Skin mucus metabolites in response to physiological challenges: a valuable non-invasive method to study teleost marine species

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Abstract

Knowledge concerning the health and welfare of fish is important to conserve species diversity. Fish mucosal surfaces, and particularly the skin, are of utmost importance to protect the integrity and homeostasis of the body and to prevent skin infections by pathogens. We performed three trials simulating different environmental and anthropogenic challenges: fish capture (air exposure), bacterial infection and fasting, with the aim of evaluating epidermal mucus as a non-invasive target of studies in fish. In this initial approach, we selected three well-known marine species: meagre (*Argyrosomus regius*), European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) for our study. Mucus viscosity was measured in order to determine its rheological properties, and mucus metabolite (glucose, lactate, protein and cortisol) levels were analysed to establish their suitability as potential biomarkers. Skin mucus appeared as a viscous fluid exhibiting clearly non-Newtonian behaviour, with its viscosity being dependent on shear rate. The highest viscosity ($p < 0.05$) was observed in sea bream. Mucus metabolite composition responded to the different challenges. In particular, glucose increased significantly due to the air exposure challenge in meagre; and it decreased during food deprivation in sea bream by a half ($p < 0.05$). In contrast, mucus protein only decreased significantly after pathogenic bacterial infection in sea bass. In addition, mucus lactate immediately reflected changes closely related to an anaerobic condition; whereas cortisol was

only modified by air exposure, doubling its mucus concentration ($p < 0.05$). The data provided herein demonstrate that mucus metabolites can be considered as good non-invasive biomarkers for evaluating fish physiological responses; with the glucose/protein ratio being the most valuable and reliable parameter. Determining these skin mucus metabolites and ratios will be very useful when studying the condition of critically threatened species whose conservation status prohibits the killing of specimens.

Keywords: Air exposure, environment, fasting, infection, viscosity.

1. Introduction

Global climate change and human activity have a great impact on marine fish and fisheries. The scientific community has become increasingly concerned about potential adverse health effects on fish, not only in terms of bio-conservation and bio-preservation (reviewed in Parsons et al., 2014), but also as fish are a valuable source of protein for human nutrition (Food and Agriculture Organisation of the United Nations [FAO], 2016). Fish physiology and performance can be challenged by both biotic and abiotic factors. These include overexploitation, pollution (from urban, industrial and agricultural areas) and the introduction of foreign species; as well as habitat loss, and alterations in water temperature and acidification. As a result, valuable aquatic resources are becoming increasingly susceptible to both natural and artificial environmental changes; and it all contributes to the declining levels of aquatic biodiversity in both freshwater and marine environments (Ficke et al., 2007; Levin et al., 2009; Nagelkerken and Munday, 2016; Pörtner and Farrell, 2008). Thus, it has become necessary to implement conservation strategies to protect and preserve aquatic life.

As fish are in intimate contact with their environment, the skin mucus has been considered a first line of defence against a wide variety of environmental conditions (Hoseinifar et al., 2017a, 2017b; Jia et al., 2016; Subramanian et al., 2007). The skin mucus is a dynamic and semipermeable barrier that performs a number of functions in fish, such as osmoregulation, respiration, nutrition or locomotion (Esteban, 2012; Hoseinifar et al., 2016a, 2016b, 2016c;

Negus, 1963; Sanahuja and Ibarz, 2015; Shephard, 1994; Subramanian et al., 2008, 2007). To gain a better understanding of how the skin mucus is involved in fish responses to environmental challenges, in the present study we reproduced three well-known situations that most of the fish species chosen for the study will face during their lifecycle. We simulated fish capture, by air exposure; we provoked a pathogenic infection; and we subjected the fish to food deprivation. The first challenge provokes a loss of available oxygen, thus simulating recurrent hypoxia, which is one of the most significant effects of global warming on fish (Pörtner and Farrell, 2008), and occurs when fish are captured by recreational fishers as a consequence of catch-and-release practices (Cooke and Schramm, 2007). To our knowledge, no data exist on the effects of air exposure or hypoxia on the skin mucus in fish. Regarding the second challenge, fish are continuously in contact with a wide variety of both non-pathogenic and pathogenic organisms. In the face of infections, animals have developed mechanisms that increase their chances of survival, and the skin mucus may be considered the first biological barrier that can prevent bacterial and viral infections via the skin. Recent studies have shown that the biochemical and immunological composition of the skin mucus affects the susceptibility to infection (Benhamed et al., 2014; Fast et al., 2002b). Finally, periods of reduced food availability, even periods of fasting, are a naturally occurring stressor in fish that is thought to influence the ultimate life-history strategy of individuals (Midwood et al., 2016). Whereas the multifaceted physiological and metabolic effects of food deprivation on fish are well documented, its consequences for skin histological properties and cutaneous mucus composition are scarcely documented (Somejo et al., 2004).

Classical diagnoses of the physiological and health status of fish are provided by haematological and clinical chemical analyses (Hrubec et al., 2000; Tavares-Dias and De Moraes, 2007). Blood analysis may become a rapid and non-lethal tool to detect early malnutrition, stress and infection situations. However, blood extraction could add an extra stress response by itself, due to skin injuries that increase the probability of suffering bacterial and fungal infections or an increase in stress, for example. In spite of numerous studies in fish,

reliable reference values for clinically normal and non-stressed animals are lacking for most species. The literature reports that feeding and diet composition induce changes in specific plasma haematological and biochemical parameters, such as glucose, lactate, proteins and the activity of some enzymes; and these could be used as potential biomarkers of the functional and nutritional status of the organism (Caruso et al., 2010; Peres et al., 2013, 1999; Shi et al., 2010). Moreover, plasma cortisol levels are the most commonly used blood parameter indicator of the stress response (reviewed in Ellis et al., 2012). However, it is also important to establish which parameters or metabolites may be of most predictive or diagnostic values for a given species. Candidate parameters would be those that show little variation under normal conditions, but respond to disturbances. For a molecule to be classified as a putative biomarker, its study and measurement should preferably also be non-invasive or non-destructive, thus allowing or facilitating the monitoring of environmental effects in protected or endangered species (Fossi and Marsili, 1997). Benninghoff (2007) established the following criteria for high-quality biomarkers: quantifiable; inducible or repressible; highly accurate; reproducible among experiments; and with a response that is sufficiently sensitive to allow for routine detection.

Although mucus plays many proposed roles, the scientific literature reports few measurements of the physical and chemical properties on which these biological functions depend (Shephard, 1994). Mucus viscosity is one of these properties, mainly attributed to mucin contents and hydration, providing the surface of the fish body with rheological, viscoelastic or adhesion characteristics. A few studies measure mucus viscosity in different fish species via rheological studies of mucus soluble components (Guardiola et al., 2015; Koch et al., 1991; Roberts and Powell, 2003), reporting the relevance of skin mucus for fish locomotion. However, to the best of our knowledge, no data exist on the study of fish raw mucus which may be of major interest in both aquaculture and wild species. Taking all the previous considerations into account, we propose analysis of the skin mucus as a non-invasive and reliable method to study the response of fish physiology when coping with environmental challenges. We selected three well-known model species, meagre (*Argyrosomus regius*), European sea bass (*Dicentrarchus labrax*) and

gilthead sea bream (*Sparus aurata*), in which to simulate three environmental or anthropogenic challenges: anoxia due to the capture process, pathogenic infection by *Vibrio anguillarum* and food deprivation for two weeks. The use of aquaculture fish species was chosen as the nutritional and environmental history of these experimental animals was known, as well as their being a large amount of literature on physiological stress responses in them. Mucus viscosity and metabolites (glucose, lactate, protein and cortisol) were analysed in order to determine their suitability as potential biomarkers of fish response to environmental challenges.

