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Original Article

The effects of starvation on some epidermal mucus immune parameters in rainbow trout, *Oncorhynchus mykiss*

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Abstract: The skin of a fish acts as the primary protective agent against biological, physical, and chemical stress. However, the effects of such stressors on fish mucosal immune responses have been hardly investigated. Fasting or feed deprivation commonly is occurred in aquaculture due to season, production policies, or disease. This research was aimed to investigate the impacts of 20-day starvation on skin mucosal immune responses of rainbow trout. The results revealed that the enzymatic activities of lysozyme (LZM) and alkaline phosphatase (ALP), as well as the total immunoglobulins (Ig) level and bactericidal activities were significantly reduced in the skin mucus of fasted fish. No significant changes were observed in the esterase and protease activities. Bactericidal activity in the mucus of starved fish was significantly lower than control group after 20 days. Therefore, it could be strongly suggested that this species should not remain under starvation stress as this kind of stress impairs mucosal immune barriers which, in turn, could make the fish more susceptible to infections or harmful agents.

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Introduction

Fasting or feed deprivation is a normal occurrence that many fish species may experience in their natural environments during migration, reproduction, and due to some adverse conditions in the environment, like red tides or cold-water masses in the summer (Cho et al., 2006; Sridee and Boonanuntanasarn, 2012; Park et al., 2012). Furthermore, feed deprivation can be used as a managing approach to decrease mortality due to disease occurrence and overproduction (Shoemaker et al., 2003) or to reduce water quality complications and decrease stress management (Davis and Gaylord, 2011). Nevertheless, in several previous researches, starvation was perceived as a stressor (Furné et al., 2009; Langer et al., 2014; Varga et al., 2014; Piccinetti et al., 2014; Arslan et al., 2015).

Unlike mammals, fish could tolerate long periods of fasting. However, extended starvation exhausts the energy reserves of the fish (Rundles, 2008) and depress their immune system. As a result, the fish become predisposed to the infectious diseases (Raina and Sachar, 2014). In fact, study of the immune defense mechanisms is of main importance for assessing fish health since a direct correlation between the functioning of the immune system and the ability to prevent disease outbreaks has been recognized (Hoseini and Ghelichpour, 2013; Hoseini et al., 2014; Hoseini et al., 2019; de Souza e Silva et al., 2019; Gholizadeh Zare Tavana et al., 2019; Foysal et al., 2020;).

The skin mucus of fish acts as the first line of defense against stressors (Jung et al., 2012; Guardiola et al., 2015; Tacchi et al., 2015; Khansari et al., 2018). Little is known regarding how the skin mucosal immune system operates in different species of fish. Fish skin mucus is composed of water, mucin, complement, C-reactive proteins, lectins, cathepsins, defensins, haemolysins, immunoglobulin M (IgM), agglutinin, proteolytic enzymes, antimicrobial peptides, acid and alkaline phosphatases, superoxide dismutase, and Lysozyme, act as inhibitory agents against various infections (Ingram, 1980; Subramanian et al., 2007; Esteban, 2012; Guardiola et al., 2014).

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Recently, numerous studies have revealed that some factors that cause stress, such as infectious agents, environmental pollutants, prolonged transport stress, and over-crowding stress can change the complete structural and cellular composition in skin of *Oncorhynchus mykiss*, *Sparus aurata*, and *Puntius tetrazona* (Lindenstrøm et al., 2004; Tacchi et al., 2015; Guardiola et al., 2015; Roosta and Hosseinifar, 2016). Furthermore, different fish species undergo some changes in their mucus structure and immune responses, following any environmental and physiological changes (Guardiola et al., 2014). Hoseini et al. (2014) revealed the serum levels of T4, T3, cortisol, glucose, lactate, triglyceride and cholesterol were significantly affected by fasting period in rainbow trout; however, there were no significant changes in serum total protein, albumin, globulin or A:G among the fish fasted 0-72 h (Hoseini et al., 2014).

The same results showed by Hoseini et al. (2019) on common carp. Three days of fasting led to a significant decrease in serum levels of lactate and thyroid hormones. Fourteen days of fasting led to initiation of physiological process to maintain serum glucose by increase in serum cortisol level and lactate utilization. These fish had lower levels of thyroid hormones suggesting suppression of basal metabolism. The fish also had lower WBC and serum lysozyme and total Ig levels indicating immunosuppression. Therefore, this research was aimed to study some immune system alteration in the skin mucus of rainbow trout following a short-term feed deprivation.

