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ORIGINAL RESEARCH

Fish epidermal mucus analytes with reference to physiological threats: non- intrusive approach to evaluate health status of carp fishes

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Abstract Fish health can influence important population-level processes, especially those trying to deal with challenges posed by the natural environment, and growth, which in turn affect stock productivity. Two experiments were conducted to evaluate the metabolites of epidermal mucus in response to two threats of environment faced by the fishes: starvation and pathogenic attack; with the aim to check the alteration in values of innate immune parameters in the epidermal mucus of three Indian major carps, *Catla catla*, *Labeo rohita*, and *Cirrhinus mrigala*. Study showed that, in all three species the amount of mucus secretion and parameters like protein, carbohydrate and lipid of skin mucus altered after both the challenge trials and their concentration differed with respect to species. In particular, immune parameters including lysozyme, alkaline phosphatase and protease increased after both challenges (starvation and pathogenic attack) to face the stress. In contrast myeloperoxidase activity decreased significantly ($P < 0.05$) after the challenge of starvation but its value increased with pathogen challenge. Variations in mucosal parameters thus could be employed as possible non-invasive biomarkers for studying fish physiology in response to environmental stresses and as an integrated approach to assess the particular species response to the specific condition.

Keywords Carp fishes . Mucus metabolites . Environmental stress . Starvation . Fish pathogen

Introduction

Both biotic and abiotic factors have the potential to affect the physiology and performance of fish. They include environmental challenges i.e. presence of pathogen, lack of foodstuff, pollution from urban, industrial, and agricultural regions, habitat loss, changes in water temperature, and hardness, as well as overexploitation (Kautsky et al. 2000). Numerous fish species have been documented to endure short moments of fasting and face pathogenic attack by activating a variety of behavioral changes as well as adaptive biochemical and physiological mechanisms that allow them to cope with this unfavorable condition (Belanger et al. 2002; McCue 2010; Benhamed et al. 2014). As a result of these challenges, rich aquatic bodies are becoming more fragile to disease and contributing to a decline in aquatic biodiversity in both freshwater and marine habitats. Fish have a variety of activities carried out by the skin mucus, including osmoregulation, respiration, feeding, protection and movement (Esteban 2012; Tiralongo et al. 2020). Epidermal mucus components of fish like lysozyme, alkaline phosphatase, antioxidant capacity and secretion rate can alter in response to exposure of pathogens in environment (Du et al. 2015; Fuochi et al. 2017). Fish maintain their health by using a complicated array of natural defensive systems of mucus to protect themselves from environmental infections. These systems, which are both constitutive and responsive (i.e., preexisting or inducible), provide protection by preventing the attachment, invasion, or growth of microbes in the tissue (Ellis 2001). Many fish infections, including *Aeromonas hydrophila*, have the capacity to adhere to

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and enter epithelial cells and affect its mucosal components (Beaz-Hidalgo and Figueras 2013; Wang and Leung 2000). Infection with *A. hydrophila* produces oxidative stress, nonspecific immunological responses, damage to digestive organs and DNA, autophagy in carps, possibly triggering apoptosis in the late stage of infection (Chen et al. 2020; Bhatnagar and Saluja 2021). However, few studies have widely documented the effects of starvation in several fish species of interest to the aquaculture industry, growing interest has been shown in fish feed deprivation and re-feeding in relation to the phenomena of compensatory growth that follows the application of brief starvation intervals (Caruso et al. 2010; McCue 2010; Davis and Gaylord 2011). Literature reports that epidermal mucus metabolites like protein, carbohydrate, lipid, lysozyme, myeloperoxidase, alkaline phosphatase and protease change when there are variations in their feeding habits, habitat, presence of pathogens or water quality (Subramanian et al. 2008; Caruso et al. 2011; Fernandez-Alacid et al. 2018). So as to better understand how the skin mucus of major carp species reacts to environmental difficulties, we recreated two conditions that the majority of the fish species encounter at some point in their lifespan; one was the pathogenic infection and other was to starve the fishes for food for a particular duration, because food supply and presence of pathogen can vary greatly in their aquatic habitats, affecting physiology of aquatic animals, including fish. Considering all of the aforementioned problems, we assumed skin mucus examination as a non-invasive and efficient procedure for studying fish physiology in response to environmental stresses and an integrated approach to assess the particular species response to the specific condition. Hence, different epidermal mucus metabolites were analysed, in relation to starvation and pathogenic attack (*A. hydrophilla*), as these parameters are affected by the environmental stresses and their range also vary with respect to species, with the aim to evaluate their propriety as potential biomarker of fish response to stresses.

Materials and methods

Experimental animals and their acclimatization

For the current study, three carp species—*Catla catla* (Hamilton 1822), *Labeo rohita* (Hamilton 1822), and *Cirrhinus mrigala* (Hamilton 1822) were chosen. Fish were procured from a nearby fish farm and acclimatized in the laboratory for 10-15 days before being placed in the Aquaculture Research Unit of the Department of Zoology at Kurukshetra University, Kurukshetra (29°58' N, 76°51' E). Experiments were carried out in plastic tanks (300 L), and every day, fresh water that has already reached the right temperature was used to refill the tubs.

During the experiment, fed group was fed with a plant based diet of 40% protein comprised of duckweed, rice bran, wheat flour, groundnut oil cake, chromic oxide and mineral mixture (Bhatnagar and Raparia 2014). During food restoration period of experiment 1, same diet was given to the fishes. The experiments were carried out with water quality parameters that were within the ideal range (Bhatnagar and Devi 2013), including dissolved oxygen (5.00 mg L^{-1} to 6.94 mg L^{-1}), pH (7.69 to 9.50), temperature (25.5º C to 28.5º C), conductivity (665.20 µmhos cm^{-1} to 667.70 µmhos cm^{-1}), chlorides (35.16 mg L^{-1} to 38.34 mg L^{-1}), calcium (4.00 mg L^{-1} to 17.80 mg L^{-1}), ortho-phosphate (0.02 mg L^{-1} to 0.14 mg L^{-1}) and ammonia (0.00 mg $L^{-1}$to 0.23 mg L^{-1}). Two experiments were conducted.