2. Material and methods

2.1 Animals and experimental procedures

Three indoor experimental trials were designed to evaluate the use of epidermal mucus metabolites as non-invasive bioindicators in fish. Meagre juveniles were submitted to a simulated "capture process", sea bass juveniles were infected with *V. anguillarum* and sea bream juveniles were fasted. Irrespective of the species selected, the experimental design aimed to identify if and how mucus metabolites respond to these stressor challenges. Meagre juveniles from the Olhão Pilot Fish Farming Station (EPPO-IPMA), and both sea bream and sea bass juveniles from local fish farms were kept in the facilities of the IFAPA Centro Agua del Pino (Huelva, Spain), IRTA – Centre de Sant Carles de la Ràpita (Sant Carles de la Ràpita, Spain) and University of Barcelona (Barcelona, Spain), respectively.

Trial 1: Meagre juveniles (105 ± 2.6 g) were reared in a flow-through system at 19°C ± 1.0°C, keeping dissolved oxygen above saturation. The culture density was 3 kg m^{-3} and the acclimation period in these conditions was 21 days, while being fed with commercial feed (Skretting L-4 Alterna) to satiety (approximately 1% of biomass, daily). Throughout the experiment, the concentrations of ammonium, nitrate and nitrite, as well as the microbial load in the culture water were periodically analysed. An intense "fish capture process" was simulated by a 3-minute air exposure, after capturing the animals with a dip net. Subsequently, the fish

were returned to their original tank and skin mucus sampled 1 h and 6 h post capture. Basal data were obtained from fish that did not undergo this air exposure. This procedure started at 10:00 AM and the animals had undergone overnight fasting. Ten fish were used for every treatment and sampling point; previously they were anaesthetized with 2-phenoxyethanol (100 ppm, Sigma-Aldrich, Spain) and skin mucus was immediately collected and stored at -80°C.

Trial 2: European sea bass juveniles (106 ± 21 g), obtained from a fish farm (Piscicultura Marina Mediterránea SL, Burriana, Spain), were reared in 500 L tanks at 20.4°C ± 0.3°C, under a natural photoperiod (March-April), at a stocking density of 2 kg m^{-3}. The fish were fed twice daily by automated feeders on a commercial diet (Microbaq 15, Dibaq SA, Spain). During this time, oxygen levels were 7.5 ± 0.2 ppm and pH values were 7.5-7.7. The water flow rate in the experimental tanks was maintained at approximately 9.0 L min^{-1} via a recirculation system (IRTAmar©; IRTA, Spain) that maintained adequate water quality (total ammonia and nitrite were ≤ 0.10 and 0.4 mg L^{-1}, respectively) through UV, biological and mechanical filtration. The fish were gently anaesthetized with tricaine methanesulfonate (MS-222, 150 mg L^{-1}) and 0.1 mL of a bacterial inoculum of the Gram negative pathogen *V. anguillarum* was injected into the peritoneal cavity (bacterial dose = $5x10^4$ CFU per fish). This bacteria species was chosen as it is one of the most menacing bacteria in aquaculture (Toranzo and Barja, 1990). After the intraperitoneal injection, the fish (n = 40) were transferred into three 100 L tanks connected to a recirculation unit and regularly monitored for ten days, when mortality stopped. During this period, the fish were fed normally and moribund fish showing erratic swimming and a loss of equilibrium were sacrificed with an anaesthetic overdose. Skin mucus samples were collected prior to both the final anaesthesia and the bacteria injection (controls). Ten mucus samples, from pools of 2-3 animals were obtained. After one week, the infection process resulted in an 80.0% ± 7.5% mortality and skin mucus was sampled from the survivors. Mucus samples from both control and survivors were obtained at 10:00 AM and the animals had undergone overnight fasting.

Trial 3: Gilthead sea bream juveniles (90.7 ± 3.6 g) were reared in 800 L open-flow tanks

at 19°C, under a 12 h light:12 h dark photoperiod, at a stocking density of 3 kg m^{-3}. The fish were fed twice daily by automated feeders on a commercial diet. A starvation period was imposed by depriving the fish of food for 2 weeks. Sampling points were: day 0 (as a control), day 7 and day 14 of starving, and then 7 days after food restoration (as a "recovery" measurement). For every sampling point, 10 fish per condition were anaesthetized with 2-phenoxyethanol (0.01%, Sigma-Aldrich) and the skin mucus was immediately collected and stored at -80°C. All samples were obtained at 10:00 AM, and both control and recovery fish had undergone overnight fasting.

IFAPA, IRTA and the University of Barcelona facilities are certified and obtained the necessary authorization for the breeding and husbandry of animals for scientific purposes. All the procedures involving the handling and treatment of the fish were approved concerning the care and use of experimental animals by the European Union (86/609/EU), the Spanish Government (RD 1201/2005) and the University of Barcelona (Spain).

2.2 Skin mucus collection

In order to characterize epidermal mucus and compare the metabolite composition of different fish species, we applied a method for collecting the samples properly. Figure 1 shows a meticulous, step-by-step epidermal mucus extraction protocol. The fish were lightly anaesthetized with 2-phenoxyethanol (0.01%, Sigma-Aldrich) to avoid the stress of manipulation. Sterile glass slides were used to carefully remove mucus from the over-lateral line in a front to caudal direction: a sterile slide was gently slid along both sides of the animal two or three times, and the skin mucus was carefully pushed and collected in a sterile tube (2 mL). It is not advisable to collect mucus by repeatedly rubbing the body surface, which would provide the maximum volume of mucus, because epidermal lesions may appear and blood and other cells can contaminate the samples. To avoid dilution of the mucus with seawater, this protocol must be performed in a precise manner, without re-wetting the animal and preventing any contact

with the non-desirable areas of the operculum, and ventral-anal and caudal fins. The mucus collected was immediately frozen in liquid nitrogen and stored at -80°C until further analysis.

2.3 Viscosity analysis

For the analyses of viscosity properties, fresh samples (without homogenization) were thawed at room temperature and vortexed for 5 seconds to resuspend the mucus. Excessive vortexing and automatic-pipette homogenization must be avoided, to maintain a reproducible protocol. Viscosity was measured in 500 μL aliquots with a cone-plate CP-40 viscometer (cone angle of 0.8°, Model DV-III Programmable rheometer, Brookfield Ametek, USA). To obtain a characteristic profile, viscosity was measured over a range of six different shear rates (2.25, 4.50, 11.25, 22.50, 45.00 and 90.00 s^{-1}). These shear rates were selected since mucus demonstrates non-Newtonian behaviour, typically at low shear rates (Antonova et al., 2003; Cone, 1999; King et al., 2001; Lopez-Vidriero et al., 1980). Due to the thixotropic characteristics of the samples, readings were performed after 1 min of shear stress application. Due to differences in temperature and the equipment used in different studies, it can be difficult to compare viscosity data without reference to a common known viscosity. Thus, the relative viscosity of mucus with regard to the viscosity of water was obtained, as suggested by Roberts and Powell (2005). Relative viscosity also makes reference to the viscous drag of the fish environment: water. The viscosity of water is 1 centipoise at 20°C and is only slightly dependent on temperature (Withers, 1992).

Casson's model transformation was used to analyse the flow properties of the samples, considering both non-linearity of the flow curve and the existence of a yield stress (Casson, 1959). Casson's equation was applied as follows:

$$\sigma^{1/2} = \sigma_o^{1/2} + K\gamma'^{1/2}$$

where σ = shear stress (Pa), σ_o = yield stress (Pa), K = constant and γ' = shear rate (s^{-1}).

In accordance with Casson's model, the square root of shear stress was plotted versus

the square root of shear rate. From the straight line thus plotted, the σ_o and K values were obtained from the square of the intercept and the slope of the straight line, respectively. The model was fitted to the experimental data using a curve fit program (CurveExpert 1.3, Copyright Daniel Hyams). The best-fit model was based on the squared correlation coefficient (R^2).

2.4 Metabolites and cortisol analyses

Before mechanical homogenization, the scales collected in the mucus samples were individually removed. The samples were diluted (1:1 v/v) with Milli-Q water to extract the mucus adhered to the scales. Mechanical homogenization was performed using a sterile Teflon sticker to desegregate mucus mesh before centrifugation at 14,000g. The resultant mucus supernatants were collected avoiding the surface lipid layer, aliquoted and stored at -80°C.