Materials and Methods

In accordance with the National Ethical Framework for Animal Research in Iran, rainbow trout was chosen for the experimentation in this study (Mobasher et al., 2008). Specimens with a body weight of 22.6±4.6 g, were selected from a local fish farm, Shiraz, Iran. In order to adaptation, fish kept in 250 L fiberglass tanks for three weeks. During this period, the water was recirculated, fish were exposed to natural photoperiod, and water temperature, dissolved oxygen, and pH were 13.2±1.8ºC, 6.2±0.9 ppm and 7.5±0.2, respectively. Fish fed twice a day with commercial pellets (Beiza Co, Iran) as 2% of their body weight.

After acclimation, fish were divided into two groups in triplicates, one maintained at starvation and the other was fed daily with the same diet to apparent satiation twice daily, as a control. The experiment lasted 20 days (Tran et al., 2018), during which 6 specimens from both the control and fasted groups were sampled at time zero (before starvation) and on day 20 after the start of the starvation for the measurement of selected skin immune parameters. In both treatments (control and starved fish), before sampling, the fish were first anesthetized by 0.1 ppm MS222 (Argent Laboratories Inc., Redmond, WA, USA). Then, a sterile plastic spatula, with enough caution to prevent contamination with blood, intestinal and urogenital secretions, was used to scratch the skin mucus from the dorso-lateral surfaces (Palaksha et al., 2008). Once the mucus was collected, an overdose (100 ppm) of MS222 was used to euthanize the fish. Mucus samples were homogenized using one volume of sterile normal saline (9‰). Afterward, the samples were centrifuged at 2000 g for 10 min at 4°C. The supernatant was stored at -80°C for further analysis.

Skin mucus total immunoglobulin: The method described by Siwiki and Anderson (1993) was used to measure the total immunoglobulins (total Ig). First, Ig was precipitated in serum using a 12% polyethylene glycol solution and remaining protein in the supernatant was assayed by Bradford method (Bradford, 1979) and it was subtracted from the total protein to give total Ig content.

Lysozyme activity: The turbidimetric assay was applied to measure the Lysozyme activity (Demers and Bayne, 1997). Briefly, 50 µL suspension of bacterium *Micrococcus luteus* (Sigma) (0.3 mg/mL of lyophilized cells dissolved in 40 mM sodium phosphate buffer, pH 6.5) and 50 µL of the mucus sample were mixed together. The incubation of the mixture was done at 30°C; then, after 0 and 15 min, the reduction in the absorbance at 450 nm was measured in a microplate reader (BioTek ELx 808

instrument, USA). The quantity of enzyme that caused a decline in the absorbance of 0.001 per minute was defined as a unit of lysozyme activity.

Alkaline phosphatase activity: Using the method of Palaksha et al. (2008), the measurement of the activity of alkaline phosphatase was done after the incubation of the mucus supernatants with 4 mM para-nitrophenyl phosphate (Sigma) in 100 mM ammonium bicarbonate buffer which contained 1 mM magnesium chloride, pH 7.8 at 30°C. The quantity of enzyme that freed 1 mmol of para-nitrophenyl product in 1 min was defined as one unit of activity.

Protease activity: Utilizing the method offered by Palaksha et al. (2008), the activity of protease was determined by azocasein hydrolysis assay. 50 µL of the mucus sample re-suspended in 100 mM ammonium bicarbonate, pH 7.8, with 50 µL azocasein substrate 0.25% (w/v) in the same buffer for 19 h at 30°C were incubated for assaying azocasein hydrolysis. Fifty µl of 20% (w/v) trichloroacetic acid was added to the process to bring the reaction to a halt. This was followed by a 5 min centrifugation at 15400 g (Equal volumes of supernatant (100 µL)). Then, 0.5 M NaOH was added to a 96 well plate and the absorbance was evaluated at 405 nm. The quantity of the enzyme that triggered a variation in the absorbance of 0.001/min was defined as one unit of activity. Each unit of activity was determined per mg of protein (specific activity).

