute handling and crowding stress (AS), as previously described [20]. In short, quickly netted fish were moved to beaker with aerated tank water, crowded (30 g of fish L^{-1}) for 20 min, and returned to the tank.

2.4.1. Effect of β-glucan on neutrophil function in non-stressed (NS) and acute-stressed (AS) fish

To investigate effects of dietary glucans on neutrophil function in NS fish, the MG diet (treatment, T) was introduced at day 0 to NS fish in the stock tank that had been previously fed the basal diet to satiation twice per day for 3 weeks (200 fish, NS treatment group, NS-T). The second stock tank continued feeding with the basal diet and served as control (200 fish, NS control group, NS-C). Water quality in stock tanks was measured daily throughout the experiment: water temperature was $20 \pm 1\,^{\circ}C$, pH was 8 ± 0.2, dissolved O_2 was $7 \pm 1\ mg\ L^{-1}$, TAN was $<0.5\ mg\ L^{-1}$ and TNN was below the detection limit. Tanks were sampled (total of four samples per tank, five fish per sample) and analyzed for neutrophil function on day 0 (before introduction of MG diet) and on days 1, 3 and 7 after MG diet was introduced.

In order to examine effect of dietary glucans on neutrophil function in fish that have been exposed to acute handling and crowding stress, at day 7, 60 out of 120 remaining fish were randomly netted from tanks and exposed to AS. Stressed fish from treatment group (AS-T) and stressed fish from control group (AS-C) were returned to their respective tanks. NS fish (NS-T and NS-C) and stressed (AS-T and AS-C) fish were separated with the net for the remainder of the experiment. Fish from NS-T, AS-T, NS-C and AS-C groups were sampled ($n = 4$ for each group) and analyzed for neutrophil function on days 1, 3 and 7 after AS (days 8, 10 and 14 after MG treatment started).

2.4.2. Effect of β-glucan on neutrophil function in chronically stressed (CS) fish with and without AS

To simulate chronic crowding stress with poor water quality conditions (CS), eight 75 L fiberglass tanks were filled with dechlorinated tap water at 20 °C, power filtered (Whisper, Tetra, USA) through active carbon and zeolite, and 5 g of basal fish diet per tank was added daily until the TAN level reached $2\ mg\ mL^{-1}$. Fish were then quickly netted from the stock tank and randomly divided into prepared tanks (40 fish per tank, 1.6 g of fish L^{-1}). Fish were fed with basal diet to satiation twice per day. Water quality parameters were measured daily throughout the experiment: water temperature was $20 \pm 1\,^{\circ}C$, pH was 7.5 ± 0.5, dissolved O_2 was $7.5 \pm 1\ mg\ L^{-1}$, TAN was $>2\ mg\ L^{-1}$ and TNN was $>0.02\ mg\ L^{-1}$. If TAN was $>2.75\ mg\ L^{-1}$ and/or TNN was $>0.330\ mg\ L^{-1}$, partial water change was performed with fresh dechlorinated tap water until TAN reached $2\ mg\ L^{-1}$. Fish were exposed to CS conditions and fed basal diet for 14 days before treatment.

In order to examine effects of dietary glucans on neutrophil functions in CS fish, we introduced MG diet (treatment, T) to four randomly selected CS tanks (CS-T) on day 0. Remaining four CS tanks served as stressed control (CS-C) and fish from stock tank served as NS-C. Each tank was sampled as described, and analyzed for neutrophil function on day 0 (before treatment), and days 1, 3 and 7 after treatment diet was introduced.

To investigate effects of glucan on neutrophil function in CS fish that were exposed to acute handling and crowding stress (AS), the described CS setup was prepared in eight 75 L tanks, 40 fish each. Water quality was measured daily and was not significantly different than described above. After 14 days of CS, MG diet (treatment) was introduced to four randomly selected tanks (CS/AS-T) at day 0. The remaining four tanks served as CS control (CS/AS-C). Seven days after MG treatment, fish were subjected to AS as described, and neutrophil

function was measured on day 0 (before AS), and days 1, 3 and 7 after AS (days 8, 10 and 14 post-MG treatment). Fish from stock tank served as NS-C.

2.5. Statistical analysis

Data are presented as means ± standard error of the mean (SEM) unless otherwise indicated. The differences among groups were analyzed using one- and two-way ANOVA with Dunnett's post-test and Student's *t*-test (GraphPad Prism 3.00, 1999; San Diego, CA). $P<0.05$ was considered significant.