Glucose concentration was determined by an enzymatic colorimetric test (LO-POD glucose, SPINREACT®, Spain). Briefly, glucose oxidase (GOD) catalyses the oxidation of glucose to gluconic acid. The hydrogen peroxide (H_2O_2) formed, is detected by a chromogenic oxygen acceptor, phenol, 4–aminophenazone (4-AP) in the presence of peroxidase (POD). Following the manufacturer's instructions for plasma determinations but with slight modifications, 10 μL of mucus extract or standard solutions (from 0 to 100 mg dL^{-1}), in triplicate, was mixed with 200 μL of working reagent and incubated for 10 min at 37°C. The OD was determined at λ = 505 nm with a microplate reader (Infinity Pro200 spectrophotometer, Tecan, Spain). The glucose values were expressed as μg glucose mL^{-1} of skin mucus.

Lactate concentration was determined by an enzymatic colorimetric test (LO-POD lactate, SPINREACT®). Briefly, lactate is oxidized by lactate oxidase (LO) to pyruvate and hydrogen peroxide (H_2O_2), which under the influence of peroxidase (POD), 4-aminophenazone (4-AP) and 4-chlorophenol, form a red quinone compound. Following the manufacturer's instructions for plasma determinations but with slight modifications, 10 μL of mucus extract or standard solutions (from 0 to 10 mg dL^{-1}), in triplicate, was mixed with 200 μL of working

reagent and incubated for 10 min at room temperature. The OD was determined at λ = 505 nm with a microplate reader (Infinity Pro200 spectrophotometer, Tecan, Spain). Lactate values were expressed as μg lactate mL^{-1} of skin mucus.

The protein concentration of homogenized mucus was determined using the Bradford assay (Bradford, 1976) with bovine serum albumin (BSA; Sigma) as the standard. Mucus extracts were previously diluted with PBS to 1:20 for sea bream, and to 1:10 for sea bass and meagre. Mucus samples or standard solutions (from 0 to 1.41 mg mL^{-1}), in triplicated, were mixed with 250 μL of Bradford reagent and incubated for 5 min at room temperature. The OD was determined at λ = 596 nm with a microplate reader (Infinity Pro200 spectrophotometer, Tecan, Spain). The protein values were expressed as mg protein mL^{-1} of skin mucus.

Cortisol levels were measured using an ELISA kit (IBL International, Germany). Briefly, an unknown amount of antigen is present in the sample and this competes with a fixed amount of enzyme-labelled antigen for the binding sites of the antibodies coated onto the wells. After incubation, the wells are washed to stop the competition reaction. Therefore, after the substrate reaction, the intensity of the colour is inversely proportional to the amount of the antigen in the sample. Following the manufacturer's instructions for saliva determinations, 50 μL of mucus extract or standard solutions (from 0 to 3 μg dL^{-1}) was mixed with enzyme conjugate (100 μL) and incubated for 2 hours at room temperature. The substrate solution (100 μL) was added after rinsing the wells with a wash solution, and incubated for 30 min. The reaction was stopped by adding 100 μL of stop solution and the OD was determined at λ = 450 nm with a microplate reader (Infinity Pro200 spectrophotometer, Tecan, Spain). The cortisol values were expressed as ng cortisol mL^{-1} of skin mucus.

2.5 Statistical analysis

Viscosity data at each shear rate were compared for the three species using one-way ANOVA. Data for all the metabolites are presented as mean values ± standard deviation (SD) and the statistical analysis between species adopted was one-way ANOVA. The effects of the "intense

capture" simulation and food deprivation for the meagre and sea bass were analyzed by one-way ANOVA, while unpaired t-tests were used to compare the two experimental sea bass groups: control *vs* survivors. Differences were considered statistically significant at $p < 0.05$. For all statistical analyses, a previous study for homogeneity of variance was performed using Levene's test. When homogeneity existed, Bonferroni's test was applied; whereas if homogeneity did not exist, the T3-Dunnet test was applied. All statistical analysis was performed using SPSS Statistics for Windows software, Version 22.0 (Armonk, NY: IBM Corp.).

3. Results

3.1 Skin mucus viscosity

Mucus obtained from epidermal exudation according to the proposed method (Figure 1) was analysed for its viscosity, without any dilution or previous homogenization. Rheograms for the three marine species revealed non-Newtonian behaviour, meaning that there were shear dependent: viscosity decreased as shear rate increased, exhibiting pseudoplastic behaviour (Fig. 2A). Sea bream mucus showed the highest viscosity at the shear rates of: 2.25 s^{-1}, 4.50 s^{-1}, 11.25 s^{-1}, 22.5 s^{-1} and 45 s^{-1}. At all these same shear rates, sea bream mucus was significantly more viscous than meagre and sea bass mucus ($p < 0.05$). No differences were found between the viscosity of meagre and sea bass mucus, except at 11.25 s^{-1}, where the meagre mucus viscosity was higher ($p < 0.05$). At the highest shear rate, 90.00 s^{-1}, no significant differences were found between any of the three species. To improve the comparison of viscosity parameters between species, the creep threshold was evaluated by adjusting the experimental data to Casson's model equation. Casson's model also provides an intercept point (σ_0; also known as the yield stress), that represents the resistance to flow at rest; and the slope (K_i), which is the plastic viscosity coefficient for non-Newtonian fluids (Fig. 2B). Whereas the intercept point was similar for sea bass and meagre, it was the highest for sea bream. Moreover, sea bream equation showed the highest slope indicating greater resistance to deformation (or movement) by friction.

3.2 Skin mucus metabolites and cortisol

In parallel to the viscosity study, the skin mucus metabolites (soluble glucose, lactate and protein) and cortisol levels were analysed. For the three species studied, soluble glucose ranged from 18 to 22 μg mL^{-1} without differences between the species (Fig. 3A). Soluble lactate levels for meagre and sea bream were 3-4-fold higher than that for sea bass (meagre: 15.6 ± 2.8 μg mL^{-1}; sea bream: 11.5 ± 0.9 μg mL^{-1}; sea bass: 3.3 ± 0.5 μg mL^{-1}) (Fig. 3B). In addition, soluble protein was species dependent, with meagre showing significantly the lowest values (3.7 ± 0.4 mg mL^{-1}) in comparison to sea bass (7.5 ± 1.4 mg mL^{-1}) and sea bream (12.8 ± 1.1 mg mL^{-1}) (Fig. 3C). Surprisingly, whereas meagre and sea bass cortisol levels were not statistically different (range: 7-12 ng mL^{-1}), cortisol levels for sea bream were significantly lower (< 1 ng mL^{-1}) (Fig. 3D).

Figure 4 shows the glucose/protein, lactate/protein and cortisol/protein ratios. Moreover, as an indicator in mucus of the aerobic/anaerobic metabolism, the glucose/lactate ratio was calculated. As protein amounts differed between the species, the glucose/protein ratio was 6.3 ± 1.1 μg mg^{-1} meagre > 3.0 ± 0.3 μg mg^{-1} sea bass > 1.5 ± 0.2 μg mg^{-1} sea bream (Fig. 4A). In the same way, the lactate/ protein ratio was fourfold higher in meagre (Fig. 4B). For the cortisol/protein ratio, the differences between the species were amplified: 1990 ± 790 ng g^{-1} in meagre, 3700 ± 1200 ng g^{-1} in sea bass and 55.2 ± 8.4 ng g^{-1} in sea bream. Finally, the glucose/lactate ratio in sea bass was approximately fourfold higher than in meagre and sea bream (Fig. 4D), due to the lower amount of mucus lactate. All these data indicate that mucus metabolites were species dependent under basal control conditions.

3.3 Response of mucus metabolites to physiological challenges

To evaluate whether the mucus metabolites would serve to measure changes in response to physiological challenges, three different trials were performed: fish capture simulated by air exposure (meagre), pathogenic bacterial infection (sea bass) and food deprivation (sea bream).