Experiment 1: Effect of stress of starvation on skin mucus metabolites: The fish were placed into two treatment groups: fed (F) and starved (S). Each tank contained eight fish, with mean weight measurements ranging between 170 g – 215 g. By limiting the fish from food for two weeks, a starving period was imposed. The following sampling points were used: day 0 (as a control), days 4, 7, 10, and 14 of starvation, followed by seven days during which food was restored (as a "recovery" assessment).

Experiment 2: Effect of stress of pathogenic bacteria (*A. hydrophilla*) on skin mucus metabolites: As *A. hydrophilla* is common fish pathogen and is an opportunistic intruder that harms the fishes, was selected as pathogenic bacteria stressor and procured from CSIR-Institute of Microbial Technology, Chandigarh, India (with MTCC No. 1739). Fishes were submerged in an *A. hydrophila* suspension containing approximately 10^9 CFU ml^{-1} for 21 days (Kumari et al. 2019), and mucus was collected following the challenge trial. Skin mucus samples were collected with a sterile plastic spatula across the entire dorso-lateral region to prevent sample contamination with scales or intestinal fluids. Individual mucus samples were then centrifuged at 9000 rpm for 5 minutes, and the mucus supernatant was stored at -80º C for the purpose of measuring

enzyme activity. Glassware, gloves were sterilised, and also sterilisation was done before disposal, are all routine safety precautions that were observed (Christian 2007).

Analytical methods of epidermal mucus

Protein

The Lowry test was used to determine the protein content of fish skin mucus (Lowry et al. 1951). 500 µL of fish mucus (collected after both the challenge) were combined with 2.5 ml of Lowry reagent in a test tube, which was then left at ambient temperature of room for 10 minutes. Then, 250 µL of folin's reagent was thoroughly combined by shaking vigorously. For 30 minutes, the mixture was left at room temperature (37º C). At 660 nanometers, the optical density (OD) was measured with a 'UV-VIS' spectrophotometer (Shimadzu UV-1900i).

Carbohydrate

The presence of carbohydrates was measured using the phenol-sulfuric acid method (Dubois et al. 1956). A sterilized test tube was filled with 0.5 ml of fish epidermal mucus, and the samples were then combined with 0.5 ml of phenol solution. Afterwards, 2.5 ml of strong sulfuric acid was added quickly. For 20 minutes, the mixture was left at ambient room temperature. At 490 nm, the OD was measured with a 'UV-VIS' spectrophotometer (Shimadzu UV-1900i).

Lipid

Folch et al. (1957) technique was used to determine lipid. To obtain the lipid, 1 ml of epidermal mucus samples were added with 20 ml of chloroform-methanol (2:1) solvent, which were then shaken continuously for 6-8 hours. The extracts were subsequently passed through a filter and its filtrate was placed in a separating funnel and rinsed with saline (0.89 %) solution before being left at room temperature overnight. The quantity of total lipid content was calculated by subtracting the sample's initial and final weights.

Acid value of fat

The acid value of fat was determined using the Zaharah et al. (2005) technique. 1ml of fish epidermal mucus was placed in a 100 ml cone-shaped flask. All the components were immersed in a 25 ml organic solvent (95% ether: alcohol). Everything was thoroughly mixed with 2-4 drops of phenolphthalein solution. Then, the solution was titrated for 10 to 15 seconds, against 0.05 M KOH until just a faint pink colour persisted for 15 to 20 seconds.

$$\text{Acid value (mg KOH ml}^{-1}\text{ of Fat)} = \frac{\text{Titrate value} \times 2.8}{\text{Volume of sample}}$$

Lysozyme activity

Lysozyme activity was determined using a modified turbidimetric technique (Sankaran and Gurnani 1972). A 100 µL volume of mucus was combined with a 2.5 ml suspension of lyophilized *Micrococcus lysodeikticus* cells (Sigma, ATCC No. 4698) (0.1 mg ml^{-1}) prepared in phosphate buffer (0.067 M) at pH 6.25. The optical density was recorded for 1-3 minutes at 37° C using a UV- 1900i UV-VIS spectrophotometer set to 450 nm. The one unit of lysozyme activity was defined as a change in absorbance of 0.001 per minute. The egg white lysozyme (SRL - No. 45822) standard curve was used to determine the concentration of samples.

Alkaline phosphatase activity

The activity of alkaline phosphatase in mucus was determined using a modified Rosalki et al. (1993) meth-

od. In a sterile test tube, 3 ml of the reagents containing magnesium chloride, ammonium bicarbonate, and p-nitrophenyl phosphate were incubated with 50 µL of mucus sample. The optical density (OD) at 405 nm with a 'UV-VIS' spectrophotometer (Shimadzu UV-1900i) was measured followed by three sequential absorbance measurements at minute intervals.

Myeloperoxidase activity (MPO)

The concentration of myeloperoxidase in epidermal mucus was determined using the Quade and Roth (1997) method with minor changes. Hank's balanced salt solution (900 µL) without Ca^{2+} or Mg^{2+} was used to dissolve mucus sample (100 µL). Next, 350 µL of 20 mM 3, 30, 5, 50 tetramethylbenzidine hydrochloride and 5 mM H_2O_2 were mixed. After 2 minutes, 350 µL of 4M sulphuric acid (H_2SO_4) was delivered to terminate the reaction. A 'UV-VIS' spectrophotometer (Shimadzu, UV-1900i) was used to measure the absorbance at 450 nm.

Protease activity

Protease activity was determined using the casein hydrolysis assay (Dash et al. 2014). Mucus sample of 100 µL was incubated for about 10 minutes at 37° C in casein substrate (5 ml), which contained potassium phosphate buffer having a pH of 7.4. This reaction was then retarded down with 110 mM trichloroacetic acid (5 ml), and then it was placed in a water bath at 37°C for about 30 minutes. After using Whatman paper to sieve the materials, an aliquot (2 ml) was then dissolved in 5 ml of sodium carbonate (500 mM) with 1 ml of Folin's reagent. Finally, these samples were incubated in a universal incubator for 30 minutes at 36° C – 37° C. The optical density was evaluated at 660 nm with a 'UV-VIS' spectrophotometer (Shimadzu UV-1900i).