Esterase activity: Based on Palaksha et al. (2008), when mucus supernatants were incubated with 0.4 mM para-nitrophenyl myristate in 100 mM ammonium bicarbonate buffer containing 0.5% Triton X-100, pH7.8 at 30°C; then, the detection of esterase activity was processed. A plate reader was used to keep a constant record of the absorbance for 2 h at 405 nm. The amount of enzyme needed to set free 1 mmol of para-nitrophenyl product in1 min was defined as the activity.

Antibacterial assay: Before the samples of mucus were analyzed, they were defrosted at room temperature. *In vitro* bactericidal activity of mucus samples were examined against *Aeromonas hydrophila, Yersinia ruckeri*, and *Lactococcus garviae*, which had been taken from the stock culture kept at the Microbiology Laboratory of the Aquatic Animal Health and Diseases Department, School of Veterinary Medicine, Shiraz University. The standard disc diffusion method was used to detect the antibacterial activity of the mucus samples (Bauer et al., 1966). At first, different species of bacteria of interest to the present study were cultured in nutrient broth medium for 24 h at 25ºC; then, 0.1 mL of broth culture medium (contains 1.5×10^8 CFU/ mL) was cultivated on Mueller Hinton agar. Aseptic paper discs with a 6 mm diameter were soaked with 200 µL of mucus sample and put on the medium. The incubation was performed at 25ºC for 24 h. Following that, the discs were checked and a ruler was used to calculate the growth inhibition area diameter in mm (minus the diameter of paper discs). The clear part which surrounded the discs was considered as an indication of the antibacterial activity.

Statistical analysis: Immunological data and antibacterial activity were analyzed using a two-sample t-test. Each data set was checked for normality using a Shapiro-Wilk test. Non-normal data were transformed for statistical comparison. All the statistical analyses were performed using SPSS software (version 16.0, Chicago, IL) and the probability of $P<0.05$ was considered statistically significant. Data are presented as mean±SD.

Results

All biomarkers were measured before the final experiment to ensure that the individual conditions of the fish tested were uniform (at 0 days). The results showed that there was no significant difference between the fish assigned to the different experimental groups (Table 1). There was no case of fish death throughout the experiment in spite of the feed deprivation. Mean±SD of the epidermal immune factors observed in the sample fish are tabulated in Figures 1. A significant reduction in the enzymatic activities of LZM (Fig. 1) and ALP (Fig. 2), as well as total Ig level (Fig. 3), was observed in the skin mucus of the starved fish compared to those measured in the fed ones ($P<0.05$). Similar results were found for skin

Table 1. Immunological and antibacterial results before the start of the final experiment.

Biomarkers	Control	Starved fish
Lysozyme	29.67±1.86	30.17±3.31
Alkaline phosphatase	8.17±1.60	9.67±1.03
Esterase	2.22±0.34	2.23±0.28
Protease	20.67±2.73	21.33±2.66
Total Immunoglobulins	5.87±0.37	6.25±0.41
Yersinia ruckeri	5.17±1.72	7.17±1.32
Aeromonas hydrophila	9.83±2.14	11±2.10
Lactococcus garviae	7.00±1.79	5.83±2.14

Table 2. Antibacterial activity of epidermal mucus in rainbow trout 20 days after subjection to starvation.

	20 days	
Bacteria	**Control**	**Starved fish**
A. hydrophila	8.8 ± 2.0^{b}	4.0 ± 2.2^{a}
Y. ruckeri	6.0 ± 1.4^{bc}	2.5 ± 1.6^{a}
L. garviae	7.7 ± 1.6^{b}	3.7 ± 2.0^{a}

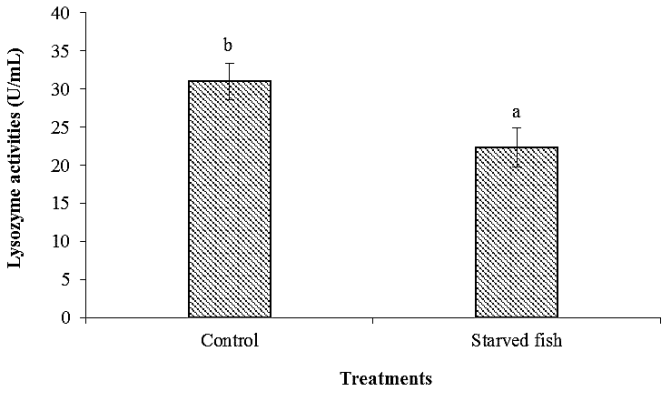

Figure 1. Alterations in skin mucus lysozyme activities of rainbow trout 20 days after subjection to starvation. (Mean±SD, n=6). Different letter notations indicate significant differences at $P<0.05$.