In meagre, we evaluated the mucus metabolites with respect to basal values, 1 and 6 h after the fish were exposed to the air (Fig. 5). There was an increase in the total mucus obtained;

315 it was approximately twofold higher (mL of collected mucus) in post-capture animals (data not
316 shown). Meanwhile, the mucus metabolites showed an early response by increasing the
317 concentration of soluble glucose (Fig. 5A), lactate (Fig. 5B) and cortisol (Fig. 5D) twofold, 1 h
318 post manipulation. However, protein values were not modified (Fig. 5C). After 6 hours, glucose
319 and cortisol levels did not increase further (Fig. 5A, 5D), but neither had they reverted; while
320 mucus lactate levels returned to basal levels (Fig. 5B). These results indicate immediate
321 exudation of the studied metabolites, but show that lactate retention in mucus is different. The
322 amount of protein reduced by 25% after 6 hours ($p < 0.05$) compared to the basal protein level
323 (Fig. 5C). As a result, the glucose/protein and cortisol/protein ratios increased threefold and
324 sixfold respectively (Fig. 6A, 6C). The glucose/lactate ratio did not change an hour post capture
325 (Fig. 6D), since both metabolites increased. Nevertheless, the decrease in lactate levels resulted
326 in an increase in the glucose/lactate ratio after 6 hours (Fig. 6D), with the relationship between
327 aerobic and anaerobic metabolism being modified over this time course.

328 The trial in sea bass aimed to study whether the same metabolites responded to an acute
329 infection of *V. anguillarum*. Table 1 shows a comparison of the mucus metabolite levels for
330 surviving animals (around 80% died within the first week of infection) and non-challenged
331 animals. We observed an increase in the total mucus obtained from the surviving animals, as in
332 meagre: approximately twofold (mL of collected mucus). However, survivors did not present
333 changes in glucose or lactate exudation, although the mucus protein concentration was threefold
334 lower than in non-challenged specimens ($p < 0.01$). Mucus cortisol levels were also
335 significantly reduced fivefold ($p < 0.05$). In consequence, the glucose/protein ratio was
336 significantly increased ($p < 0.01$) 7 days after the bacterial pathogen infection (Table 1). In
337 contrast, no significant changes were detected for the lactate/protein, glucose/lactate or
338 cortisol/protein ratios.

339 The trial proposed in sea bream evaluated changes in the same mucus metabolites after
340 two weeks of fasting and recovery as, in their natural habitats, fish are often challenged by a
341 variety of environmental stressors that cause nutritional challenges. In contrast to the first and

second trials, the volume of mucus obtained from each fasted animal was lower than in corresponding normally fed specimens (data not shown). Figure 7 shows the values of mucus metabolites during 2 weeks of starvation and 1 week of recovery (food restoration). Glucose levels were significantly decreased, by a half, ($p < 0.05$) after 7 and 14 days of fasting, and rapidly recovered in a week (Fig. 7A). Mucus lactate levels were decreased at 7 days of fasting ($p < 0.05$) and reverted at 14 days of fasting; thus showing, as in the meagre trial, a response that is different from that of glucose. Moreover, food restoration also supposed a reduction in the mucus lactate levels, in relation to the initial values (Fig. 7B). In this way, soluble protein in skin mucus did not change significantly throughout the trial (Fig. 7C)**.** Mucus cortisol was transiently increased at 7 days of fasting. As a result of the reduction in glucose exudation, the glucose/protein ratio decreased twofold after 7 and 14 days of fasting with respect to basal levels ($p < 0.05$, Fig. 8A). Metabolite/protein ratios (Fig. 8) responded exactly the same as the metabolite levels, since no changes in protein concentration were found. The glucose/lactate ratio indicates alterations in the aerobic metabolism during the trial, mainly during the recovery.

4. Discussion

In natural environments, fish are challenged by several types of biotic and abiotic stressors simultaneously. In the present study, we have proposed a non-invasive method to monitor fish welfare via skin mucus. Few studies have highlighted the importance of understanding skin mucus functionality in fish (Cordero et al., 2017; De Mercado et al., 2018; Guardiola et al., 2014; Micallef et al., 2017; Sanahuja and Ibarz, 2015). According to some authors, mucus samples can be collected by placing fish in individual plastic bags containing ammonium bicarbonate buffer (Ross et al., 2000); by placing a small piece of pre-cut glass fiber filter paper on the side of the fish (Ekman et al., 2015); by gently scraping with a slide and collecting without further actions (Guardiola et al., 2014; Sanahuja and Ibarz, 2015); or by scraping with a plastic spatula and subsequently placing in phosphate buffer (Dzul-Caamal et al., 2016a, 2016b, 2013). We propose collecting mucus after a light anaesthesia and by gently rubbing (2-3 times per side) with a sterile slide, causing no injuries to the epidermis and minimizing contamination

by epidermal cells. To prevent contamination, there is a consensus to avoid the gill, anal and caudal areas. Moreover, we have recommended the rapid freezing of mucus (at -80°C) in sterile tubes, as most other authors have also suggested. This standard and proposed method for collecting fish skin mucus could be applied to perform non-invasive studies on fish in farms or in the field, for ecological and conservation purposes. This procedure allows us to obtain mucus samples easily, which can then be further analysed in specialized laboratories. Following this method, we have collected skin mucus from three different species (meagre, sea bass and sea bream), under different conditions (a capture challenge, an infection challenge and starvation, respectively) and at different research laboratories (IFAPA, IRTA, UB), while ensuring minimal dilution of the samples. Mucus metabolite analysis has confirmed the reproducible and reliable method of extraction, as explained below.

Mucus is a viscous biological secretion with physicochemical properties such as elastic deformability. Few studies have even reported apparent mucus viscosity in several fish species (Guardiola et al., 2017, 2015; Koch et al., 1991; Roberts and Powell, 2005, 2003) and have been carried out after centrifugation of the mucus samples. Mucins, high-molecular-weight and highly glycosylated glycoproteins, are the most common molecules in mucus, but sample centrifugation may precipitate them. Since the viscosity of mucus depends on its hydration state and mucin content, sample centrifugation provokes the loss of its physicochemical characteristics (adhesion, viscoelasticity and rheological properties). For this reason, here, for first time, we have analysed mucus viscosity in raw (non-centrifuged) mucus from three model marine species: meagre, sea bream and sea bass.

Mucus from these three species showed clear non-Newtonian viscous behaviour, whereby it exhibited greater viscosity at lower shear rates (2.25 s^{-1}, 4.50 s^{-1}, 11.25 s^{-1}) than at higher ones (45 s^{-1}, 90 s^{-1}), when it adopted pseudo-plastic behaviour. For a deeper understanding of this characteristic, we treated mucus viscosity using Casson's model (Casson, 1959). That model describes non-Newtonian fluids acting under a yield stress and is widely used in industrial applications; but it has also been applied to biological fluids, such as to model blood flow in

narrow arteries (Venkatesan et al., 2013). The Casson equations obtained from the mucus demonstrated that sea bream is the species with the most viscous mucus. Roberts and Powell (2005) suggested that when fish increase their swimming speed, mucins aggregate, creating a slippage plane and reducing flow resistance, so the skin mucus works as a drag-reducing agent. If we accept this premise, sea bream skin mucus would show higher resistance to swimming than that of sea bass and meagre at lower speeds. In fact, it has been suggested that this property of skin mucus may help fish locomotion by reducing fluid friction and enhancing movement through water (Lebedeva, 1999; Rosen and Cornford, 1971). The study of non-soluble components of the fish skin mucus, such as mucins-net, is difficult due to their specific characteristics. The rheological approach via viscosity determination would be useful to determine global mucus response to environmental challenges. Changes in mucus viscosity, as is explained above for differences between sea bream mucus and sea bass and meagre mucus, could respond to different mucins-net conformations to cope with locomotion needs, physical protection or adhesion properties. In this way, the analysis of viscosity properties from raw mucus, instead of the soluble fraction, should be of mayor interest in bioconservation and ecology studies of wild fish, such as commercial and endangered species, or in comparing aquaculture and wild species, benthonic and pelagic species, large swimmers and small swimmers, migrators and non-migrators, or sea water and fresh water species.