Statistical analysis

Utilizing SPSS (version 16.0), a variance analysis was performed on the data. Tukey's test was used to determine the difference between the data. Data with a p value exceeding 0.05 regarded as non-significant, whereas those with a p value below 0.05 are regarded to have significant value. Mean ± SEM are used to express data.

Results

In the current study, which included *C. catla*, *L. rohita* and *C. mrigala* carp species, several skin mucus metabolites were assessed and compared. Values of constituents of mucus varied significantly after both the stress challenges, among studied freshwater carp species.

Weight and length of fishes

During challenge trial of starvation, no mortality was recorded during the experimentation. Body weight and length of all the carp species showed reduction after 14 days of starvation (Table 1 and 2), however weight of fishes increased after recovery period (re-fed) of 7 days but values were still less than the fed ones. Weight of *C. catla* decreased by 12.85% at the end of experimentation, while *L. rohita* and *C. mrigala* showed weight loss of 6.07% and 8.74% compared with the control fishes. Body length of the starved fishes decreased than the control but the differences were non-significant. However, no such changes of weight and length were recorded during the stress challenge of pathogenic bacteria i.e. *A. hydrophilla* (hence, data not shown).

Mucus volume

The volume of mucus obtained after both the stress challenges and single day scrapping depicted that each fasted fish was secreting less mucus than the corresponding normally fed specimens (Table 3). However,

Table 1 Weight (g) of experimental fishes before and after challenge (starvation)

Fish	At Day 0	Fed (F)	Starved (S)	(F) vs (S)
C. catla	208.05 ± 4.039 ^{A}	215.89 ± 5.010^{A}	188.13 ± 2.870^{AB}	**
L. rohita	173.07 ± 5.048 ^{B}	182.17 ± 3.791^{B}	171.11 ± 3.499 ^{B}	**
C. mrigala	203.66 ± 6.985 ^{A}	210.43 ± 8.762 ^{A}	192.02 ± 8.728 ^{A}	Ns

All values are Mean ± S.E of mean (n=8).
Means with different letters in the same column are significantly (P < 0.05) different (Tukey's test). The asterisks (*P < 0.05) and (**P < 0.01) indicate the differences were significant with respect to treatment, while, 'ns' indicate that differences between fed (F) and starved fish (S) at each sampling time were statistically non-significant (Student's t- test).

Table 2 Length (cm) of experimental fishes before and after challenge (Starvation)

Fish	At Day 0	Fed(F) (length)	Starved (S) (length)	(F) vs (S)
C. catla	27.752 ± 0.781 ^{A}	28.211 ± 0.842^{A}	25.953 ± 0.666^{AB}	Ns
L. rohita	24.177 ± 0.770 ^{B}	24.581 ± 0.748^{B}	23.922 ± 0.805^{B}	Ns
C. mrigala	27.653 ± 0.874 ^{A}	27.951 ± 0.881^{A}	27.164 ± 0.971^{A}	Ns

All values are Mean± S.E of mean (n=8).
Means with different letters in the same column are significantly (P < 0.05) different (Tukey's test). The 'ns' indicate that differences between fed (F) and starved fish (S) at each sampling time were statistically non-significant (Student's t- test).

Table 3 Variation in the amount of mucus produced by control and challenged fishes

Fish Species	Amount of Mucus (ml)		
	Control Fishes	Starved Fishes (Experiment 1)	Pathogen challenged Fishes (Experiment 2)
C. catla	3-6	3-4	6-9
L. rohita	3-5	2-3	4-6
C. mrigala	2-4	1-2	3-5

after following a 21- days challenge trial of fish pathogen, more mucus was secreted by the carp species under examination as compared to the control group.

Biochemical parameters

Experiment 1 (Effect of stress of starvation on skin mucus metabolites): Results of starvation as challenge trial showed that protein content of mucus declined in all the three carp species from day 0 to day 14 and recovered after re-feeding for 7 days (Fig. 1). Among the three species studied highest reduction of 34.31% (1.02 ± 0.001 mg ml^{-1} to 0.67 ± 0.0012 mg ml^{-1}) in protein level was observed in *C. catla* followed by *L. rohita* with reduction of 14.58% (0.96 ± 0.003 mg ml^{-1} to 0.815 ± 0.005 mg ml^{-1}) and *C. mrigala* with decline of 15.94% (0.69 ± 0.006 mg ml^{-1} to 0.583 ± 0.002 mg ml^{-1}).

Carbohydrate levels of epidermal mucus of all the three species were significantly (P < 0.05) decreased after starvation. The decline was about 50% in *C. catla* (from 0.077 ± 0.003 mg ml^{-1}to 0.039 ± 0.001 mg ml^{-1}) after 14 days of fasting, and rapidly recovered within a week (Fig. 1). *L. rohita* (0.097 ± 0.002 mg ml^{-1} to 0.054 ± 0.0004 mg ml^{-1}) and *C. mrigala* (0.133 ± 0.002 mg ml^{-1}to 0.074 ± 0.001 mg ml^{-1}) also showed reduction in carbohydrate concentration after two weeks of starvation (Fig. 1). Both the lipid and acid value of fat declined more in *C. catla* than the other studied fishes after starvation, and almost recovered after 7 days of re-feeding, however, their recovery varied with respect to the species (Fig. 2).