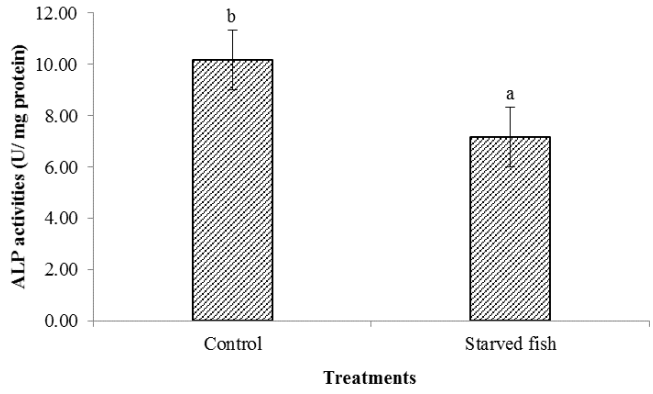

Figure 2. Alterations in skin mucus alkaline phosphatase activities of rainbow trout 20 days after subjection to starvation. (Mean±SD, n=6). Different letter notations indicate significant differences at $P<0.05$.

mucus bactericidal activities against selected bacterial pathogens (Table 2). However, the protease and esterase activities did not show any differences after exposure to starvation (Figs. 4, 5).

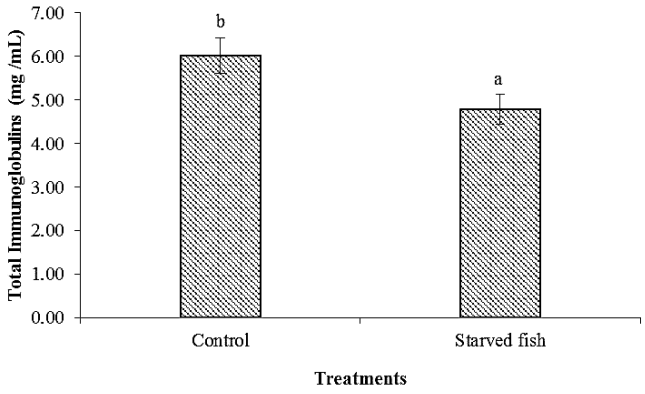

Figure 3. Alterations in skin mucus total immunoglobulin levels of rainbow trout 20 days after subjection to starvation. (Mean±SD, n=6). Different letter notations indicate significant differences at $P<0.05$.

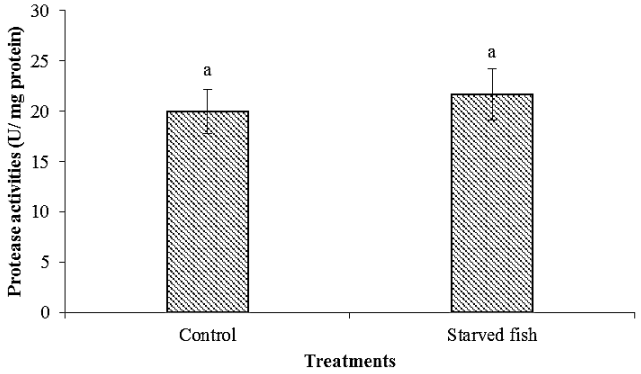

Figure 4. Alterations in skin mucus protease activities of rainbow trout 20 days after subjection to starvation. (Mean±SD, n=6). Different letter notations indicate significant differences at $P<0.05$.