The present work also aimed to evaluate the potential use of skin mucus as an easy, non-invasive and reliable method for ecosystem environmental studies. In intensive fish production, haematology and clinical chemistry may also provide important diagnostic information concerning the physiological and health status of fish (Hrubec et al., 2000; Tavares-Dias and De Moraes, 2007). Currently, the most commonly used physiological indicators in fish are plasma metabolites and hormones, together with enzyme activities (Ellis et al., 2012; Peres et al., 2013). We propose analysis of soluble metabolites (glucose, lactate and protein) and cortisol in the skin mucus to determine physiological response via a non-invasive system. Whereas mucus glucose concentration was similar for the 3 species studied, lactate, protein and cortisol differed. To our

423 knowledge, no data exist on soluble glucose and protein in the mucus of marine species. Only
424 De Mercado et al., (2018) reported mucus lactate and cortisol in trout (*Onchorhynchus mykiss*),
425 and Guardiola et al., (2016) reported mucus cortisol in sea bream. Comparing those results with
426 the species in the present study, sea bass and sea bream exhibited similar ranges of lactate levels
427 ($\mu g\ mg^{-1}$) to those of trout, whereas meagre was > 5-fold higher. Mucus cortisol levels were also
428 revealed to be species dependent: sea bream around 55 ng g^{-1}, meagre around 2000 ng g^{-1} and
429 sea bass around 3700 ng g^{-1}. In trout, the mucus cortisol levels reported by De Mercado et al.,
430 (2018) were 5-55 ng g^{-1}. However, the cortisol levels provided for sea bream by Guardiola et al.,
431 (2016) are not comparable to ours, since those authors presented them as $\mu g\ mL^{-1}$ and no data
432 for mucus proteins were provided. During the collection process, the mucus samples may have
433 been affected by water diluting them. Thus, normalization of data through mucus protein
434 concentration is recommendable. In agreement with those previous results, glucose and lactate
435 contents expressed as $\mu g\ mg^{-1}$ of protein instead of $mg\ mL^{-1}$ mucus were different among
436 species, leading to different conclusions. Meagre mucus showed the lowest values of soluble
437 protein, resulting in higher glucose/protein and lactate/protein ratios than those for sea bass and
438 sea bream.

439 To verify the validity of mucus metabolites as bioindicators of fish condition (following
440 criteria described by Benninghoff (2007), three physiological challenges were proposed,
441 simulating possible environmental and anthropogenic situations: an intense capture process, a
442 bacterial infection and food deprivation. These approaches were initially performed here with
443 model species in aquaculture, in order to be extrapolated to other marine fish and environmental
444 conditions, since they are easy to obtain in aquaculture and their life history is traceable. It has
445 been much reported that, to cope with infection challenges, healthy fish continuously secrete
446 and replace their mucus layer (reviewed in Benhamed et al., 2014). Moreover, increased
447 production of mucus and higher mucous cell density have previously been reported following
448 infection in salmonids (Buchmann and Bresciani, 1998; Fast et al., 2002a; Holm et al., 2015).
449 Although no data were reported for air exposure or fasting challenges, the mucus volume

collected differed in each challenge; volume being doubled for capture and infection, and decreased by a half for fasting.

The glucose/protein ratio seems to be the parameter that best reflects the skin mucus response; it increases after air exposure (1 and 6 h) and following a bacterial infection (1 week), but decreases during fasting (1 and 2 weeks). It is well known that fish mobilize and elevate glucose production through gluconeogenesis and glycogenolysis pathways to cope with the energy demand produced by an environmental challenge, the so-called "fight of flight" reaction (Iwama et al., 1999). Post-stress increases in plasma glucose and lactate are sometimes used as measurements for activation of the HPI axis (reviewed in Pankhurst, 2011) but the burst of both metabolites may need to be taken into account as rises in plasma glucose are restricted in species with limited hepatic glycogen stores or according to nutritional status (Martinez-Porchas et al., 2009).

The air exposure challenge reflected this situation in mucus after just 1 hour, as should be expected from plasma values (Barton, 2002; Martinez-Porchas et al., 2009; Pankhurst, 2011). However, while higher glycaemia decreased when the acute stress stopped, mucus glucose 6 h post stress still reflected the immediate response: neither diminishing nor increasing. After an infection with *V. anguillarum*, the surviving sea basses increased their glucose/protein ratio via protein reduction. Possibly, the biological needs to cope with a lethal infection (over 80% mortality, similar to reports by Azeredo et al., (2015) modified the protein turnover in goblet mucous cells affecting protein exudation in the medium- or long-term. It also seems important to maintain soluble carbohydrates in fish mucus, as they are recognized by the surface lectins of bacteria, thereby blocking bacterial adhesion to animal cells *in vitro* (Sharon, 2006). Thus, while an increase of mucus glucose marked an acute stress response, the reduction in mucus glucose would indicate a compromised state in fish. We have showed that the reduction in the glucose/protein ratio in sea bream under fasting could respond to an energy-sparing process, by reducing glucose exudation. Thus, natural fasting reported in fish could be reflected in mucus levels and, then, these could provide information on fish performance and infection

susceptibility. As both mucus glucose and protein depend on the status of the fish and environmental conditions, further studies tackling metabolite turnover at the epidermal level are necessary to elucidate exudation capacity.

After a stressful condition, increases in plasma lactate and cortisol concentrations have been widely reported (Barton, 2002; Martinez-Porchas et al., 2009). The mucus lactate/protein ratio does not seem to be a powerful indicator of fish response. Our air exposure trial provoked an immediate rise (1 h) which was not detectable after 6 h; whereas no significant differences were detected after infection. Beyond the reported lactatemia, lactate may be produced at the level of epidermal cells as a consequence of the anaerobic cellular metabolism produced by hypoxia (Omlin and Weber, 2010). This would be the case of the air exposure challenge. Instead, De Mercado et al., (2018) also reported recovered levels of mucus lactate 1 h after air exposure in trout. Thus, in contrast to mucus glucose, lactate is not an adequate candidate for measuring sustained stress responses. However, although no references exist for the mucus glucose/lactate ratio, this parameter could be an interesting biomarker of the aerobic/anaerobic response and can be extrapolated, if confirmed by further analysis, to analyse fish from aquatic hypoxic environments non-invasively.

With regard to mucus cortisol exudation, its mechanism of secretion has not been addressed yet, though it has already been detected in several fish species at the skin mucus level (Bertotto et al., 2010; Ellis et al., 2005; Guardiola et al., 2016). Under stress conditions, the hypothalamus releases corticotropin-releasing factor towards blood circulation. This polypeptide further stimulates secretion of adrenocorticotrophic hormone from the anterior pituitary gland, which finally activates the release of cortisol by the inter-renal tissue (reviewed in fish by Mommsen et al., 1999). Although the control levels for the three species studied differed greatly, all responded to challenges; as also occurs in the plasma of most fish where, cortisol reaches its highest concentration 1 hour after being stressed, and returns to basal levels after a few hours (reviewed in Barton, 2002; Bertotto et al., 2010; Martinez-Porchas et al., 2009). As had been already reported in sea bream (Guardiola et al., 2016) and trout (De

Mercado et al., 2018), mucus cortisol increased in response to air exposure, similarly to mucus glucose. However, in response to an infection or fasting, cortisol levels did not lead to the same conclusion as the glucose/protein ratio. Indeed, in chronic-stress experiments, some fish showed only a slight increase in plasma cortisol or even a decrease; probably caused by exhaustion of the endocrine system (Barton, 2002). Mucus cortisol levels decreased under infection, showing the same trend as soluble protein; whereas no significant changes were observed during 2 weeks of fasting. These data indicate that further studies are necessary to extend reference values, to provide a better interpretation of mucus metabolites, since their levels varied depending on the stressor considered.

