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Original Article

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

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Abstract: Fish epidermis functions as the first defense barrier against physical, chemical, and biological stressors. However, the effects of hand-stripping on fish mucosal immune responses have been hardly investigated. The present study investigated the effects of stripping procedures on skin mucosal immune responses of rainbow trout (*Oncorhynchus mykiss*) breeders. The skin mucus was sampled from six male and six female trout before and one week after the stripping handling. The results showed that stripping had significant effect on all parameters except protease activity, but gender had a significant effect only on the mucosal protease and alkaline phosphatase activity. The results did not show any effect of the interaction between stripping and gender on mucosal lysozyme activity. The data revealed that enzymatic activities of alkaline phosphatase, lysozyme, esterase, as well as the total immunoglobulin level and bactericidal activity were significantly reduced in the skin mucus of fish one week after the stripping. The reduction of immune parameters in the skin mucus could be related to immunosuppression caused by stripping stress which, in turn, might have made the fish more susceptible to microbial infections and diseases. Therefore, care should be taken during stripping to minimize the manipulation stress.

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Introduction

Fish are in continuous interaction with different challenges, such as variations in water quality (*e.g.* dissolved oxygen, temperature, salinity, etc.), culture conditions (*e.g.* feed supply and fish population density), and regular handling (*e.g.* transportation and grading) (Guardiola et al., 2016). Generally, breeders are highly sensitive to stress due to manipulation during breeding. Fish catches, air exposure, transportation, hormone therapy, confinement, and hand-stripping can suppress the fish's immune system. Several studies have been performed on the effects of stressors on mucosal parameters in different fish species (Vatsos et al., 2010; Tacchi et al., 2015; Guardiola et al., 2015).

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; Parra et al., 2015; Khansari et al., 2018). Fish skin mucus is composed of several immune and antimicrobial molecules, such as lysozymes, immunoglobulins, complements, lectins, and antimicrobial peptides (Ingram, 1980; Subramanian et al., 2007; Esteban, 2012; Guardiola et al., 2014; Salinas, 2015). The structure and cellular composition of fish skin are affected by various stressors, such as environmental contaminants, pathogens, transportation, and crowding density (Lindenstrøm et al., 2004; Tacchi et al., 2015; Guardiola et al., 2015; Roosta and Hosseinifar, 2016). Moreover, the mucus composition and immune functions change with the fish species, alternation in the environment, and the fish's physiology (Guardiola et al., 2014). While the fish skin plays a vital role in mucosal immune reactions, there are no available data on the changes to epidermal immune parameters under stripping conditions. Although most fish species show a generalized stress reaction in mucosal membranes, the pattern and scale of the response may be influenced not only by environmental factors (such as temperature and salinity) but also by the nature of

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Table 1. The weights of male and female rainbow trout brooders.

Brooder's weight immediately after stripping (g)		Brooder's weight one week after stripping (g)	
male	female	male	female
673	695	714	734
782	806	826	846
864	886	912	934
976	997	1182	1203
1163	1185	1268	1291
1282	1305	1337	1358

stressors and specific evolutionary life stories (Khansari et al., 2017, 2018). Therefore, the present study aimed to evaluate the effects of stripping manipulation on some immune parameters in the skin mucus of rainbow trout breeders.

Materials and Methods

Experimental fish and mucus collection: The trial was conducted during the spawning season at a private rainbow trout hatchery farm in Fars Province, southwestern Iran. During the sampling, water temperature, pH, and dissolved oxygen were monitored as 12ºC, 7.3, and 6 ppm, respectively. The broodstock (with average weight of 967.8±221.2 g) were held in raceway ponds under natural photoperiod and fed with a commercial trout diet (Beyza Technology Co. Ltd., Iran) at 2% of their body weight once a day. After being kept 24 h without feeding, six male and six female fish with different weights (Table 1) were randomly netted, individually placed in a bathtub tank, and anesthetized with clove powder (150 mg/L). Different weights were selected for an easy and stress-free individual recognition because tagging or marking techniques could be invasive and potentially add stress (Sharpe et al., 1998; Carballo et al., 2018) to the normal stripping manipulation. In this study, a factorial design (2×2) was used to evaluate the effect of stripping (fish sampled before and after stripping), and gender (male and female) on some mucosal immune parameters in the rainbow trout breeders.