3. Results

3.1. Yeast β-glucan is potent stimulant of degranulation in vitro

Kidney cell suspensions used during in vitro assays had a mean neutrophil purity of 71.3 ± 1.8%. The increase in degranulation was dose-dependent and is shown in Fig. 1. Stimulation with GY and MG caused a significant increase in MPO release ($P<0.001$; 70%) when compared to Z and GB (<10%). The MPO release from primary granules reached a plateau when 200 μg mL^{-1} GY and MG were used, and a concentration of 200 μg mL^{-1} of MG was selected for time trials. The release of MPO from primary granules depended on incubation time as well as stimulant used, MG causing significantly rapid and higher maximal degranulation ($P<0.001$; 5 min; 90%) than CaI (40 min, 65%) (Fig. 2). Pre-incubation of neutrophils for 20 min with different concentrations of soluble MG caused a significant, dose-dependent, increase in CaI stimulated degranulation ($P<0.01$; CaI control, 100%; soluble MG 8.25 μg mL^{-1}, 120%; soluble MG 16.5 μg mL^{-1}, 170%; soluble MG 33 μg mL^{-1}, 215% of control) (Fig. 3).

Neutrophil oxidative burst was significantly higher from HBSS control ($P<0.01$; 2.5 nmol O_2^- per 10^6 neutrophils) when PMA was used as the stimulant. When 600 μg mL^{-1} MG was used, concentrations did not exceed 0.75 nmol. In all, 150 μg mL^{-1} MG induced O_2^- production not different from HBSS (Fig. 4A). Pre-incubation of neutrophils for 20 min with different concentrations of soluble MG caused dose-dependent significant increases in superoxide production after stimulation with PMA ($P<0.01$; PMA control, 1.5 nmol O_2^- per 10^6 neutrophils at 10 min; soluble MG 4.125 μg mL^{-1}, 2 nmol; 8.25 μg mL^{-1}, 2.4 nmol; soluble MG 16.5 μg mL^{-1} and soluble MG 33 μg mL^{-1}, 3 nmol), increasing PMA stimulated burst two-fold, starting at 10 min and continuing for the remaining time points (Fig. 4B).

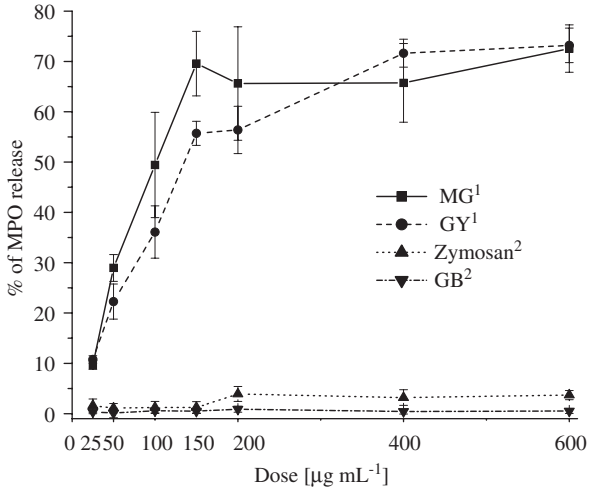

Fig. 1. Dose-dependent increase in β-glucan stimulated degranulation of neutrophil primary granules. MG, β-glucan from baker's yeast cell wall (MacroGard Feed Ingredient, Biotec, Norway); GY, β-glucan from baker's yeast cell wall (Sigma); zymosan (non-opsonized zymosan from yeast cell wall, Sigma); GB, β-glucan from barley. Different numbers indicate significant differences between groups ($P<0.001$, $n=6$).

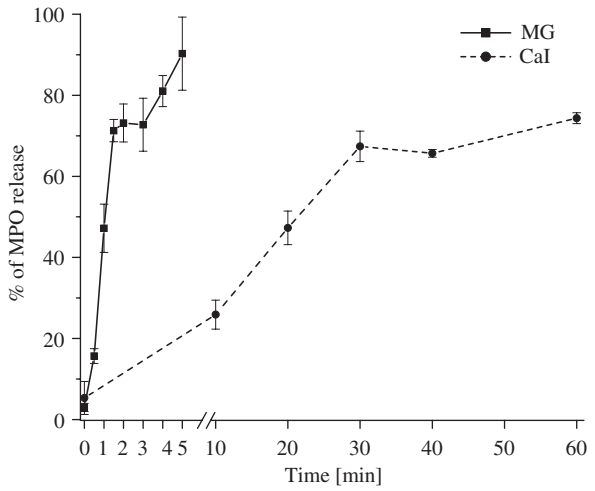

Fig. 2. Time-dependent β-glucan activation of degranulation. MG, β-glucan from baker's yeast cell wall (MacroGard Feed Ingredient, Biotec, Norway); CaI, calcium ionophore. Stimulation of neutrophils with MG causes significantly quicker activation of degranulation then when CaI is used ($P<0.001$, $n=6$). Viability of cells before and after application of MG and CaI for 30 min is not significantly different.