5. Conclusion

Being in direct contact with their environment, fish have developed effective strategies to overcome all types of environmental challenges; the modification of skin mucus exudation and composition is one of them. Thus, our air exposure trial aimed to simulate an intense capture process for fish, as well as a drop in oxygen in marine environments. Mucus metabolites in meagre demonstrated that the increase of the glucose/protein ratio reflected acute stress through a large exudation of glucose in the mucus. Moreover, both glucose and lactate permitted us to evaluate aerobic and anaerobic affectations. Mucus response in the face of a pathogenic infection provoked, in surviving sea bass, a higher mucus exudation with a loss of soluble protein, indicating changes in protein turnover preferences to cope with the challenge. That trial allowed us to predict putative responses to natural infectious processes in wild or water polluted areas. In this way, natural fasting and low food availability were reproduced in the sea bream trial. Energy sparing was demonstrated at the mucus level by reduced glucose, while protein was maintained and would compromise bacterial adhesion defences. All the data presented here allow us to propose these skin mucus-associated biomarkers or SMABs as non-invasive indicators of fish status, because the proposed challenges are reflected in the exuded mucus. Moreover, if sample dilution or concentration during mucus collection occurs, referring the resulting values to protein levels (ratios) provided normalized data that proved comparable.

Although pending further studies, this method based on mucus metabolites could be applied to environmental studies such as climate change effects, human impact, alterations in trophic networks or habitat degradation.

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Bibliography

Antonova, Todorov, Exerowa, 2003. Rheological behavior and parameters of the in vitro model of lung surfactant systems: The role of the main phospholipid component, Biorheology. Pergamon Press.

Azeredo, R., Pérez-Sánchez, J., Sitjà-Bobadilla, A., Fouz, B., Tort, L., Aragão, C., Oliva-Teles, A., Costas, B., 2015. European sea bass (Dicentrarchus labrax) immune status and disease resistance are impaired by Arginine dietary supplementation. PLoS One 10, 1–19. https://doi.org/10.1371/journal.pone.0139967

Barton, B., 2002. Stress in Fishes: A Diversity of Responses with Particular Reference to Changes in Circulating Corticosteroids. Integr. Comp. Biol. 42, 517–525. https://doi.org/10.1093/icb/42.3.517

Benhamed, S., Guardiola, F., Mars, M., Esteban, M., 2014. Pathogen bacteria adhesion to skin mucus of fishes. Vet. Microbiol. 171, 1–12. https://doi.org/10.1016/j.vetmic.2014.03.008

Benninghoff, A., 2007. Toxicoproteomics--the next step in the evolution of environmental biomarkers. Toxicol. Sci. 95, 1–4.

556 Bertotto, D., Poltronieri, C., Negrato, E., Majolini, D., Radaelli, G., Simontacchi, C., Bertotto,
557 D., 2010. Alternative matrices for cortisol measurement in fish. Aquac. Res. 41, 1261–
558 1267. https://doi.org/10.1111/j.1365-2109.2009.02417.x

559 Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities
560 of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
561 https://doi.org/10.1016/0003-2697(76)90527-3

562 Buchmann, K., Bresciani, J., 1998. Microenvironment of Gyrodactylus derjavini on rainbow
563 trout Oncorhynchus mykiss: association between mucous cell density in skin and site
564 selection. Parasitol. Res. 84, 17–24.

565 Caruso, G., Maricchiolo, A., Micale, A., Genovese, A., Caruso, A., Denaro, A., 2010.
566 Physiological responses to starvation in the European eel (Anguilla anguilla): effects on
567 haematological, biochemical, non-specific immune parameters and skin structures. Fish
568 Physiol Biochem 36, 71–83. https://doi.org/10.1007/s10695-008-9290-6

569 Casson, 1959. A Flow Equation for Pigment Oil Suspensions of the Printing Ink Type,
570 Rheology of Disperse Systems, Pergamon Press, Oxford.

571 Cone, R., 1999. Mucus. Mucosal Immunol. 43–64.

572 Cooke, S., Schramm, H., 2007. Catch-and-release science and its application to conservation
573 and management of recreational fisheries. Fish. Manag. Ecol. 14, 73–79.

574 Cordero, H., Brinchmann, M., Cuesta, A., Esteban, M., 2017. Chronic wounds alter the
575 proteome profile in skin mucus of farmed gilthead seabream. BMC Genomics.
576 https://doi.org/10.1186/s12864-017-4349-3

577 De Mercado, E., Larrán, A., Pinedo, J., Tomás-Almenar, C., 2018. Skin mucous: A new
578 approach to assess stress in rainbow trout. Aquaculture 484, 90–97.
579 https://doi.org/10.1016/j.aquaculture.2017.10.031

580 Dzul-Caamal, Olivares-Rubio, H.F., Salazar-Coria, L., Rocha-Gómez, M.A., Vega-López, A.,

2016a. Multivariate analysis of biochemical responses using non-invasive methods to evaluate the health status of the endangered blackfin goodeid (Girardinichthys viviparus). Ecol. Indic. 60, 1118–1129. https://doi.org/10.1016/j.ecolind.2015.09.017

Dzul-Caamal, R., Olivares-Rubio, H.F., López-Tapia, P., Vega-López, A., 2013. Pro-oxidant and antioxidant response elicited by CH2Cl2, CHCl3 and BrCHCl2 in Goodea gracilis using non-invasive methods. Comp. Biochem. Physiol. - A Mol. Integr. Physiol. 165, 515–527. https://doi.org/10.1016/j.cbpa.2013.03.005

Dzul-Caamal, Salazar-Coria, L., Olivares-Rubio, H.F., Rocha-Gómez, M.A., Girón-Pérez, M.I., Vega-López, A., 2016b. Oxidative stress response in the skin mucus layer of Goodea gracilis (Hubbs and Turner, 1939) exposed to crude oil: A non-invasive approach. Comp. Biochem. Physiol. -Part A Mol. Integr. Physiol. 200, 9–20. https://doi.org/10.1016/j.cbpa.2016.05.008

Ekman, D., Skelton, D., Davis, J., Villeneuve, D., Cavallin, J., Schroeder, A., Jensen, K., Ankley, G., Collette, T., 2015. Metabolite profiling of fish skin mucus: A novel approach for minimally-invasive environmental exposure monitoring and surveillance. Environ. Sci. Technol. 49, 3091–3100. https://doi.org/10.1021/es505054f

Ellis, T., James, J., Scott, A., 2005. Branchial release of free cortisol and melatonin by rainbow trout. J. Fish Biol. 67, 535–540. https://doi.org/10.1111/j.1095-8649.2005.00740.x

Ellis, T., Yildiz, H., López-Olmeda, J., Spedicato, M., Tort, L., Øverli, Ø., Martins, C., 2012. Cortisol and finfish welfare. Fish Physiol. Biochem. 38, 163–188. https://doi.org/10.1007/s10695-011-9568-y

Esteban, M., 2012. An Overview of the Immunological Defenses in Fish Skin. ISRN Immunol. 2012, 1–29. https://doi.org/10.5402/2012/853470

Fast, M., Ross, N., Mustafa, A., Sims, D., Johnson, S., Conboy, G., Speare, D., Johnson, G., Burka, J., 2002a. Susceptibility of rainbow trout Oncorhynchus mykiss, Atlantic salmon

606 Salmo salar and coho salmon Oncorhynchus kisutch to experimental infection with sea
607 lice Lepeophtheirus salmonis. Dis. Aquat. Organ. 52, 57–68.
608 https://doi.org/10.3354/dao052057

609 Fast, M., Sims, D., Burka, J., Mustafa, A., Ross, N., 2002b. Skin morphology and humoral non-
610 specific defence parameters of mucus and plasma in rainbow trout, coho and Atlantic
611 salmon. Comp. Biochem. Physiol. Part A 132, 645–657.

612 Ficke, A., Myrick, C., Hansen, L., 2007. Potential impacts of global climate change on
613 freshwater fisheries. Rev. Fish Biol. Fish. 17, 581–613. https://doi.org/10.1007/s11160-
614 007-9059-5

615 Food and Agriculture Organisation of the United Nations [FAO], 2016.

616 Fossi, Marsili, 1997. The use of non-destructive biomarkers in the study of marine mammals.
617 Biomarkers 2, 205–216.

