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# Effects of the prebiotic mannan-oligosaccharide on the stress response of feed deprived zebrafish (*Danio rerio*)

Mohammad Navid Forsatkar[a], Mohammad Ali Nematollahi[a,*], Gholamreza Rafiee[a], Hamid Farahmand[a], Christian Lawrence[b]

[a] Department of Fisheries, Faculty of Natural Resources, University of Tehran, Karaj, Iran
[b] Aquatic Resources Program, Boston Children's Hospital, Boston, MA, USA

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A B S T R A C T

Feed deprivation has deleterious effects on fish behavior and stress physiology which may susceptible them to disease outbreak. Functional ingredients in diets may substantially impact the physiology and stress responses of host organisms. Here, we hypothesized that the administration of a dietary prebiotic might attenuate the negative influences of feed deprivation on the behavioral profile of anxiety and physiological responses to stress in zebrafish (*Danio rerio*). Fish were fed with either basal or mannan-oligosaccharide supplemented (0.4% MOS/kg diet) diets, once per day (normal-control: CN, and normal-prebiotic: PN) or once every other day (starved-control: CS, and starved-prebiotic: PS) for 8 weeks. Afterwards, fish were subjected to a novel tank test to measure anxiety. Fish from the CS treatment exhibited more pronounced bottom-dwelling behavior than the other treatments. The number of transitions from the bottom to the top third of the novel tank was significantly higher in PN fish than the CS specimens. No significant differences were found between the CN and PS treatments in all of the anxiety behaviors. CS fish showed higher baseline cortisol levels than the other treatments, which was in line with higher expression of *CRH* gene in fish subjected to this treatment. Cortisol levels and *CRH* gene expression of the subjects were also measured after induction of two routine aquaculture stressors. CN and PS fish exhibited similar patterns of cortisol responses at most of the sampling times after stress, and PN specimens showed a significantly lower concentration of cortisol than the other treatments in most cases. Expression of the *CRH* gene was higher in feed deprived fish immediately after stress induction. Overall, the results show that feed deprivation in some cases influenced anxiety-like behaviors and elevated stress response in zebrafish juveniles; however, the addition of MOS to the diet helped deprived fish exhibit behaviors more typical of normally fed animals.

## 1. Introduction

Various stressors are inevitable in commercial fish farming, and may influence the physiology, behavior, and welfare of fish [1]. Routine manipulations of fish in a farm, such as handling, tank/container cleaning and live transport have all been shown to promote stress in fish [2]. Fish respond to external stimuli via modulation of the endocrine system and altering their behaviors [3]. In fish, the perception of a stressful signal activates a neuroendocrine cascade response that culminates in secretion and release of corticosteroids (cortisol in teleost) that ultimately function to mobilize energy resources necessary for flight, fight, or coping [4]. Cortisol, as well as upstream factors in the pathway, hypothalamic corticotropin-releasing hormone (CRH), and the adrenocorticotropic hormone (ACTH), are all suitable indicators of the endocrine response to acute stress [5].

Despite a large number of studies on the physiological and behavioral effects of myriad stressors on fish species, relatively few of them have addressed the impact of starvation on the stress response. Feed deprivation is a phenomenon that occurs both in the wild and in aquaculture settings [6]. It may be a chronic condition, as individuals are not able to meet their nutritional requirements for extended periods of time. It is worth noting that nutritional status may have far-reaching effects on metabolic, hormonal and behavioral pathways in fish [7–9]. Interestingly, it has been reported that feed deprivation elevated the cortisol response in jundiá (*Rhamdia quelen*; [10]), Senegalese sole (*Solea senegalensis*; [11]), and red porgy (*Pagrus pagrus*; [12]), but had no effects on cortisol production in sunshine bass (*Morone chrysops* × *Morone saxatilis*; [13]). However, because a limited form of feed deprivation could be employed to help reduce operational costs, there is a great deal of interest in exploring whether this practice could be

* Corresponding author at: Department of Fisheries, Faculty of Natural Resources, University of Tehran, Chamran St., PO Box: 31585-4314, Karaj, Iran.
*E-mail address:* malahi@ut.ac.ir (M.A. Nematollahi).

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tenable in fish production settings.

On the other hand, behavioral profiles of the stress response may serve as important indicators as alterations in behavior may take place long before physiological changes occur [14]. The relationship between chronic stress and mood disorders such as anxiety is well established in a few fish species [3,15]. In this regard Chakravarty et al. [16] found that a variety of chronic stressors induced anxiety in zebrafish. Therefore, it is expected that disturbances in homeostatic equilibrium, i.e. a chronically stressful condition, might influence the anxiety responses of individuals. In zebrafish, for instance, anxiety can be simply induced by exposing animals to a novel environment [3,17]. The hallmark anxiety behaviors, such as bottom-swimming, a longer latency to enter the upper half of the water column, and freezing/immobility are useful indicators of anxiety in zebrafish (reviewed in [18]). However, we are not aware if stress arising from the negative nutritional status (i.e. starvation) can induce anxiety-like behaviors in fish. Some evidence shows that there is an increase in physical activity levels after a short term caloric restriction [9], but behaviors associated with anxiety are beyond a simple change in the levels of activity.

The sometimes stressful conditions experienced by fish in aquaculture may have profound effects on their physiology and behavior, making them more susceptible to disease outbreaks (e.g. [19]). Stress induced by feed deprivation may reduce innate resistance to pathogens as reported in previous studies [20,21]. The search for nutraceutical products that can be used as alternatives to antibiotics in aquaculture has demonstrated the promise of prebiotics to improve fish health and resistance to disease (e.g. [22]). Prebiotics, which are non-digestible food ingredients that selectively stimulate the growth of health-promoting bacteria within the intestine [23,24], have the potential to improve the well-being of the host and counteract the negative impacts of stressors experienced in culture.