The skin mucus was scraped from the dorso-lateral surface using a plastic spatula with enough caution to avoid contamination with blood and urino-genital and intestinal excretions (Palaksha et al., 2008). The mucus samples were transferred to 15 ml sterile falcon tubes. After mucus collection, the fish were hand-stripped. The collection of semen and egg samples was carried out applying massage from the anterior portion of the belly (testis or ovary region) towards the genital papilla.

After stripping, the fish were released back into the same ponds and fed for one week. Mucus sampling was repeated of the same individuals one week after stripping based on the procedure applied in the first sampling. Identical volumes of mucus samples were then homogenized using one volume of sterile normal saline (0.9%) following the method of Kuppulakshmi et al. (2008). The samples were well shaken, and then centrifuged (2000 × g (equivalent to 4226 rpm) for 10 min at 4°C, following Guardiola et al. (2014, 2016). The supernatant was kept at -80°C before use.

Skin mucus total immunoglobulin: The immune-globulin concentration was measured based on Siwicki and Anderson (1993). Immunoglobulins were precipitated with 10,000 kDa polyethylene glycol (PEG, Sigma). Mucus (100 µL) was mixed with an equal volume of 12% PEG solution for 2 h at room temperature under constant shaking. After centrifugation at 5000 g for 15 min, the supernatant was collected and the concentration of proteins was determined based on Bradford (1979). The difference in protein contents prior to and after the immunoglobulin molecules precipitation was considered as the Ig content.

Lysozyme activity: Lysozyme activity was measured based on a turbidimetric assay (Demers and Bayne, 1997). In brief, 50 µL suspension of bacterium *Micrococcus luteus* (Sigma, St Louis, MO) (0.3 mg/mL of lyophilized cells dissolved in 40 mM sodium phosphate buffer, pH 6.5) was mixed with 50 µL of the mucus sample. The mixture was then incubated at 30°C and the decline in absorbance at 450

nm was detected after 0 and 15 min in a microplate reader (BioTekELx 808 instrument, USA). A unit of lysozyme activity was defined as the quantity of enzyme that induced a reduction in the absorbance of 0.001 per minute. Enzyme activity was expressed as U mg^{-1} of protein.

Alkaline phosphatase activity: Alkaline phosphatase activity was measured over the incubation of mucus supernatants with 4 mM para-nitrophenyl phosphate (Sigma) in 100 mM ammonium bicarbonate buffer containing 1 mM magnesium chloride, pH 7.8 at 30°C, based on the method described by Palaksha et al. (2008). One unit of activity was defined as the amount of enzyme that released 1 mmol of para-nitrophenyl product in 1 min. The activity unit was expressed per mg of protein (specific activity).

Protease activity: Protease activity was determined using the azocasein hydrolysis assay as described by Palaksha et al. (2008). Azocasein hydrolysis was assayed by incubating 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. The reaction was stopped by adding 50 µl of 20% (w/v) trichloroacetic acid followed by a 5 min centrifugation at 15400 × g. Equal volumes (100 µL) of the resultant supernatant and 0.5 M NaOH were added to a 96-well plate and the absorbance was measured at 405 nm. One unit of activity was defined as the amount of enzyme that caused a change in absorbance of 0.001/min. The activity unit was expressed per mg of protein (specific activity).

Esterase activity: Esterase activity was detected following the incubation of mucus supernatants with 0.4 mM para-nitrophenyl myristate in 100 mM ammonium bicarbonate buffer containing 0.5% Triton X-100, pH7.8 at 30°C, based on the method described by Palaksha et al. (2008). The absorbance was recorded constantly for 2 h at 405 nm by a plate reader. The activity was defined as the quantity of enzyme needed to release 1 mmol of para-nitrophenyl product in1 min. Enzyme activity was expressed as U mg^{-1} of protein.