618 Guardiola, F., Cuartero, M., Collado-González, M., Arizcún, M., Diaz Baños, F., Meseguer, J.,
619 Cuesta, A., Esteban, M., 2015. Description and comparative study of physico-chemical
620 parameters of the teleost fish skin mucus. Biorheology 52, 247–256.
621 https://doi.org/10.3233/BIR-15052

622 Guardiola, F., Cuartero, M., Collado-Gonzalez, M., Díaz Baños, F., Cuesta, A., Moriñigo, M.,
623 Esteban, M., 2017. Terminal carbohydrates abundance, immune related enzymes,
624 bactericidal activity and physico-chemical parameters of the Senegalese sole (Solea
625 senegalensis, Kaup) skin mucus. Fish Shellfish Immunol. 60, 483–491.
626 https://doi.org/10.1016/j.fsi.2016.11.025

627 Guardiola, F., Cuesta, A., Arizcun, M., Meseguer, J., Esteban, M., 2014. Comparative skin
628 mucus and serum humoral defence mechanisms in the teleost gilthead seabream (Sparus
629 aurata). Fish Shellfish Immunol. 36, 545–551. https://doi.org/10.1016/j.fsi.2014.01.001

630 Guardiola, F., Cuesta, A., Esteban, M., 2016. Using skin mucus to evaluate stress in gilthead

631 seabream (Sparus aurata L.). Fish Shellfish Immunol. 59, 323–330.
632 https://doi.org/10.1016/j.fsi.2016.11.005

633 Holm, H., Santi, N., Kjøglum, S., Perisic, N., Skugor, S., Evensen, Ø., 2015. Difference in skin
634 immune responses to infection with salmon louse (Lepeophtheirus salmonis) in Atlantic
635 salmon (Salmo salar L.) of families selected for resistance and susceptibility. Fish
636 Shellfish Immunol. 42, 384–394. https://doi.org/10.1016/j.fsi.2014.10.038

637 Hoseinifar, S.H., Ahmadi, A., Raeisi, M., Hoseini, S.M., Khalili, M., Behnampour, N., 2017a.
638 Comparative study on immunomodulatory and growth enhancing effects of three
639 prebiotics (galactooligosaccharide, fructooligosaccharide and inulin) in common carp
640 (Cyprinus carpio). Aquac. Res. 48, 3298–3307. https://doi.org/10.1111/are.13156

641 Hoseinifar, S.H., Khodadadian Zou, H., Kolangi Miandare, H., Van Doan, H., Romano, N.,
642 Dadar, M., 2017b. Enrichment of common carp (Cyprinus carpio) diet with medlar
643 (Mespilus germanica) leaf extract: Effects on skin mucosal immunity and growth
644 performance. Fish Shellfish Immunol. 67, 346–352.
645 https://doi.org/10.1016/j.fsi.2017.06.023

646 Hoseinifar, S.H., Zoheiri, F., Caipang, C.M., 2016a. Dietary sodium propionate improved
647 performance, mucosal and humoral immune responses in Caspian white fish (Rutilus frisii
648 kutum) fry. Fish Shellfish Immunol. 55, 523–528. https://doi.org/10.1016/j.fsi.2016.06.027

649 Hoseinifar, S.H., Zoheiri, F., Dadar, M., Rufchaei, R., Ringø, E., 2016b. Dietary
650 galactooligosaccharide elicits positive effects on non-specific immune parameters and
651 growth performance in Caspian white fish (Rutilus frisii kutum) fry. Fish Shellfish
652 Immunol. 56, 467–472. https://doi.org/10.1016/j.fsi.2016.08.001

653 Hoseinifar, S.H., Zoheiri, F., Lazado, C.C., 2016c. Dietary phytoimmunostimulant Persian
654 hogweed (Heracleum persicum) has more remarkable impacts on skin mucus than on
655 serum in common carp (Cyprinus carpio). Fish Shellfish Immunol. 59, 77–82.
656 https://doi.org/10.1016/j.fsi.2016.10.025

Hrubec, T., Cardinale, J., Smith, S., 2000. Hematology and Plasma Chemistry Reference Intervals for Cultured Tilapia (Oreochromis Hybrid). Vet. Clin. Pathol. 29, 7–12. https://doi.org/10.1111/j.1939-165X.2000.tb00389.x

Iwama, G., Vijayan, M., Forsyth, R., Ackerman, P., 1999. Heat Shock Proteins and Physiological Stress in Fish 1. Amer. Zool. 39, 901–909.

Jia, R., Liu, B., Feng, W., Han, C., Huang, B., Lei, J., 2016. Stress and immune responses in skin of turbot (Scophthalmus maximus) under different stocking densities. Fish Shellfish Immunol. 55, 131–139. https://doi.org/10.1016/j.fsi.2016.05.032

King, D., Wang, Z., Kendig, J., Palmer, H., Holm, B., Notter, R., 2001. Concentration-dependent, temperature-dependent non-Newtonian viscosity of lung surfactant dispersions. Chem. Phys. Lipids 112, 11–19.

Koch, E., Spitzer, R., Pithawalla, R., Downing, S., 1991. Keratin-like components of gland thread cells modulate the properties of mucus from hagfish (Eptatretus stouti). Cell Tissue Res. 264, 79–86.

Lebedeva, 1999. Skin and superficial mucus of fish: biochemical structure and functional role. Recent Res. Adv. D.N. Saksena, Oxford IBN Publ. CO. PTV.LTD, New Dehli, Calcutta 177–193.

Levin, L., Ekau, W., Gooday, A., Jorissen, F., Middelburg, J., Naqvi, S., Neira, C., Rabalais, N., Zhang, J., 2009. Effects of natural and human-induced hypoxia on coastal benthos. Biogeosciences 6, 2063–2098. https://doi.org/10.5194/bg-6-2063-2009

Lopez-Vidriero, M., Jones, R., Reid, L., 1980. Analysis of skin mucus of plaice Pleuronectes platessa L. J. Comp. Pathol. 90, 415–20.

Martinez-Porchas, M., Martinez-Cordova, L., Ramos-Enriquez, R., 2009. Cortisol and Glucose : Reliable indicators of fish stress ? J. Aquat. Sci. 4, 158–178. https://doi.org/10.1016/j.aquaculture.2010.01.025

Micallef, G., Cash, P., Fernandes, J., Rajan, B., Tinsley, J., Bickerdike, R., Martin, S., Bowman, A., 2017. Dietary Yeast Cell Wall Extract Alters the Proteome of the Skin Mucous Barrier in Atlantic Salmon (Salmo salar): Increased Abundance and Expression of a Calreticulin-Like Protein. PLoS One. https://doi.org/10.1371/journal.pone.0169075

Midwood, J., Larsen, M., Aarestrup, K., Cooke, S., 2016. Stress and food deprivation: linking physiological state to migration success in a teleost fish. J. Exp. Biol. 219, 3712–3718. https://doi.org/10.1242/jeb.140665

Mommsen, Vijayan, Monn, 1999. Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation.

Nagelkerken, I., Munday, P., 2016. Animal behaviour shapes the ecological effects of ocean acidification and warming: moving from individual to community-level responses. Glob. Chang. Biol. 22, 974–89. https://doi.org/10.1111/gcb.13167

Negus, V., 1963. The function of mucus. Acta Otolaryngol. 56, 204–14.

Omlin, T., Weber, J., 2010. Hypoxia stimulates lactate disposal in rainbow trout. J. Exp. Biol. 213, 3802–9. https://doi.org/10.1242/jeb.048512

Pankhurst, N., 2011. The endocrinology of stress in fish: An environmental perspective. Gen. Comp. Endocrinol. 170, 265–275. https://doi.org/10.1016/j.ygcen.2010.07.017

Parsons, E.C.M., Favaro, B., Aguirre, A.A., Bauer, A.L., Blight, L.K., Cigliano, J.A., Coleman, M.A., Côté, I.M., Draheim, M., Fletcher, S., Foley, M.M., Jefferson, R., Jones, M.C., Kelaher, B.P., Lundquist, C.J., Mccarthy, J.B., Nelson, A., Patterson, K., Walsh, L., Wright, A.J., Sutherland, W.J., 2014. Seventy-one important questions for the conservation of marine biodiversity. Conserv. Biol. 28, 1206–1214. https://doi.org/10.1111/cobi.12303

Peres, H., Goncalves, P., Oliva-Teles, A., 1999. Glucose tolerance in gilthead seabream (Sparus aurata) and European seabass (Dicentrarchus labrax). Aquaculture 179, 415–423.