In our study, mucus lysozyme showed significant (P < 0.05) changes during 14 days of starvation period. Starvation affect the level of lysozyme more in *C. catla* (2.599 ± 0.107 µg ml^{-1} to 4.407 ± 0.0979 µg ml^{-1}) in mucus than *L. rohita* and *C. mrigala* which also showed enhancement in the level of lysozyme, while values were back to normal after one week of re-feeding (Fig. 3). However, in contrast, myeloperoxidase values of epidermal mucus decreased after two weeks of starvation period. *C. catla* showed reduction in MPO value (OD_{450}=1.407 ± 0.013 to 0.189 ± 0.002) after the stress of starvation, while values of myeloperoxidase of *L. rohita* (0.980 ± 0.013 to 0.222 ± 0.004) and *C. mrigala* (0.516 ± 0.023 to 0.158 ± 0.002) also decreased (Fig. 3). Both *C. catla* and *L. rohita* recovered rapidly than *C. mrigala* which was not able to cope even after re-feeding for 7 days. Alkaline phosphatase (AP) activity of skin mucus differed significantly between the three fresh water carp species after the effect of starvation and showed enhancement in level of AP (Fig. 4). Changes in the level of protease was found 30.30% higher in *L. rohita* (0.920 ± 0.026 U ml^{-1} to 1.328 ± 0.026 U ml^{-1}), whereas *C. catla* and *C. mrigala* also showed enhancement of 14.76% and 21.77%

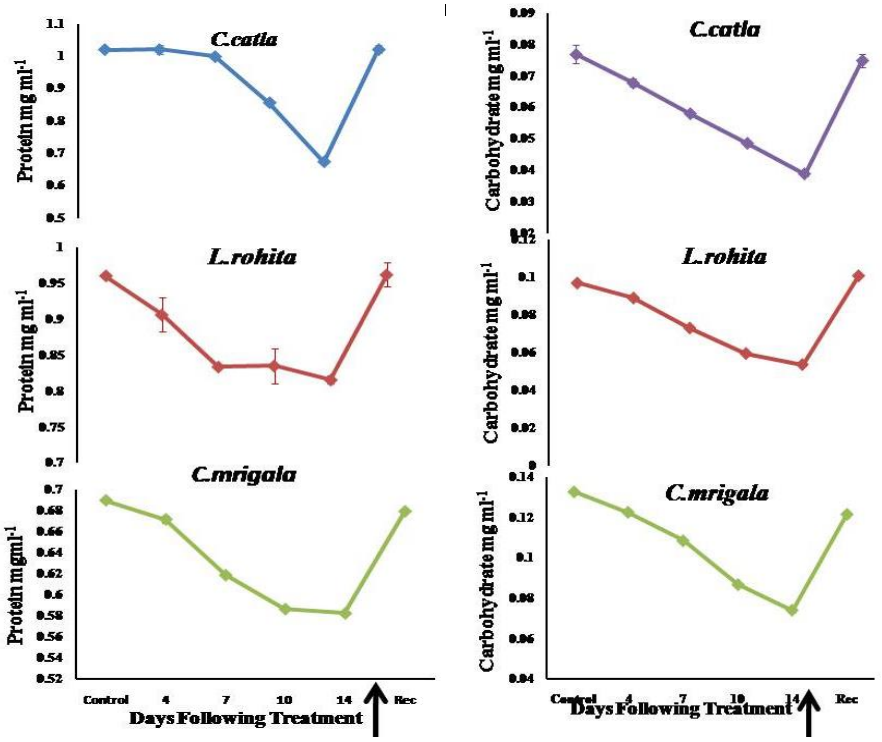

Fig. 1 Variation in Protein (a) and Carbohydrate (b) concentration of mucus components in experimental fishes, *C. catla*, *L. rohita* and *C. mrigala* after challenge of starvation from day 0 to 14 and re-feeding for 7 days (Experiment 1). Arrow at the x-axis indicating re-feeding started after the starvation period. The values represent the mean and standard error (S.E.) of mean for each fish ($P < 0.05$, one-way ANOVA).

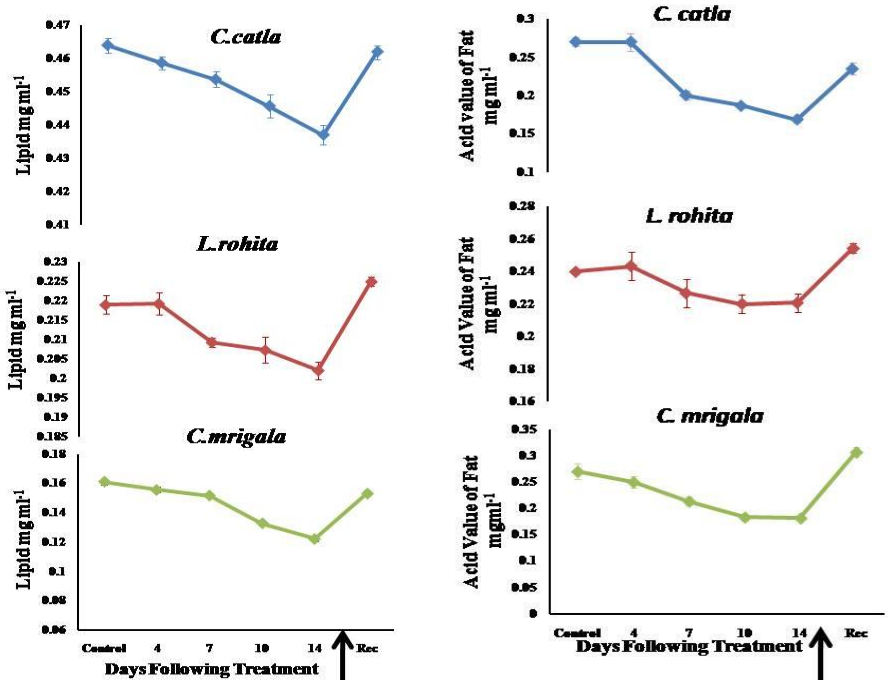

Fig. 2 Variation in Lipid (a) and Acid value of Fat (b) of mucus components in experimental fish i.e. *C. catla*, *L. rohita* and *C. mrigala* after challenge trial of starvation from day 0 to 14 and re-feeding for 7 days (Experiment 1). Arrow at the x-axis indicating re-feeding started after the starvation period. The values represent the mean and standard error (S.E.) of mean for each fish ($P < 0.05$, one-way ANOVA).