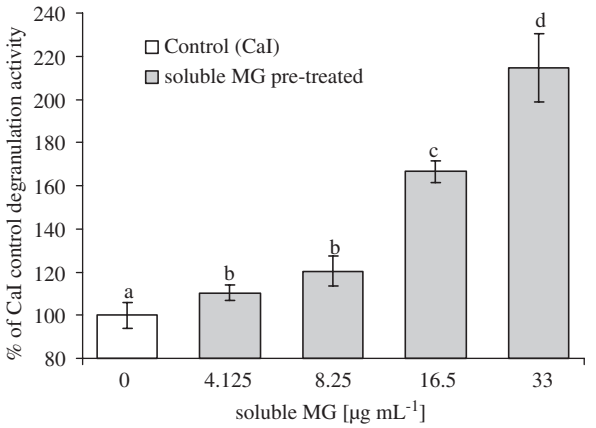

Fig. 3. Degranulation of neutrophils after pre-incubation with water soluble β-glucan in vitro. CaI stimulated degranulation (open bar); pre-treatment with different concentrations of soluble MG for 20 min (gray bar). Different letters (a–d) indicate significant difference between groups ($P<0.05$; mean ± SEM, $n=4$).

3.2. Dietary β-glucan enhanced degranulation in NS fish

Neutrophil functional parameters were measured on day 0 (before diet), and after the diet was supplemented with 10 g kg^{-1} (1%) MG for treatment groups (NS-T) on days 1, 3 and 7 (Fig. 5). CaI stimulated degranulation of primary granules was significantly increased in MG treated fish (NS-T; $P<0.01$; 170%) when compared to non-treated control (100%) after 7 days of treatment. Neutrophil degranulation was significantly decreased at day 1 post-treatment ($P<0.01$; 82% of control), followed by significant increase in degranulation observed after fish were fed with MG for 3 days ($P<0.01$; 120%). A significant increase in superoxide production ($P<0.01$; 190% of control) was observed 1 day after MG diet was introduced, but reduced to control (100%) level at day 3 and for the duration of experiment. Total MPO content in lysed cells was significantly increased 1–3 days after MG diet was introduced ($P<0.01$; 125%) and returned to control (100%) values at day 7. The neutrophil ratio in kidney cell suspensions significantly increased 3 days after MG diet was introduced ($P<0.01$; 125%).

3.3. Dietary β-glucan prevented degranulation decrease in AS fish

Degranulation in fish that were exposed to acute handling and crowding stress after feeding MG

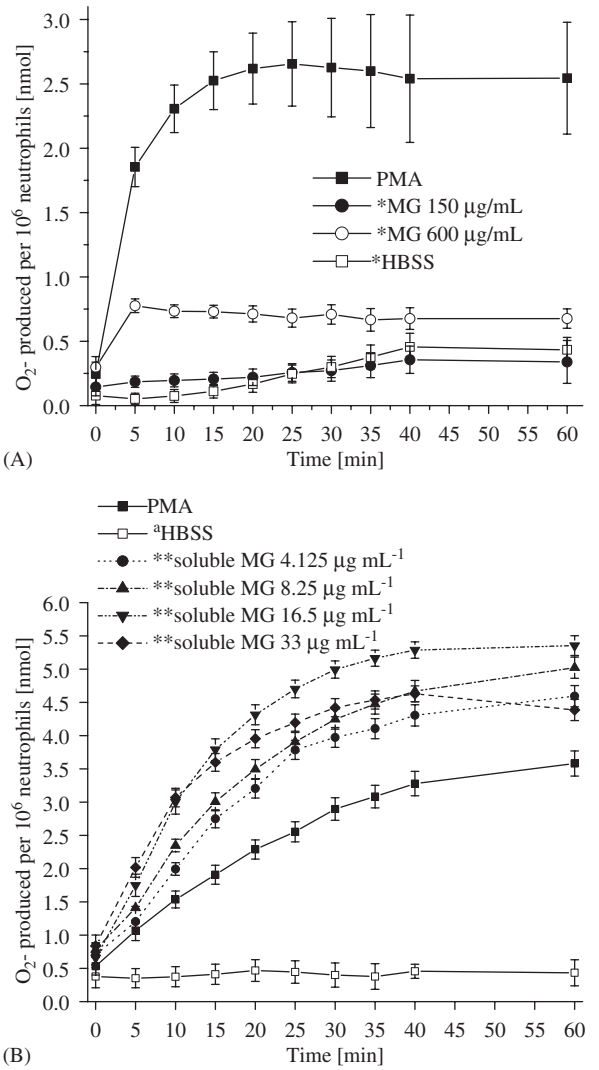

Fig. 4. Effect of PMA and particulate β-glucan on oxidative burst (A) and effect of pre-incubation with water soluble β-glucan in vitro on PMA stimulated superoxide production (B). * Significant difference from PMA stimulated respiratory burst ($P<0.01$; mean ± SEM, $n=4$). ** Significant difference from PMA stimulated respiratory burst of cells without pre-treatment ($P<0.01$; mean ± SEM, $n=4$) starting at 10 min and continuing through all time points. [a]HBSS caused negligible stimulation of respiratory burst at all time points.