Mannan-oligosaccharides (MOS) are one of the well-studied prebiotics in several cultured fish species. Improved growth, feed conversion, stress resistance, and immune function are the main effects of MOS in fish [24–26]. In addition, gut development, stimulation of intestinal microbiota, and increased stamina and survival during stress are all the effects of MOS in fish species (reviewed in [24]). It is therefore not unreasonable to theorize that the inclusion of MOS in the diet may make fish more resistant to starvation stress.

The aim of the present study was to examine i) how feed deprivation impacts the anxiety and stress responses of zebrafish (*Danio rerio*), and ii) whether or not the inclusion of MOS in the diet would alter those responses. Zebrafish is a good model to study behavior and stress, as the stress response and anxiety like behaviors are extremely well defined in this species [18]. Furthermore, the zebrafish has excellent utility as a model for finfish aquaculture, as it shares many of the same characteristics with numerous fish species of economic interest [27]. Therefore, the results of the present study are expected to have relevance to other fish species.

## 2. Materials and methods

### 2.1. Animals and housing

In a local zebrafish hatchery (Karaj, Iran; where zebrafish are produced for ornamental fish industry), 300 twenty day old long-fin albino zebrafish were sorted and subsequently transported to laboratory. Fish were kept in two 100 L glass rectangular tanks for 10 days to allow them to acclimate. Water temperature was 27 ± 1 °C and the photoperiod was regulated to provide a 14 h light: 10 h dark cycle, with lights on at 0800H. The tanks were continuously aerated by a central pump equipped with a sponge filter to maintain the water parameters within the following range: dissolved $O_2$: 6.9–8.2 mg/L; pH: 7.3–7.7; hardness: 150–180 mg/L as $CaCO_3$. A commercially available extruded granular pellet (BioMar group: proximate composition of 35% crude protein, 12% total lipid, 10% ash, 4% moisture; 20.5 kJ/g energy) was used to feed fish twice daily to apparent satiation. This food was ground down to a particle size such that it would suitable to feed juvenile stage zebrafish (600–800 μm). This feed, prepared in the same way, was used in the following main experiments.

### 2.2. Prebiotic and diet preparation

The prebiotic Agrimos® mannan-oligosaccharides (Agrimos® MOS) was used in the present experiments. Agrimos® MOS is a specific combination of mannan-oligosaccharides and glucose (B-glucans) extracted from the yeast cell walls of *Saccharomyces cerevisiae* (Lallemand Animal Nutrition, France). According to the manufacturer, Agrimos® MOS is obtained by the autolysis of yeast cells at high temperature and a controlled pH. After yeast autolysis is completed, cell wall and yeast extracts are separated by centrifugation, and the cell wall is spray dried.

Diet preparation in this study was after Akrami et al. [28]. Diets were prepared weekly, by diluting the appropriate amount of Agrimos® MOS in distilled water and gently mixing it with the crushed BioMar food to make a paste that was then spread on a plastic sheet, air dried, slightly ground and sieved to produce a suitable crumble size of 800 μm. The same procedure was used to prepare the control diet, except that no prebiotic was added.

### 2.3. Experimental design

In a pre-test experiment, among the concentrations of 0, 0.2, 0.4, 0.6 and 0.8% MOS/kg diet for 6 weeks, we found that inclusion of 0.4% Agrimos® MOS results in higher final body weight, food intake, and specific growth rate of juvenile zebrafish [29]. Accordingly, we developed four treatments to assess the stress response of the subjects under starvation conditions:

i) normal control (CN): fed daily with the control diet,
ii) starved control (CS): fed every other day with the control diet,
iii) normal prebiotic (PN): fed daily with prebiotic supplemented diet (0.4%),
iv) starved prebiotic (PS): fed every other day with the prebiotic supplemented diet (0.4%).

Fish with an initial body-weight of 43.27–51.83 mg (One-way ANOVA: $p > 0.05$) were distributed into 18 L glass rectangular tanks (30 cm × 20 cm × 25 cm water height). Each treatment contained 25 juvenile fish in triplicate (75 fish per treatment). The duration of feeding period was 8 weeks, as proposed by Torrecillas et al. [24]. Subjects were fed twice daily (3% body weight) at 1000 h and 1600 h throughout the experiment, and the laboratory conditions were similar to what the fish experienced during the acclimation period (water temperature: 27 ± 1 °C, photoperiod: 14 h light: 10 h dark cycle, dissolved $O_2$: 6.9–8.2 mg/L; pH: 7.3–7.7; hardness: 150–180 mg/L as $CaCO_3$.).

### 2.4. Intestinal microbiota analysis

Autochthonous lactic acid bacteria (LAB) levels were determined at the start and end of the trial of 9 fish per treatment (3 per replicate). Samples preparation was performed after Hoseinifar et al. [30] and the resulted homogenates were spread in triplicate onto deMan, Rogosa and Sharpe (MRS) agar media (Merck, Germany). For 5 days, plates were incubated at 25 °C, and colony forming units (CFU/g) were calculated from statistically viable plates (i.e. plates containing 30–300 colonies; [30]).

### 2.5. Behavioral assay

Anxiety-like behaviors of 8 fish in each treatment (2–3 per replicate) were assessed a day after the eight weeks of the feeding trials, i.e. on

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