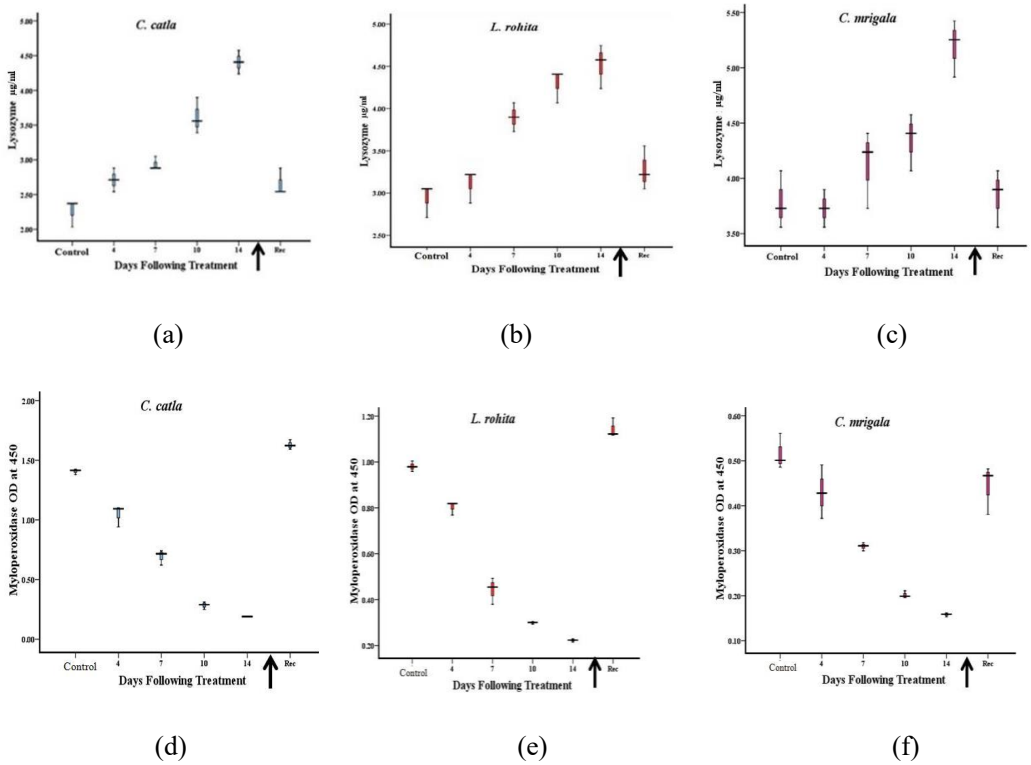

Fig. 3 Variation in Lysozyme (a to c) and Myeloperoxidase (d to f) activity of mucus components in experimental fishes, *C. catla*, *L. rohita* and *C. mrigala* after challenge trial of starvation from day 0 to 14 and re-feeding for 7 days (Experiment 1). Arrow at the x-axis indicating re-feeding started after the starvation period. The values represent the mean and standard error (S.E.) of mean for each fish ($P < 0.05$, one-way ANOVA).

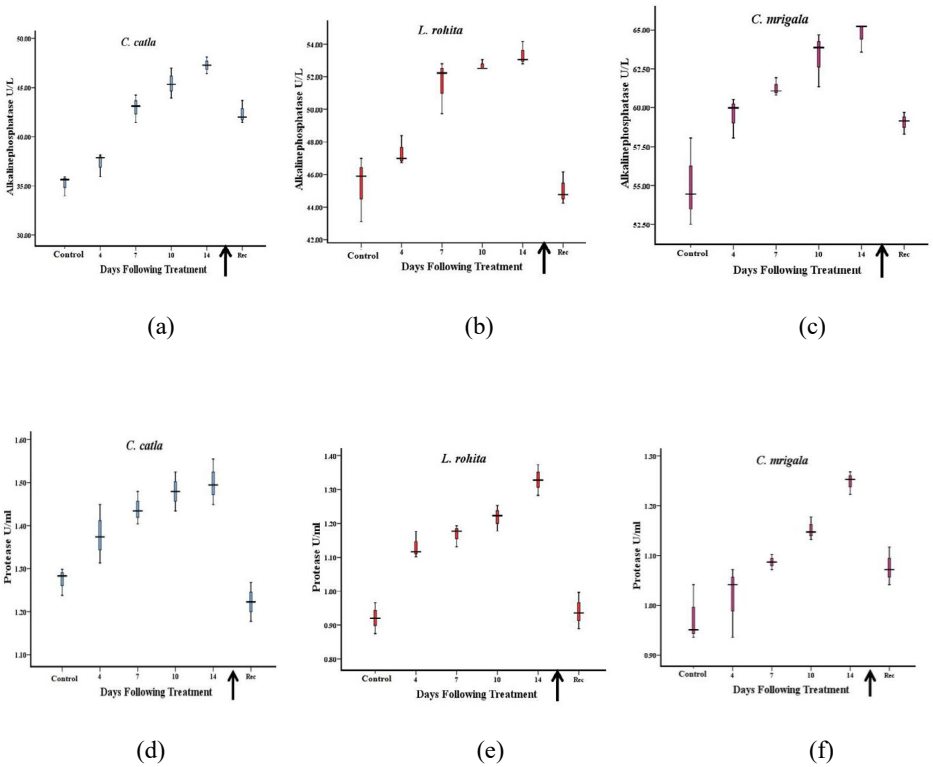

Fig. 4 Variation in Alkaline Phosphatase (a to c) and Protease (d to f) activity of mucus components in experimental fish i.e. *C. catla*, *L. rohita* and *C. mrigala* after challenge trial of starvation from day 0 to 14 and re-feeding for 7 days (Experiment 1). Arrow at the x-axis indicating re-feeding started after the starvation period. The values represent the mean and standard error (S.E.) of mean for each fish ($P < 0.05$, one-way ANOVA).

after challenge trial of starvation (Fig. 4).