supplemented diet (AS-T) for 7 days was significantly higher ($P<0.01$, 125% of control) than degranulation level in control fish at day 3, and was not significantly different from control level (100%) at 7 days after AS (Fig. 5A). AS in fish fed the basal diet (AS-C) significantly reduced degranulation ($P<0.05$, 80% of control) at day 3. MG supplemented fish had a decrease in degranulation 1

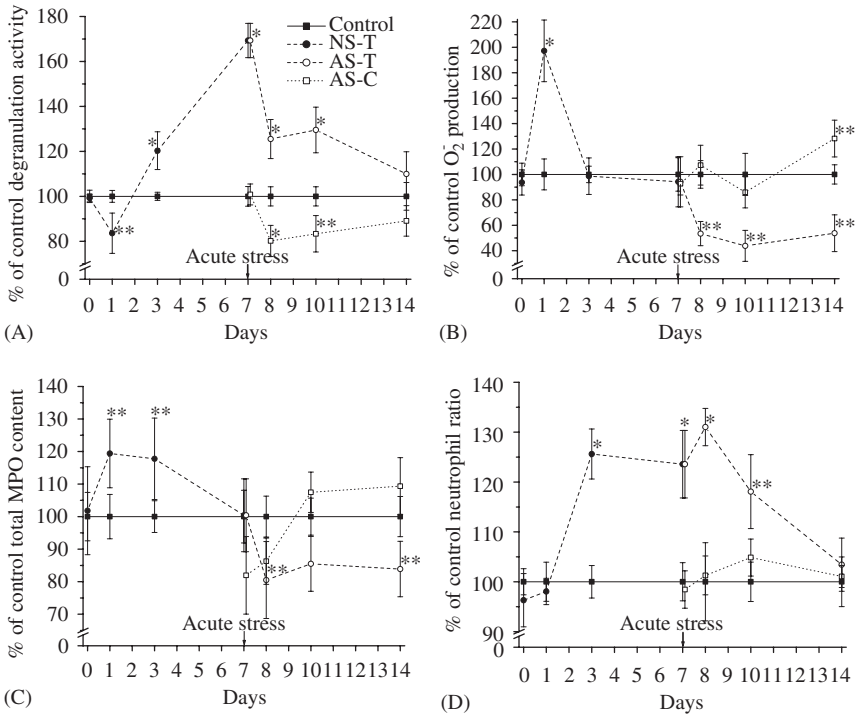

Fig. 5. Effect of dietary β-glucan on degranulation, oxidative burst, total MPO content and neutrophil ratio in kidney cell suspensions of non-stressed fish (days 0–7) and acute stress (days 8–14). Effect of dietary β-glucan on CaI stimulated degranulation (A), PMA stimulated oxidative burst (B), total MPO content (C) and neutrophil ratio (D) in kidney cell suspensions of non-stressed (NS) and acute-stressed (AS) fish. Treated fish (NS-T and AS-T) were fed the MG day starting on day 0. Non-treated fish (NS-C and AS-C) served as controls. Fish were examined for neutrophil function during first week (days 0, 1, 3 and 7), exposed to acute stress on day 7, and neutrophil function was measured during second week of the experiment (days 8, 10 and 14). *,** Significant difference from control ($^*P<0.01$, $^{**}P<0.05$; mean ± SEM, $n \geqslant 4$).

day after stressful procedure (from 170% to 125% of control), but it remained above control. Superoxide production in AS-T fish decreased to 60% of control after AS, while AS-C fish dropped to 85% of control at 3 days, and then increased to 130% of control O_2^- production (100%) 7 days after stress (Fig. 5B). Total MPO content in AS-T fish dropped to 75% of control 1 day after stress, while AS-C fish did not differ significantly from controls for the observation period (Fig. 5C). The neutrophil to non-neutrophil ratio in AS-T fish remained significantly higher ($P<0.01$, 130% of control) than in control and AS-C fish for 3 days, and then returned to near control level (100%) by 7 days post-stress (Fig. 5D).