Antibacterial assay: Prior to analysis, the mucus samples were thawed in room temperature. Preliminary screening for the antimicrobial activity of mucus samples was carried out against different bacterial species, including *Aeromonas hydrophila, Yersinia ruckeri*, and *Lactococcus garviae,* which were obtained from the stock cultures maintained at the Microbiology Laboratory of the Aquatic Animal Health and Diseases Department, School of Veterinary Medicine, Shiraz University. These bacteria were selected based on their influence in causing diseases and mortalities in rainbow trout. The antimicrobial activity was studied following the slightly modified method offered by Subramanian et al. (2008). All the bacterial species were grown in Mueller-Hinton (MH) broth medium for 24 h at 25ºC. One milliliter of MH broth and one milliliter of each bacterial culture (containing 1.5×10^6 $CFUmL^{-1}$) were added into a tube containing a different concentration of mucus samples. Assays were carried out in triplicate for each mucus sample. The antimicrobial activity was confirmed by the visual inspection, absorbance at 595 nm using microplate reader (BMG Labtech, Germany). The antimicrobial activity was further confirmed by spread plating on MH agar plates. All the plates were performed in triplicate. The minimal bactericidal concentrations (MBCs) of mucus extracts were defined as the minimum mucus concentrations (μLmL^{-1}) that could cause complete inhibition of bacterial growth.

Statistical analysis: The normality of data was assessed by Shapiro-Wilk test. Then the effects of the time of stripping and sexuality on skin mucus immune parameters were analyzed through two-way full factorial analysis of variance (ANOVA). Tukey's multiple comparison tests were used for comparing the means of treatments following two-way ANOVA. All statistical analyses were tested at the 0.05 level of probability ($P<0.05$), using SPSS 16.0 for Windows. Data are presented as mean ±SD.

Results

Mean values±SD of the skin mucosal immune parameters recorded in the experimental fish are shown in Tables 2 and 3 and Figures 1 and 2. No

Table 2. Enzymatic activities of protease, esterase and alkaline phosphatase (ALP) (Unit/mg protein) found in skin mucus of male and female rainbow trout brooders before and one week after stripping manipulation. (Mean±SD, n=6). Values in the same rows showing the same superscript letters are not significantly different (P>0.05).

Parameters	Before stripping		After stripping	
	male	female	male	female
Protease activity (Unit/mg protein)	20.5±2.5[a]	21.8±2.4[a]	19.5±1.5[a]	22.2±2.1[a]
Esterase activity (Unit/mg protein)	3.00±0.35[b]	3.15±0.40[b]	2.23±0.34[a]	2.20±0.30[a]
ALP activity (Unit/mg protein)	11.8±1.7[b]	13.6±1.7[b]	8.5±1.4[a]	9.9±1.3[a]

Table 3. Minimum bactericidal concentration (MBC) (µL/mL) found in skin mucus of male and female rainbow trout brooders against selected bacterial pathogens before and one week after stripping manipulation (Mean±SD, n=6). MBC was measured using broth dilution method. Values in the same rows showing the same superscript letters are not significantly different (P>0.05).

Bacteria	Before stripping		After stripping	
	male	female	male	female
A. hydrophila	225±52[a]	200±45[a]	358±74[b]	325±52[b]
Y. ruckeri	208±38[a]	200±55[a]	317±52[b]	300±45[b]
L. garviae	275±27[a]	250±45[a]	392±38[b]	375±69[b]