Experiment 2 (Effect of stress of pathogenic bacteria, *A. hydrophilla*): All the mucosal parameters were analysed and found that their values vary after 21 days challenge trial of fish pathogen. Results of fish pathogen stress showed that the mucus protein values of all the three challenged fish were higher than those of the control. Among the three species, *C. catla* showed the highest increment of 16.52% in value of protein (from 0.964 ± 0.003 mg ml^{-1} to 1.159 ± 0.002 mg ml^{-1}), while, L. *rohita (*0.951 ± 0.0007 mg ml^{-1} to 1.043 ± 0.001 mg ml^{-1}) and *C. mrigala* (0.681 ± 0.001 mg ml^{-1} to 0.732 ± 0.001 mg ml^{-1}) also showed enhancement by 8.6% and 6.8% (Fig. 5a). Carbohydrate levels were also elevated in all the studied fishes, *C. catla* showed increment of 18.26% in comparison to control whereas in *L. rohita* and *C. mrigala* values were 9.8% and 8.08% high (Fig. 5b). After the challenge trial, the lipid level increased approximately 50% in epidermal mucus (Fig. 5c) and acid value of Fat also elevated significantly ($P < 0.05$*)* in all the studied major carp (Fig. 5d). Among the studied species, acid value of Fat in *C. catla* increased by 38.23% (from 0.21 ± 0.011 mg ml^{-1} to 0.343 ± 0.012 mg ml^{-1}), in *L. rohita* increased by almost 50% (0.203 ± 0.012 mg $ml^{-1}$to 0.456 ± 0.011 mg ml^{-1}) and in *C. mrigala* increased by 34.21% (0.25 ± 0.011 mg ml^{-1} to 0.386 ± 0.008 mg ml^{-1}).

The level of lysozyme, protease, alkaline phosphatase, and myeloperoxidase levels were significantly ($P < 0.05$) different in epidermal mucus of *C. mrigala*, *L. rohita*, and *C. catla* and after the stress challenge of fish pathogen. The values of lysozyme revealed that *L. rohita* showed the highest elevation of almost 60.91%, ranged between 2.429 µg ml^{-1} and 6.214 µg ml^{-1} in controlled and challenged fishes. In *C. catla* and *C. mrigala*, the increase was 52.94% and 58.54% respectively after the stress challenge (Fig. 6a). Myeloperoxidase, an essential enzyme having antibacterial properties increased after the challenge of fish pathogen. The activity of myeloperoxidase increased from 1.391 ± 0.002 to 2.057 ± 0.032 (OD_{at} 450) in epidermal mucus of *C. catla* after the challenge trial. The values were also significantly high in other two species (Fig. 6b). Level of alkaline phosphatase activity also increased among the studied fishes. Among the challenged fishes, alkaline phosphatase activity increased by 28.64% (from 62.650 ± 0.437 U L^{-1} to 87.803 ± 0.803 U L^{-1}) in the mucus of *C. mrigala*. However, the value of alkaline phosphatase activity was elevated by only 10.83% (from 45.513 U L^{-1} to 51.041 U L^{-1}) in *L. rohita* and least enhancement of 8.31% was found in *C. catla* (Fig. 6c). Similarly, after the stress challenge of pathogen, *A. hydrophilla*, a significant ($P < 0.05$) change was also observed among the values of protease activity of all the studied carp fishes (Fig. 6d).

Discussion

Fish are exposed to a variety of biotic and abiotic stresses in their natural surroundings. Several researches have emphasized the significance of knowing fish skin mucus function (Guardiola et al. 2014; Cordero et al. 2017; De Mercado et al. 2018). The mucus can be evaluated as a non-intrusive bioindicator of fish health status according to recent research. Two different stress conditions; fasting and bacterial infection, were administered to the three major carp species. It has been widely documented that in order to deal with stress and infection issues, healthy fish secrete and renew their mucus layer on a continual basis (Benhamed et al. 2014). The current findings of experiment 1 showed that after 14 days of starvation, the body weight reduced and after re-feeding, starved fishes restored their body mass, but slightly lower than that recorded in fed fish. Changes in body weight and length of fishes during a stressful environment have been earlier reported (Caruso 2011). The current research showed that collected mucus after experimentation differed in both the challenges. All the studied carp species generated a large amount of mucus and volume of mucus varied among the three fish species after exposure to fish pathogen i.e. *A. hydrophila*. According to earlier investigations, the mucus output of a few species of catfish rise following the challenge trial of fish pathogen, (Nigam et al. 2012; Bhatnagar et al. 2023) supporting the present results. Fish without scales released a greater volume of mucus than fish with scales (Dash et al. 2018), however in the present study, although the fishes were with scales the significant increase in the amount of mucus was observed after challenge with *A. hydrophila* clearly depicting a significant response to stress. After the starvation challenge, in our study, amount of mucus decreased by almost half that might be because of unavailability of food, fishes were facing low metabolic activities to cope with food deprivation. Furthermore, alteration in mucus production could be a sign of high stress levels and the start of a natural defense mechanism against bacterial attack and food deprivation. However, no data is available on Indian major carps for fasting challenge. Previous

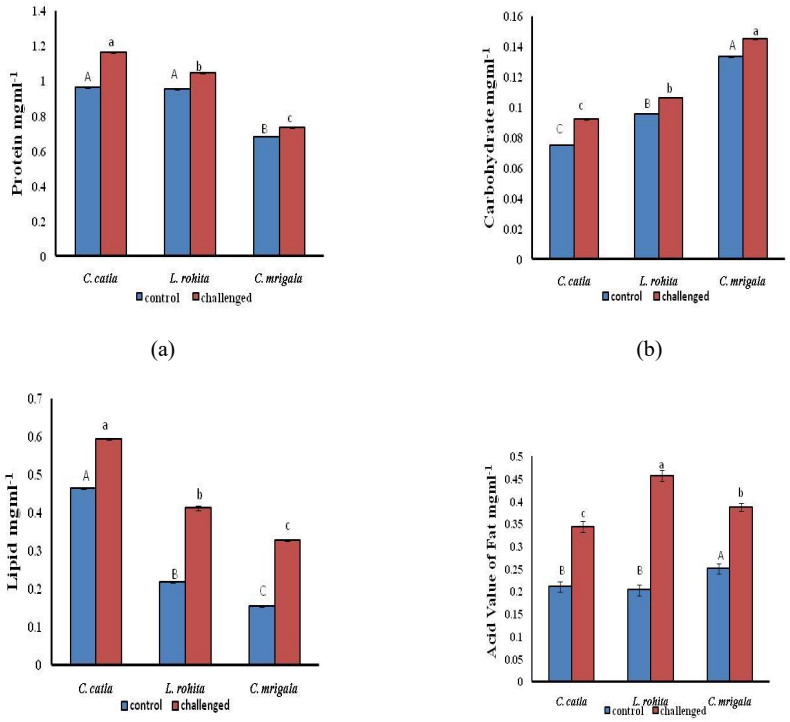

Fig. 5 Response of mucus metabolites to 21 days of fish pathogen (*A. hydrophilla*) challenge. (a) Protein (b) Carbohydrate (c) Lipid (d) Acid value of Fat. Mean bars with different capital letters in the same graph are significantly ($P < 0.05$) different for control group of studied fish species. Mean bars with different small letters in the same graph are significantly ($P < 0.05$) different for challenged group of studied fish species (Tukey's test).