3.4. Dietary β-glucan prevented degranulation decrease in CS fish without AS

Fish exposed to 2 weeks of chronic stress, and fed a basal diet (CS-C) had significantly lower degranulation ($P<0.01$, 65% of control) than control fish for the duration of experiment (Fig. 6A). Degranulation in CS fish fed MG supplemented diet (CS-T) at day 0, showed transient decrease at day 1 after the administration of the treatment diet, but returned to control levels (100%) at day 3, and remained at control levels 7 days after MG diet was introduced. Oxidative burst in CS-T fish was significantly increased ($P<0.01$, 140% of control) at day 1, dropped to 65% of control at day 3 and returned to control level (100%) at day 7 after MG diet was introduced (Fig. 6B). Total MPO content and neutrophil ratio in CS-T and CS-C fish were not significantly affected by MG treatment (Fig. 6C and D).

3.5. Dietary β-glucan prevented neutrophil function decrease in CS fish exposed to AS

CS fish fed MG for 7 days were exposed to AS (CS/AS-T), and a significant increase in degranulation

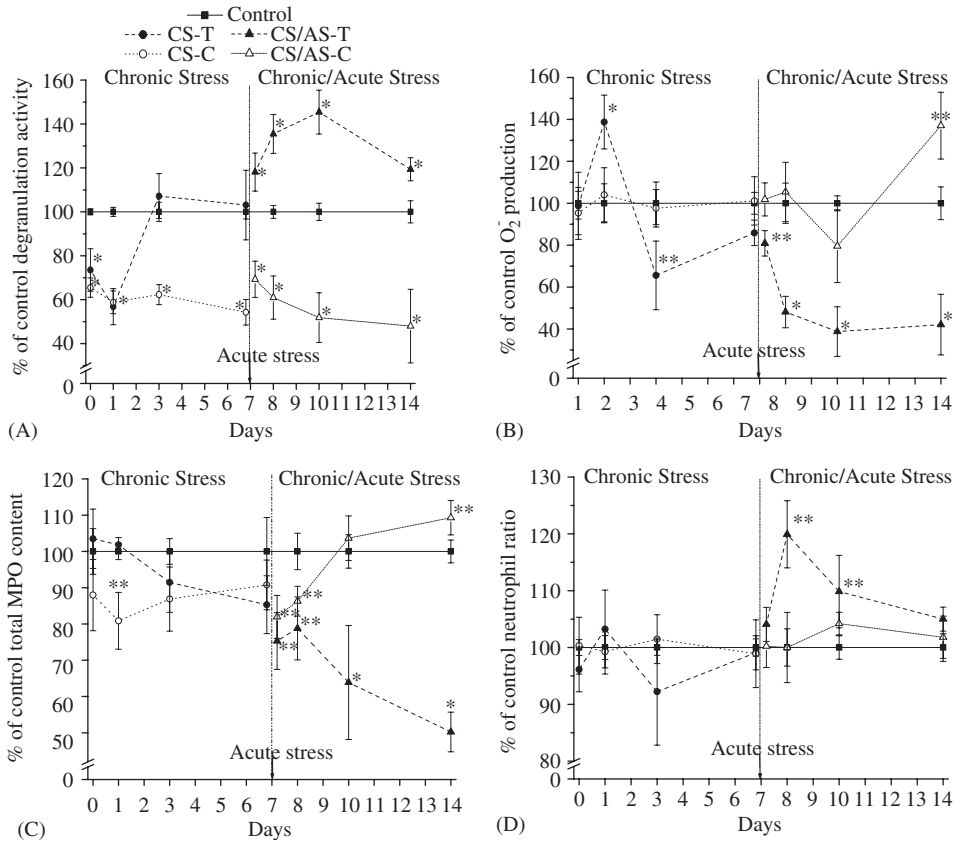

Fig. 6. Effect of dietary β-glucan on degranulation, oxidative burst, total MPO content and neutrophil ratio in kidney cell suspensions of chronically stressed fish (days 0–7) with and without exposure to acute stress (days 8–14). Effect of dietary β-glucan on CaI stimulated degranulation (A), PMA stimulated oxidative burst (B), total MPO content (C) and neutrophil ratio (D) in kidney cell suspensions of chronically stressed (CS) fish with and without exposure to acute stress (AS). Control fish (NS-C), chronically stressed fish with glucan treatment (CS-T, days 0–7), chronically and acutely stressed fish with glucan treatment (CS/AS-T, days 8–14). Fish without glucan treatment (chronic stress, CS-C; chronic and acute stress CS/AS-C). Two experiments are represented on the same graph separated with dashed vertical line. In first experiment, the effect of 10 g kg^{-1} MG on neutrophil function in CS fish was monitored for 7 days after introduction of supplemented diet (day 0). In the second experiment, CS fish have been fed MG supplemented diet for 7 days and exposed to AS on day 7. The effect of 10 g kg^{-1} MG (1%) on neutrophil function in CS/AS fish was monitored for 7 days after AS. *·** Significant difference from control (*$P<0.01$, **$P<0.05$; mean ± SEM, $n \geqslant 4$).