Discussions

Although it is clear that nutritional disturbance can interfere with the resistance to pathogens in

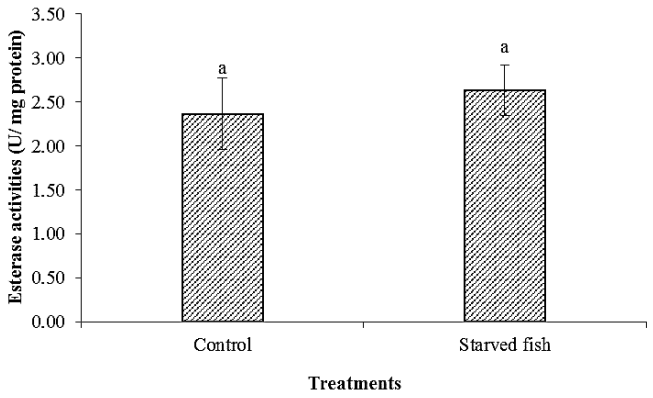

Figure 5. Alterations in skin mucus esterase activities of rainbow trout before and 20 days after subjection to starvation. (Mean±SD, n=6). Different letter notations indicate significant differences at $P<0.05$.

domesticated animals (Klasing, 1998), the mechanisms by which nutritional variations modulate the fish immune system are largely unidentified. On the other hand, the significance of the mucosal surfaces, as the first defending position and the initial target in an attack by infectious agents, has been increasingly highlighted (Rombout et al., 2011; Esteban, 2012). Nevertheless, the vulnerability of the mucosal defensive system to alterations in nutrients has just recently been noticed in fish species (Vieira et al., 2011; Landeira-Dabarca et al., 2013).

Different enzymes with recognized functions in the immune reactions have been shown in numerous species of fish as they were noticed in the present research. Of these enzymes, ALP in mucus has been reported to function as a factor with antibacterial properties because it is an enzyme involved in hydrolysis (Ross et al., 2000). Also, it has been reported that ALP plays a pivotal role in the primary phases of wound healing (Rai and Mittal, 1991; Iger and Abraham, 1990, 1997) and against stress (Iger and Abraham, 1990, 1997).

No available data were found regarding the effects of starvation on mucosal ALP activity in fish species. Our findings pointed to a significant reduction in the mucosal activity of ALP in the fasted fish. Similar results were found in turbot (*Scophthalmus maximus*) reared in high density for 120 days (Jia et al., 2016). However, despite to the results of the present work, it has been shown that some stressors namely high stocking densities (Roosta and Hosseinifar., 2016), aluminum acidity (Ledy et al., 2003), and hypoxia (Vatsos et al., 2010), significantly enhanced the ALP activity in the mucus of the tiger barbs (*Pentius tetrazona*), brown trouts (*Salmo truta fario*), and sea bass (*Dicentrarchus labrax*), respectively. Such alteration in the activity of ALP could be used as an indication of stress in some circumstances (Ross et al., 2000), for instance in the conditions tested in the current work. However, further studies are required to approve this hypothesis. Since ALP activity is dependent on maintaining the homeostasis of ions, starvation may affect ALP activity by reducing the level of uptake of ions such as calcium.

Proteases and esterase were also measured in the current work because both of them have been linked with skin immune responses and defense against microbial infections (Esteban, 2012). In skin mucus, proteases can act as a defense means against infectious agents both directly, via splitting their proteins (Subramanian et al., 2007), and indirectly, via hindering their colonization and invasion mechanisms (Aranishi et al., 1998). Furthermore, proteases may activate other immune parameters such as complement, immunoglobulins or antibacterial peptides (Hjelmeland et al., 1983; Fernandes and Smith., 2002; Kennedy et al., 2009). Some studies have documented the alterations that take place in the mucus proteases in the face of stress (Aranishi and Nakane., 1997; Aranishi et al., 1999; Easy and Ross., 2010) and infection (Ross et al., 2000; Aranishi and Mano, 2000) and pointed their importance in mucosal immunity. However, our data showed no changes in the fish epidermal proteases activities following 20 days of starvation. In a similar way, Jia et al. (2016) found no changes in protease activity in the epidermal mucus of turbots (*Scophthalmus maximus*) exposed to crowding stress.

Furthermore, in the current research, the effects of feed deprivation on esterase level in the skin mucus were examined. Even though the function of the esterase in fish mucus is not known, it is known that it can function separately or in collaboration with other mucosal immune components defending against the

infectious agents (Sheikhzadeh et al., 2012). Previously, increased esterase activity was demonstrated in gilthead sea bream fish (*Sparus aurata*) following waterborne exposure to some heavy metals (Guardiola et al., 2015). Conversely, a significant reduction in the esterase activity was reported in the skin mucus of turbots reared in high-density conditions (Jia et al., 2016). However, in the current work, no change in the esterase activity was found in the mucus of the starved fish.