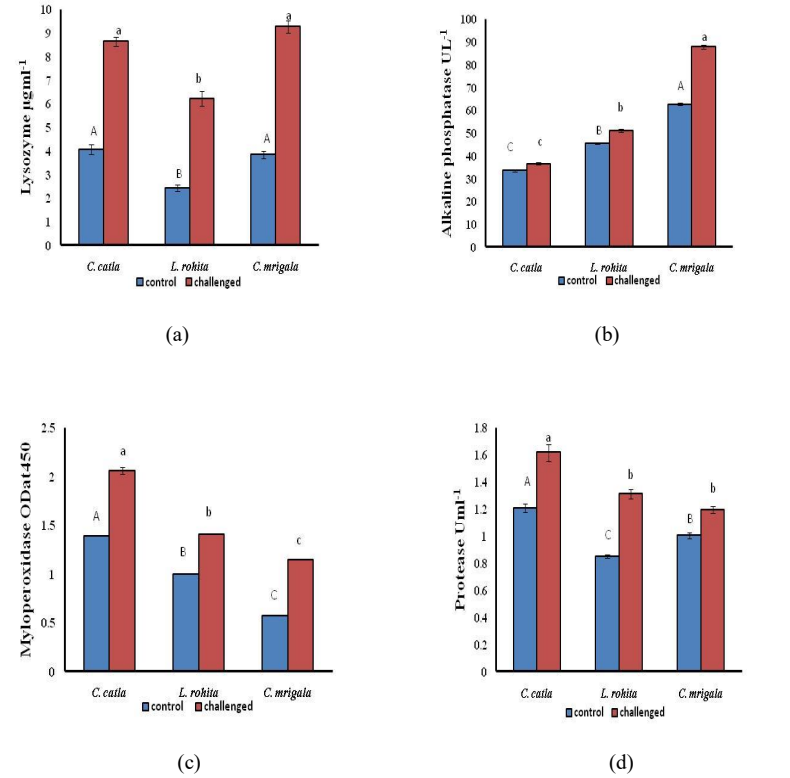

Fig. 6 Response of mucus metabolites to 21 days of fish pathogen (*A. hydrophilla*) challenge.Lysozyme (a), Alkaline Phosphatase (b), Myeloperoxidase (c) and Protease (d). Mean bars with different capital letters in the same graph are significantly ($P < 0.05$) different for control group of studied fish species. Mean bars with different small letters in the same graph are significantly ($P < 0.05$) different for challenged group of studied fish species (Tukey's test).

studies by some authors, including Subramanian et al. (2008) and Nigam et al. (2012), showed that environmental changes like pH, dissolved oxygen, temperature, and others, as well as differences in their habitat, stress of bacteria, reproduction, and developmental ages, affect the quantity of mucus secretion. According to Jones (2001), infected fish with *Gyrodactylus* also produced more mucus than usual, which is consistent with our finding that higher bacterial stress increased mucus output in the challenged group. Mucus is the first physical barrier that protects fish from environmental pathogens, microbes and works as a mechanical barrier between fish and viruses by lying at the interface between both (Reverter et al. 2018; Bhatnagar and Rathi 2021), hence alteration in the amount of mucus secretion can be considered as indicator of stress.

In our study, we investigated how the innate immune parameters and enzyme activity of epidermal mucus of *C. catla*, *L. rohita* and *C. mrigala* changed in response to challenges. Earlier studies of our laboratory showed that mucus metabolites of skin vary between the Indian major carps and their values differed with respect to the species (Bhatnagar and Budhalia 2022). Results of present study showed that mucus metabolites differed from the control fishes to cope with the stress condition. Food deprived fishes showed that both the protein and carbohydrate metabolites of skin mucus decreased after every sampling and recovered up to some extent after 7 days. According to Azeredo et al. (2015), the biological necessities to cope with a deadly infection (above 80% mortality) affect protein cycle in goblet cells of mucus, influencing protein secretion in the medium or long-term. Present studies demonstrated that a decrease in the protein and carbohydrate levels in fasting major carps could respond to an energy-saving strategy. Fish mucus levels may reflect natural fasting and reveal details about the functionality and infection risk of fish. Fernandez-Alacid et al. (2018) studied epidermal mucus of Sea bass after the challenge of starvation and showed that after two weeks of fasting, levels of glucose were reduced by almost 'half' and protein levels were also decreased but both the metabolites quickly recovered in a week. In addition, our findings showed significant changes in level of lipid metabolism and acid value of fat which decreased gradually after every sampling, a response that is consistent with carbohydrates. Some studies have reported that starvation induces transcriptional responses at gene level and affect several other activities like oxidative stress, energy metabolism, stress response, lipid metabolism, and protein breakdown (Morales et al. 2004; Martin et al. 2010; Antonopoulou et al. 2013). This might be the reason of decrease in protein and carbohydrate contents in epidermal mucus of studied fishes in the present work.

After the fish pathogen challenge (Experiment 2), different biochemical components were analyzed in which protein content in epidermal mucus was significantly higher in *C. catla* than *L. rohita* and *C. mrigala* in both the control and pathogen challenged state. Similarly, Bhatnagar et al. (2023) revealed that due to the effect of both the gram positive and gram negative bacteria challenge, protein concentration of skin mucus increased among the two Asian catfish species viz. *Clarias batrachus* and *Heteropneustes fossilis*. Furthermore, level of carbohydrate and lipid were also present in adequate amount and vary after the challenge trial. Li et al. (2013) suggested that *A. hydrophila* coordinately modifies mucosal variables via a variety of cellular pathways to improve its capacity to cling to and infect the blue catfish, *Ictalurus furcatus* host. Results of our study also showed that after the infection of fish pathogen, all the studied carps modified their level of mucus to cope with the challenge. In addition, lipids found in mucus discharges contain fatty acids with covalent bonds, which improve the gel's visco-elasticity. However, to the best of authors' knowledge no data have been reported about the level of carbohydrate and lipid after the effect of *A. hydrophila* challenge.