was observed ($P<0.01$, 120–140% of control) 1 day after AS and remained above control levels for 7 days. CS-C fish exposed to AS (CS/AS-C) had significantly less degranulation ($P<0.01$, 50–60% of control) for the duration of the experiment (Fig. 6A). Oxidative burst in CS/AS-T fish was significantly lower ($P<0.01$, 40–80% of control) from control fish at all time points, while CS/AS-C fish showed significant increase in superoxide production ($P<0.05$, 140% of control) at day 7 after AS (Fig. 6B). Total MPO content in CS/AS-T fish was significantly lower from control ($P<0.01$, 50–80% of control) at all time points, while CS/AS-C fish increased from 80% of control on day 0 to 110% of control on day 7 ($P<0.05$) after AS (Fig. 6C). The neutrophil ratio increased significantly in CS/AS-T fish ($P<0.05$, 115–125%) at days 1 and 3, while CS/AS-C fish were not significantly different from the control (Fig. 6D).

4. Discussion

β-1,3–1,6-Glucans from baker's yeast and other bacterial, fungal and plant sources have been recognized as potent immunomodulators in different fish species, and recent breakthroughs in industrial manufacturing of yeast β-glucans allow for its use as an affordable dietary supplement for aquaculture and pet fish [1,8]. Stimulatory effects of

β-glucans on neutrophil function and disease resistance have been reported in mammals, amphibians, fish and crustaceans [8–10,31]. It has been demonstrated that large molecular weight or particulate β-glucans activate transcription factor NF-κB, and increase production of IFN-γ, IL-8, IL-1β, IL-6, TNF-α, monocyte tissue factor and nitric oxide [3,31–34], and lower molecular weight and soluble glucans have been shown to induce cytokines (IL-8 and IL-6) and nuclear transcription factors (NF-κB and NF-IL-6) [35,36]. Use of highly purified particulate β-1,3–1,6-glucans from baker's yeast in diets stimulated cellular and humoral immune responses, increased disease resistance in a number of fish species [8] and enhanced anticancer treatments in mouse models [2]. Negative effects of higher β-glucan doses or prolonged oral application of β-glucans on disease resistance and general condition of fish have also been reported, indicating need for further investigation before recommendations for duration and concentration of β-glucan treatments are made [24,26,37].

The effects of glucans on neutrophil degranulation in fish have not been previously reported. Fathead minnows are a useful fish model for studying degranulation of neutrophil primary granules [20], are a well-established laboratory animal in toxicology research [23] and a significant aquaculture (bait fish) and ecological (forage) species [38]. Handling and crowding stress, often combined with poor water quality in intensive aquaculture operations, results in poor performance, disease outbreaks, and in severe cases, increased mortality [18]. Immunomodulators, such as β-glucans, can be used to increase or to prevent decrease of neutrophil function in stressed animals [1,10].

The first part of this study was to determine effects of different glucan stimulants on fathead minnow kidney neutrophil degranulation and oxidative burst. Particulate β-1,3–1,6-glucans from baker's yeast (Sigma and MacroGard Feed Ingredient®) proved to be a potent stimulant of degranulation in vitro, while non-opsonized Z, and β-1,3–1,6-glucans from barley failed to induce degranulation in this species. Yeast glucan induced significantly stronger (>80%) and more rapid (<2 min) degranulation than CaI (65%, 40 min), suggesting that a different induction mechanism may be involved in β-glucan stimulated degranulation. This finding is in accordance with described responses of human, murine and seabream phagocytes [31,39,40], and could be related to the possible presence of a specific β-glucan receptor on fathead minnow neutrophils. Specific β-glucan receptors (CR3, Dectin-1), and Z-binding motifs on Toll-like receptors (TLR2 and TLR4), have been described on immune cells (neutrophils, macrophages, dendritic cells, NK cells and subsets of T-cells) in human beings, mice and catfish [4,5,41–43], and β-glucans can modulate neutrophil responses in mice and human beings in vitro [44,45]. The molecular mechanisms behind cellular responses to β-glucans are not well understood, partly because most of the earlier studies utilized impure, or β-glucan-rich particles, such as Z, rather than purified β-glucan [42]. In this study, two different (barley and yeast) highly purified β-glucans (>99% purity by manufacturers' claims), as well as Z, were used as stimulants of degranulation. The TLRs have been identified as major signal transducers of pathogen associated molecular patterns and TLR2 and TLR4 have been shown to induce cytokine production in response to Z [46]. Although able to interact with Z, the TLRs do not appear to recognize β-glucan structures, and the recognition and response to purified β-glucans was shown to require the β-glucan receptor Dectin-1. Furthermore, Dectin-1 has been shown to induce intracellular signaling independently of TLRs and is the most likely candidate for mediating immune responses to β-glucans [47–50]. The observed activation of fathead minnow neutrophils with purified particulate yeast β-glucans, and lack of the response when Z was used as stimulant suggest that specific β-glucan receptor is involved in degranulation of primary granules in fathead minnow neutrophils.