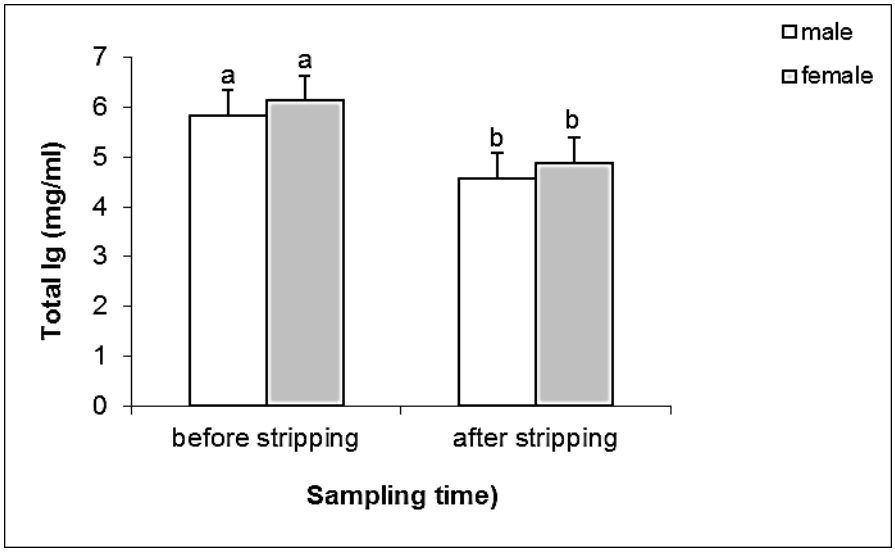

Figure 1. Alterations in skin mucus total immunoglobulin of rainbow trout brooders before and one week after stripping manipulation. (Mean ± SD, n=6). Different letter notations indicate significant differences at P<0.05.

significant difference was noted in the skin mucosal immune parameters between male and female fish either before or after stripping, except for lysozyme activity that showed significantly higher values (P<0.05) in female before and after stripping (Fig. 2). However, the results did not show any effect of gender alone or any effect of the interaction between stripping and gender on mucosal lysozyme activity (Table 4). The skin mucus lysozyme activity significantly decreased one week after the stripping in male and female fish.

The results also showed that gender had a significant effect on the mucosal protease activity. However, there was no significant change in the activity of protease under stress induced by stripping. Although the effects of stripping and gender alone on the activity of mucosal alkaline phosphatase were significant, the results showed that the interaction between these two factors had no significant effects on the activity of ALP (Table 4).

The enzymatic activity of esterase, as well as the level of total Ig, was significantly reduced in the skin mucus of fish one week after the stripping (P<0.05). Similar results were found for skin mucus bactericidal

Table 4. Statistical significances of immune modulations; Results of two-way ANOVA performed on skin mucus immune parameters considering male and female rainbow trout brooders before and one week after stripping manipulation. Differences were considered significant for $P>0.05$.

Parameters	Factors	Sig
Lysozyme activity (Unit/mg)	Stripping	<0.0001
	Sex	0.347
	Stripping × Sex	0.949
Total Ig (mg/ml)	Stripping	<0.0001
	Sex	0.224
	Stripping × Sex	0.851
ALP activity (Unit/mg protein)	Stripping	<0.0001
	Sex	<0.05
	Stripping × Sex	0.672
Esterase activity (Unit/mg protein)	Stripping	<0.0001
	Sex	0.691
	Stripping × Sex	0.533
Protease activity (Unit/mg protein)	Stripping	0.711
	Sex	<0.05
	Stripping × Sex	0.462
MBC (µl/ml) (*Yersinia ruckeri*)	Stripping	<0.0001
	Sex	0.528
	Stripping × Sex	0.833
MBC (µl/ml) (*Aeromonas hydrophila*)	Stripping	<0.0001
	Sex	0.223
	Stripping × Sex	0.859
MBC (µl/ml) (*Lactococcus garviae*)	Stripping	<0.0001
	Sex	0.293
	Stripping × Sex	0.831