One of the most examined innate responses in fish is lysozyme. In this study, the value of lysozyme activity and alkaline phosphatase activity in epidermal mucus of three different carp species increased after both the challenge trials and was shown to be significantly different. Increased values of lysozyme activity and a change in alkaline phosphatase activity could be interpreted as a sign of stress that activates the immune system and their enhancement level depends on fish species. Previous reports showed that fasting and introduction of fish pathogen (*Aeromonas salmonicida*) has effects on fish health and range of lysozyme and alkaline phosphatase activity in mucus and skin, as well as their mRNA expression in skin, were significantly higher in the challenged fish than in the control, indicating lysozyme and alkaline phosphatase appears to be involved in innate immunity (Caruso et al. 2008 and 2011; Du et al. 2015). Our results also showed that both the lysozyme and alkaline phosphatase activity increased with the stress challenges and both of them played critical roles in shielding the carp species during starvation and infection of *A. hydrophila*, that might be due to infection with *A. hydrophila* or other challenges in fishes quickly changed a number of

chemokines, lectins, interleukins, and other mucosal factors that may be essential for the pathogen's capacity to stick to or penetrate which is also supported by the study of Li et al. (2013).

In addition, increased alkaline phosphatase levels in mucus or mucous secretary cells have been observed in Atlantic salmon following parasite infection (Ross et al. 2000). Furthermore, in the current study, it was observed that many enzymes with known activities in immunological reactions have been found in studied fish species. One of them is myeloperoxidase activity attributed to the stimulatory effect of microbial and parasitic compounds on neutrophilic granulocytes observed among sick carp fishes (Stosik et al. 2001). There were no data available on the impact of food deprivation on activity of mucosal myeloperoxidase among fish species. Our data demonstrated that fasting fish had significantly ($P < 0.05$) lower mucosal myeloperoxidase activity. However, results showed that activity of this mucosal component activity increased after the inoculation of pathogenic bacteria (*A. hydrophilla*) because myeloperoxidase is a vital enzyme having antibacterial properties and thus, we might infer that the high myeloperoxidase may be caused by antimicrobial substances found in skin mucus. Kumari et al. (2019) and Bhatnagar et al. (2023) revealed that after challenge with *A. hydrophila*, all three carp species and Asian catfishes showed increased mucus secretion, and extracts of skin mucus (crude and aqueous) prepared from 'healthy' and 'challenged' fish species displayed high antibacterial activity against all the examined microbial strains. Our findings are consistent with these investigations as the myeloperoxidase activity of fish skin mucus is concerned with antibacterial activity. According to Easy and Ross (2010), activity of protease changed in *S. salar* during prolonged stress. Proteases were also examined in the current study since they have been associated to skin immunological responses and microbial infection defense (Esteban 2012). Moreover, proteases may cause the activation of additional immune components such as complement, immunoglobulins, or antimicrobial peptides (Fernandes and Smith 2002; Kennedy et al. 2009). However, after 14 days of food deprivation (Experiment 1), our findings showed modifications in the activity of epidermal proteases of studied carp fishes and re-feeding (7 days) induce significant change to recover. Chen et al. (2022) also conclude that the presence and rise of *A. hydrophilla* results in mucosal microbiota dysregulation, which can promote cutaneous immune responses. But Soltanian and Gholamhosseini (2019) found no change in protease level of epidermal mucus of rainbow trout after 20 days of starvation. It appears that a variety of parameters, including season, gene mutation, stress management, species, food, maturation, sex, diameter of epidermis and number of mucosal cells, may influence the mucosal activity (Schrock et al. 2001; Balfry and Iwama 2004; Subramanian et al. 2007; Caruso et al. 2011). Thus, changes in the studied parameters could be used as biomarkers to generate a base line data for analyzing the status of fish; however, more research is needed, with different commercially significant species, to fully understand the role of skin mucus after various stress circumstances.

Conclusion

The interactions between environmental challenges and the epidermal mucosal microbiota, along with the immune responses of fish skin in the existence of different stress, were studied in this work. Since they are constantly interacting with their surroundings, fish have evolved efficient coping mechanisms. One of them is the changing of the content and exudation of skin mucus. Alteration of mucus secretion could be a sign of elevated stress and the start of a natural defense mechanism against bacterial assault and food deprivation. Thus, our food deprivation challenge showed significant decline in concentration of biochemical parameters like protein, carbohydrate, lipid and acid value of fat during starvation indicating that carp fishes were not able to maintain their level at the time of food shortage. However, enzymatic activities of lysozyme, alkaline phosphatase and protease are associated with immune response of skin mucus of carp fishes. Mucus response during inoculation of *A. hydrophila*, in particular, rapidly altered a number of potentially mucosal variables, enhancing its ability to cling to and penetrate the carp host. Our findings showed that *A. hydrophila* infection produced stress, increased the concentration of innate immune responses of epidermal mucus, to protect itself against the infection. According to the stated problems being represented in the secreted mucus, all the findings presented above enable us to suggest these skin mucus-associated biomarkers, as non-invasive measures of fish health status. Further research is necessary to know about the state of skin mucus prior to infection, impact of changing climate and interference of humans.

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Authors' contributions AB conceived the idea, designed the experiment and participated in the preparation of the manuscript. KB collected the material, carried out the experiment and analyzed the samples/data. Both authors read and approved the final manuscript.

Availability of data and materials All data sets generated and/or analyzed during the current study are available with the corresponding author

Ethics approval Permission was obtained from Institutional Animal Ethical committee vide No. IPS/IAEC/314/22 and ethical guidelines for the use of animal care were followed.

Competing interests The authors declare that they have no competing interests.

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