Particulate yeast β-glucan stimulation of in vitro oxidative burst of fathead minnow neutrophils was significantly lower than PMA induced superoxide production. Strong respiratory burst response of phagocytes to glucans in doses from 50 to 1000 μg mL^{-1} was reported previously in other fish species [51,52], but fathead minnow neutrophils were not responsive to 150 μg mL^{-1} of particulate β-glucan, while 600 μg mL^{-1} caused only a low increase in superoxide anion production (0.75 nmol, compared to 2.5 nmol O_2^- produced with PMA). Unlike direct stimulation, pre-treatment of neutrophils with soluble β-glucan caused a significant increase in CaI stimulated degranulation and in PMA stimulated oxidative burst. This finding is in agreement with a reported increase in functional parameters in fish phagocytes pre-incubated with glucans [51,52]. β-Glucan mediated induction of

NF-κB, as well as production of cytokines, such as IL-8, could lead to activation of neutrophil killing mechanisms measured as increase in degranulation and oxidative burst. Degranulation of neutrophil primary granules and release of MPO is an important step in oxygen mediated killing and formation of halide ions [29], and it appears to be the essential step in exocytosis of neutrophil extracellular traps, a newly discovered neutrophil defense mechanism [53]. Yeast β-glucan proved to be a potent direct stimulator of neutrophil degranulation, and at lower concentrations an efficient priming agent of neutrophil degranulation and oxidative burst in vitro.

The second part of this study was to examine the effects of dietary administration of particulate β-1,3–1,6-glucans from baker's yeast on neutrophil function in NS fish, and fish exposed to acute handling and crowding stress. AS is often associated with routine fish handling and transport [44], and negative effects on degranulation after stress have been reported in fathead minnows [20]. Effects of dietary glucan on fathead minnows have not been reported previously. In this study, the dose of dietary glucan (MG, 1% or $10\,g\,kg^{-1}$ of feed) followed recommendations for other fish species, and was selected based on reported effects on different immunological responses [24–26]. MG treated, NS, fish (NS-T) showed a significant increase in neutrophil degranulation and neutrophil ratios for the duration of treatment (14 days), and remained significantly higher than controls (NS and AS-C) after exposure to AS. Oxidative burst and total MPO content showed a transient increase during treatment of NS fish (day 1, and days 1–3, respectively), followed by a decrease when fish were exposed to AS (AS-T). Findings of this study are in general agreement with the limited information available about glucan effects on phagocytic cells in stressed fish [26,54], where oxidative burst was increased before stress, but decreased in stressed fish fed 1% glucan diet. The increased neutrophil ratio observed in fathead minnows is in agreement with reported elevated blood neutrophil counts during dietary glucan administration [54].

The mechanism responsible for the dietary β-glucan induced increase of neutrophil degranulation and oxidative burst is not completely understood [55]. Studies in mice suggested that the large particulate β-1,3–1,6-glucans molecules were taken up by gastrointestinal macrophages, degraded within macrophages to smaller soluble β-glucan fragments, and exocytosed in the vicinity of bone marrow granulocytes, allowing for CR3 or Dectin-1 receptors on the granulocyte surface to interact with soluble β-glucan and elicit a stronger C3b response in granulocytes [2]. Presence of glucanases has not been reported in macrophages, and the observed mechanism for degradation of glucans within macrophages is not yet understood. In this study, direct stimulation of neutrophils with particulate β-glucans elicited stronger and more rapid degranulation responses, while application of soluble β-glucans caused significant priming of neutrophils and increased response to conventional stimulants (CaI and PMA). Recent studies demonstrated significant role of CR3 activation of neutrophils with orally administered β-glucans in conjunction with monoclonal antitumor antibodies [2]. Uptake and degradation of particulate β-glucans by intestinal macrophages through Dectin-1 receptor, as well as uptake of soluble β-glucans through GALT and intestinal mucosa independent of Dectin-1 receptor, suggest that soluble, rather than particulate glucans are responsible for biological actions of glucans in vivo [2,55,56]. The increased neutrophil degranulation and respiratory burst after short (20 min) priming with soluble β-glucan observed in this study agrees with studies performed on phagocytes in other fish species [51,52].