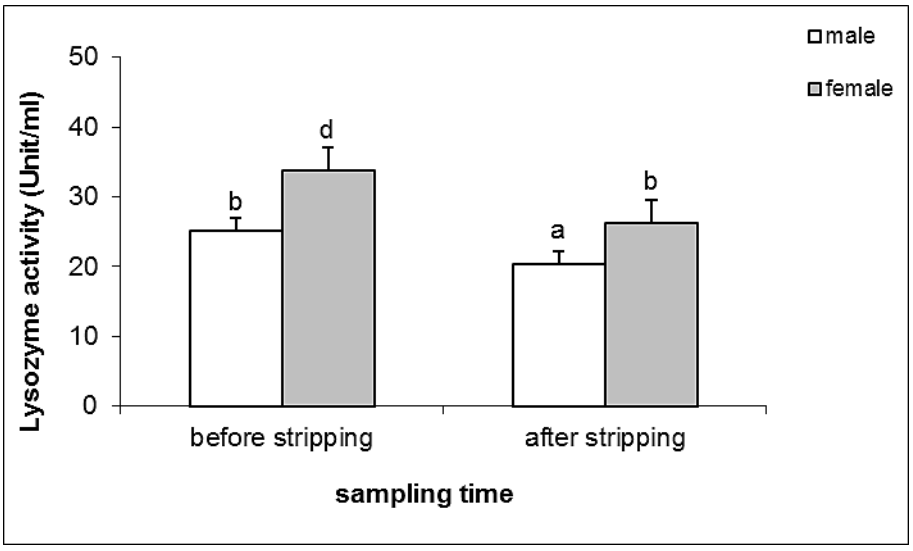

Figure 2. Alterations in skin mucus lysozyme activity of rainbow trout brooders before and one week after stripping manipulation. (Mean ± SD, n=6). Different letter notations indicate significant differences at $P<0.05$.

activities against selected bacterial pathogens (Table 3). The results revealed that neither gender nor the interaction between stripping and gender had a significant effect on total Ig level, skin mucus esterase, and bactericidal activities (Table 4).

Discussions

There is some evidence that the mucus composition

varies with stress, disease, and parasite attack (Schrock et al., 2001; Mustafa, 2005; Easy and Ross, 2010; Lü et al., 2012). The skin mucus comprises different biologically active molecules such as complement factors, hydrolytic enzymes (e.g., lysozyme, cathepsin B, proteases), immunoglobulins, lectins, interferons and antimicrobial peptides which plays a vital role in mucosal immune reactions (Böckelmann et al., 2010; Huang et al., 2011; Esteban, 2012; Khansari et al., 2018). Different enzymes with recognized functions in the immune reactions have been identified in numerous fish species, a finding which was also noticed in our study. Among them, ALP in mucus has been reported to act as an antibacterial factor because of its hydrolytic activity (Ross et al., 2000). Also, it has been reported that ALP has a protective role both in the initial stages of wound healing (Iger and Abraham, 1990, 1997; Rai and Mittal, 1991) and against stress (Iger and Abraham, 1990, 1997). Our results revealed a significant reduction in the ALP activity in the mucus of fish one week after exposure to stripping manipulation. In addition, a gender effect on ALP activity was noticed that might be associated with different levels of sex hormones in male and female fish (Thongprajukaew and Kovitvadhi, 2013; Dash et al., 2018; Reverter et al., 2018). Similar results were found in turbot (*Scophthalmus maximus*) reared in high density for 120 days (Jia et al., 2016). However, despite the results of the present study, it has been shown that some stressors, such as high stocking densities (Roosta and Hosseinifar, 2016), hypoxia (Vatsos et al., 2010), and aluminum exposure at lower pH (Ledy et al., 2003) could significantly enhance mucosal ALP activity in tiger barbs (*Puntigrus tetrazona*), sea bass (*Dicentrarchus labrax*), and brown trout (*Salmo trutta fario*), respectively. Moreover, a significant variation in ALP activity was reported in the skin mucus of gilthead sea bream (*Sparus aurata*) depending on the waterborne metal tested and the exposure time (Guardiola et al., 2015). Such alteration in ALP activity could be used as a stress indicator in some circumstances (Ross et al., 2000).

Proteases and esterase were measured in the current work because both of them have been linked with skin immune response and defense against microbial infections (Esteban, 2012). In skin mucus, proteases may play a defensive role against pathogens by direct way i.e. splitting their proteins (Subramanian et al., 2007), and indirect ways i.e. hindering their colonization and invasion mechanisms (Aranishi et al., 1998). Furthermore, the 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 describing changes in mucus proteases following stress (Aranishi and Nakane, 1997; Aranishi et al., 1999; Easy and Ross, 2010) and infection (Ross et al., 2000; Aranishi and Mano, 2000) pointed out its importance in mucosal immunity. In the current study, no significant change was noticed on mucosal protease activity following the stripping, although a gender effect was detected. The effect of gender on mucosal protease activity may be due to the difference between the level of proteases and antiproteases in the mucus of male and female trout. Hormonal changes, especially for sex hormones during the reproductive season, may have a significant effect on the activity of mucus protease (Thongprajukaew and Kovitvadhi, 2013; Dash et al., 2018; Reverter et al., 2018). Likewise, Jia et al. (2016) found no changes in the protease activity in epidermal mucus of turbot exposed to crowding stress. It seems that the protease level in the skin mucus differs depending on the specific stressor conditions (Guardiola et al., 2016). Although the function of esterase in fish mucus is not known, it could act separately or in collaboration with other immune components in the mucus in defending against pathogens (Sheikhzadeh et al., 2012). Previously, increased esterase activity was demonstrated in gilthead seabream following waterborne exposure to mercury, arsenic, and cadmium (Guardiola et al., 2015). In the current work, the esterase activity reduced in the mucus of fish after exposure to stripping manipulation, which is consistent with a previous report in the epidermal mucus of turbot cultured in high-density conditions