In addition, the total Ig was measured in the present research. Immunoglobulins have crucial roles to play in defending the fish from serious infectious agents and conserving the mucosal homeostasis (Esteban, 2012). IgM and teleost polymeric immunoglobulin receptor (pIgR) found locally in the skin play an essential role in the mucosal immunity of fish (Esteban, 2012). Our data revealed that total Ig took a remarkable reduction in the epidermal mucus of the fasted fish. Hormonal changes during starvation may affect the production of immunoglobulins. Furthermore, starvation can affect the stimulation of B cells and the secretion of T cell-derived cytokines (Ota et al., 2016). Interestingly, the value of the Ig measured in the skin mucus of the sea bream (*Sparus aurata*) which had been exposed to heavy metals reduced on the second day of exposure; however, it elevated later (Guardiola et al., 2015). Nevertheless, no change in the serum IgM values was recorded after starvation in either European sea bass (*Dicentrarchus labrax*) (Caruso et al., 2011) or *Mesopotamichthys sharpeyi* (Najafi et al., 2015).

In fish immunity studies, serum lysozyme activity has been mainly evaluated (Ellis, 2001), yet the lysozyme functions in the mucosal surfaces have received less attention comparatively (Bergsson et al., 2005; Nigam et al., 2012). For supporting the importance of lysozyme for disease resistance, in a study carried out by Yazawa et al. (2006), they generated a transgenic zebrafish (*Danio rerio*) strain expressing a chicken lysozyme gene and the results showed that the transgenic strain survived 65% when confronted with *Flexibacter columnare* while the wild-type fish survived 0% when challenged with the same bacterial agent.

We revealed that lysozyme content in the skin mucus significantly decreased after starvation. Likewise, the mucus lysozyme activity obviously decreased in turbots raised in high-density condition (Jia et al., 2016). Similarly, in blackspot sea bream (*Pagellus bogaraveo*), which had been starved for 31 days, a significant reduction was found in the lysozyme activity of their skin mucus. However, the activity of lysozyme was doubled in the skin mucus of European seabass, *Dicentrarchus labrax* reared at the same condition (Caruso et al., 2011). It seems that the difference in the mucosal activity of lysozyme could be associated with many factors such as season (Schrock et al., 2001), type of species, stress management, gene mutation, diet, maturity, sex (Balfry and Iwama., 2004; Caruso et al., 2011), and even epidermis thickness and the number of the mucous cells (Subramanian et al., 2007).

Regardless the effector components and the mechanisms participating in the bacterial killing, the assessment of bactericidal activity of skin mucus could be more important in practice than single enzymatic activities (Guardiola et al., 2014). Several studies have shown that the skin mucus of some fish species presented a strong antibacterial and antifungal activity against a wide range of microbial pathogens (Hellio et al., 2002; Subramanian et al., 2008; Dhanaraj et al., 2009). In a study, one-week starvation in channel catfish significantly increased the mortality to *F. columnare* which normally penetrates through surface mucosa (Liu et al., 2013).

The results of the present study showed that when the sample fish were exposed to starvation, some antimicrobial activities started to operate against specific infectious agents and a remarkable reduction was observed in the antibacterial activity. Our results conflict with those obtained by Roosta and Hoseinifar (2016) who found the elevation of antibacterial activity in the skin mucus of the tiger barbs (*Pentius tetrazona*) subjected to crowding stress. In addition, the exposure of sea bream to heavy metals caused a significant increase in their skin mucus bactericidal activity (Guardiola et al., 2015).

In conclusion, the current work demonstrated a converse relationship between starvation and immune response in the skin mucus of rainbow trout. The enzymatic activities of LZM, ALP as well as Ig level and bactericidal activity were significantly reduced in the skin mucus of fish after 20 days of starvation. Moreover, the dramatic variations observed between the findings of the present study and those of others could possibly be attributed to factors such as season, length of feed deprivation, evolutionary adaptation to starvation, and pathogen dynamics (prevalence, routes of transmission, and so on). The present findings suggest the important role of nutrition in maintaining the immunological functioning of the skin mucus in the rainbow trout. Thus, it is strongly recommended that this fish species should not remain under starvation stress since this kind of stress can impair the mucosal immune barriers which, in turn, could make the fish more susceptible to infections or harmful agents.

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