The general stimulative effects of dietary administration of β-glucan on the immune system have been reported, but specific responses varied with glucan type, and dose and duration of the treatment [8]. Further, investigations are necessary to determine the appropriate dose and duration of treatment to achieve optimal immunostimulation, and challenge studies with specific pathogens will be required to investigate the effects of glucans on disease resistance in fathead minnows. The diet supplemented with $10\,g\,kg^{-1}$ MG for 1 week, significantly increased neutrophil degranulation and the ratio of neutrophils to non-neutrophils in kidney cell suspensions; transiently increased oxidative burst and total MPO content; and prevented a decrease in degranulation after acute handling and crowding stress.

The third part of the study was to investigate the effects of dietary administration of particulate β-1,3–1,6-glucans from baker's yeast (MG) on neutrophil function in CS fish with and without exposure to acute handling and crowding stress. Negative effects of chronic environmental stress on

the immune system of fish have been reported [57], and recent investigations indicated that dietary administration of β-glucans can increase immune responses in fish exhibiting immunosuppression due to overwintering stress [58,59]. The effect of dietary glucans on neutrophil function in fish exposed to chronic overcrowding and poor water quality has not been previously described. Fathead minnows exposed to poor water quality and overcrowding for 2 weeks (CS-C) had significantly lower neutrophil degranulation than NS-Cs. Application of dietary MG to CS fish (CS-T) increased degranulation to control levels after 3 days, while the oxidative burst followed a pattern described in NS fish (transient increase, followed by decrease). These findings are similar to the limited results describing dietary β-glucan enhancement of respiratory burst in fish exposed to long-term stress, such as winter low temperatures [59]. When CS fathead minnows were exposed to AS after 7 days of MG treatment (CS/AS-T), neutrophil degranulation was significantly higher than in NS-C fish and in CS fish exposed to AS without MG diet (CS/AS-C). A significant decrease in oxidative burst and total MPO content was observed after AS in CS/AS-T fish. Effects of dietary glucans on phagocytic cell function and disease resistance in stressed fish have been reported to depend on dose and duration of the treatment [10,26,54,58], and long-term feeding with high doses of glucan can reduce oxidative burst [54]. Dietary application of MG increased neutrophil degranulation in CS fathead minnows to the levels observed in NS fish.

Short-term glucan application in vivo enhanced overall neutrophil activity, while prolonged application (over 7 days) and AS caused decrease in oxidative burst, and total MPO content, but increased percent degranulation. It has been demonstrated that higher doses of glucans applied for longer periods of time can cause a decrease in neutrophil oxidative burst and disease resistance [24,26,37]. When mouse strains that are genetically altered to have strong acute inflammatory responses have primed (pre-stimulated) neutrophils that are subsequently exposed to another stimulant, these mice have increased concentrations of pro-inflammatory cytokines and increased responsiveness of neutrophils that lead to rapid neutrophil maturation [60]. It also has been shown that the glucan immunomodulating effect on neutrophils can be direct (specific membrane receptors), as well as indirect (cytokines produced by activated macrophages) [42]. In NS fish, it is possible that dietary glucans primed kidney-derived neutrophils in vivo both directly and indirectly, causing the majority of mature neutrophils to degranulate more readily in vivo to natural stimuli in the aquatic environment. However, prolonged glucan treatment stimulation that primes neutrophils combined with acute and/or chronic stress may cause the subsequent exhaustion of MPO and oxidative metabolism seen as an overall reduction of total MPO and superoxide generation in isolated neutrophils (Figs. 5 and 6). Future research should focus on physiological mechanisms behind glucan mediated immunomodulation and the interaction with stress.

This study is the first report on effects of particulate and water soluble β-1,3–1,6-glucans from baker's yeast on fish neutrophil degranulation as a strong stimulant in vitro and dietary immune enhancer in vivo. It was shown that a dietary administration of $10\,g\,kg^{-1}$ of yeast particulate β-glucan to fathead minnows can increase neutrophil function in NS and CS fish, and prevent a decrease in degranulation in AS and CS fish exposed to AS. The degranulation assay measuring MPO release from fathead minnow neutrophil primary granules appears to be useful in investigating effects of in vitro stimulants, as well as dietary immunomodulators of neutrophil function, allowing for fast and relatively inexpensive screening of treatments prior to more expensive and elaborate studies, such as challenge tests. Use of β-glucans in fish diets prior to AS and during chronic stress may enhance aspects of neutrophil function, and potentially increase disease resistance and survival rates after transportation or exposure to poor water quality.

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