(Jia et al., 2016).

Total Ig remarkably decreased in the epidermal mucus of fish after stripping procedures. Contrary to the usual expectation, the level of Ig measured in the skin mucus of fish exposed to heavy metals was reduced on day 2 of exposure; however, it increased afterward (Guardiola et al., 2015). Studies conducted pertinent to fish immunity has primarily evaluated serum lysozyme activity (Ellis, 2001), but, comparatively, less attention has been paid to the function of lysozyme in the mucosal surfaces (Bergsson et al., 2005; Nigam et al., 2012). In the current study, the female brooders exhibited higher lysozyme activity in their skin mucus. The results are in agreement with the findings of Ghafoori et al. (2014), who reported a significant higher lysozyme level in the skin mucus of female Caspian kutum (*Rutilus kutum*). Studnika et al. (1986) observed an increase in the lysozyme level in the spawning stage of the common carp during the development of oocyte. In another study, lysozyme activity in the serum of female tilapia increased with the development of oocyte, especially during the vitellogenesis (Takemura et al., 1995). In the spawning stage, the lysozyme trend shows an exponential increase, particularly in the skin, due to its involvement in overcoming probable pathogenic factors (Ghafoori et al., 2014). The mucus lysozyme activity diminished in turbot raised in a high-density condition (Jia et al., 2016). Similarly, in blackspot seabream (*Pagellus bogaraveo*), a significant decrease in lysozyme values was found in the mucus subjected to a 31-day starvation (Caruso et al., 2011). However, in the Atlantic salmon (*Salmo salar*), short-term stress did not cause any significant changes in the mucus lysozyme activity 3 and 24 h post-stress (Easy and Ross, 2010). It seems that the discrepancy in the mucus lysozyme activity could be connected to several factors such as season (Schrock et al., 2001), species and genetic variations, sex, maturity, diet, stress handling (Balfry and Iwama, 2004; Caruso et al., 2011), and even the thickness of the epidermis and the number of mucus cells (Subramanian et al., 2007).

Regardless of the effector components and the mechanisms involved in bacterial killing, the assessment of the bactericidal activity of skin mucus could be more important in practice than the enzymatic activities alone (Guardiola et al., 2014). Several studies have shown that the skin mucus of some fish species presents 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 the present study, a remarkable decrease in the bactericidal activity was detected in the mucus of fish subjected to stripping manipulation. Our results conflict with those of Roosta and Hosseinifar (2016), who found the elevation of antibacterial activity in the skin mucus of tiger barbs subjected to crowding stress. Moreover, a significant increase in the bactericidal activity of the skin mucus of seabream was observed following exposure to heavy metals (Guardiola et al., 2015).

The present study demonstrated a negative relationship between stripping handling and the immune response in the skin mucus of rainbow trout breeders. The enzymatic activities of LZM, ALP, esterase, Ig level and bactericidal activity were significantly reduced in the skin mucus one week after exposure to stripping manipulation. The reduction of the immune parameters in the mucus might suggest immunosuppression due to the stripping stress which, in turn, might have made the fish more susceptible to infections and diseases. Overall, the dramatic variations observed between the results of the current study with those of others can be ascribed to factors such as differences in sex, species, maturity, diet, stressor types, and stress duration (Guardiola et al., 2014).

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