Peres, H., Santos, S., Oliva-Teles, A., 2013. Selected plasma biochemistry parameters in gilthead seabream (Sparus aurata) juveniles. J. Appl. Ichthyol. 29, 630–636. https://doi.org/10.1111/j.1439-0426.2012.02049.x

Pörtner, H., Farrell, A., 2008. Ecology. Physiology and climate change. Science 322, 690–2. https://doi.org/10.1126/science.1163156

Roberts, S., Powell, M., 2005. The viscosity and glycoprotein biochemistry of salmonid mucus varies with species, salinity and the presence of amoebic gill disease. J. Comp. Physiol. B Biochem. Syst. Environ. Physiol. 175, 1–11. https://doi.org/10.1007/s00360-004-0453-1

Roberts, S., Powell, M., 2003. Comparative ionic flux and gill mucous cell histochemistry: effects of salinity and disease status in Atlantic salmon (Salmo salar L.). Comp. Biochem. Physiol. 525–537.

Rosen, Cornford, 1971. Fluid Friction of Fish Slimes. Nature 234, 49–51. https://doi.org/10.1038/234049a0

Ross, N.W., Firth, K.J., Wang, A., Burka, J.F., Johnson, S.C., 2000. Changes in hydrolytic enzyme activities of naive Atlantic salmon Salmo salar skin mucus due to infection with the salmon louse Lepeophtheirus salmonis and cortisol implantation. Dis. Aquat. Organ. 41, 43–51. https://doi.org/10.3354/dao041043

Sanahuja, I., Ibarz, A., 2015. Skin mucus proteome of gilthead sea bream: A non-invasive method to screen for welfare indicators. Fish Shellfish Immunol. 46, 426–35. https://doi.org/10.1016/j.fsi.2015.05.056

Sharon, N., 2006. Carbohydrates as future anti-adhesion drugs for infectious diseases. Biochim. Biophys. Acta 1760, 527–537. https://doi.org/10.1016/j.bbagen.2005.12.008

Shephard, K., 1994. Functions for fish mucus. Rev. Fish Biol. Fish. 4, 401–429. https://doi.org/10.1007/BF00042888

Shi, X., Zhuang, P., Zhang, L., Chen, L., Xu, B., Feng, G., Huang, X., 2010. Optimal starvation

time before blood sampling to get baseline data on several blood biochemical parameters in Amur sturgeon, Acipenser schrenckii. Aquac. Nutr. https://doi.org/10.1111/j.1365-2095.2009.00711.x

Somejo, Herrera, Fabillo, Abucay, 2004. The development of integumentary and skeletal systems of starved Nile Tilapia, Oreochromis niloticus L. New Dimens. farmed Tilapia Proc. sixth Int. Symp. Tilapia Aquac. Am. Tilapia Assoc. Aquac. CRSP, Minist. Agric. Manila 733–740.

Subramanian, S., MacKinnon, S., Ross, N., 2007. A comparative study on innate immune parameters in the epidermal mucus of various fish species. Comp. Biochem. Physiol. B. Biochem. Mol. Biol. 148, 256–63. https://doi.org/10.1016/j.cbpb.2007.06.003

Subramanian, S., Ross, N., Mackinnon, S., 2008. Comparison of antimicrobial activity in the epidermal mucus extracts of fish. Comp. Biochem. Physiol. 150, 85–92. https://doi.org/10.1016/j.cbpb.2008.01.011

Tavares-Dias, M., De Moraes, F., 2007. Leukocyte and thrombocyte reference values for channel catfish (Ictalurus punctatus Raf), with an assessment of morphologic, cytochemical, and ultrastructural features. Vet. Clin. Pathol. 36, 49–54.

Toranzo, A., Barja, J., 1990. A review of the taxonomy and seroepizootiology of Vibrio anguillarum, with special reference to aquaculture in the northwest of Spain. Dis. Aquat. Organ. 9, 73–82.

Venkatesan, Sankar, Hemalatha, Yatim, 2013. Mathematical Analysis of Casson Fluid Model for Blood Rheology in Stenosed Narrow Arteries. J. Appl. Math. 2013, 1–11. https://doi.org/10.1155/2013/583809

Withers, P., 1992. Comparative animal physiology. Saunders College Pub.

Figure captions

Figure 1. Scheme of mucus collection procedure.

Figure 2. Analyses of mucus viscosity in sea bream, sea bass and meagre. Rheograms (A) and Casson's model transformation plots (B). Values are mean ± standard deviation (SD) from individual fish analyses. Lowercase letters (a, b, c) indicate significant differences between species in each shear rate ($p<0.05$, one-way ANOVA).

Figure 3. Comparison of control mucus metabolites in meagre, sea bass and sea bream juveniles. Glucose (A), lactate (B), protein (C) and cortisol (D). Values are mean ± standard deviation (SD) from individual fish. Lowercase letters (a, b, c) indicate significant differences between species ($p<0.05$, one-way ANOVA).

Figure 4. Mucus metabolite ratios of meagre, sea bass and sea bream juveniles. Glucose/protein ratio (A), lactate/protein ratio (B), cortisol/protein ratio (C) and glucose/lactate ratio (D). Values are mean ± standard deviation (SD) from individual fish. Lowercase letters (a, b, c) indicate significant differences between species ($p<0.05$, one-way ANOVA).

Figure 5. Response of mucus metabolites to 3-minute air exposure in meagre (Trial 1). Glucose (A), lactate (B), protein (C) and cortisol (D). Values are mean ± standard deviation (SD) from individual fish. Lowercase letters indicate significant differences between samplings ($p<0.05$, one-way ANOVA).

Figure 6. Response of mucus metabolite ratios to 3-minute air exposure in meagre (Trial 1). Glucose/protein ratio (A), lactate/protein ratio (B), cortisol/protein ratio (C) and glucose/lactate ratio (D). Values are mean ± standard deviation (SD) from individual fish. Lowercase letters indicate significant differences between samplings ($p<0.05$, one-way ANOVA).

Figure 7. Response of mucus metabolites to food deprivation and recovery in sea bream (Trial 3). Glucose (A), lactate (B), protein (C) and cortisol (D). Values are mean ± standard deviation (SD) from individual fish. Lowercase letters indicate significant differences between samplings ($p<0.05$, one-way ANOVA).

782 **Figure 8. Response of mucus metabolite ratios to food deprivation and recovery in sea**
783 **bream (Trial 3).** Glucose/protein ratio (A), lactate/protein ratio (B), cortisol/protein ratio (C)
784 and glucose/lactate ratio (D). Values are mean ± standard deviation (SD) from individual fish.
785 Lowercase letters indicate significant differences between samplings ($p<0.05$, one-way
786 ANOVA).

Table 1. Response of mucus metabolites and their ratios after *V. anguillarum* infection in sea bass (Trial 2).

	Control		Survivors		
	Mean	SD	Mean	SD	
Metabolites					
Glucose ($\mu g\ mL^{-1}$)	22.90	16.20	24.01	2.85	
Lactate ($\mu g\ mL^{-1}$)	3.30	1.50	2.18	1.13	
Protein ($mg\ mL^{-1}$)	7.46	4.89	1.98	2.15	**
Cortisol ($ng\ mL^{-1}$)	7.53	4.15	1.41	1.21	*
Ratios					
Glucose/Protein ($\mu g\ mg^{-1}$)	2.97	0.89	6.77	1.78	**
Lactate/Protein ($\mu g\ mg^{-1}$)	0.70	0.41	1.31	0.98	
Cortisol/Protein ($ng\ g^{-1}$)	2081	1502	1950	1789	
Glucose/Lactate ($mg\ mg^{-1}$)	6.39	4.83	9.78	6.02	

Values are mean ± standard deviation (SD) from individual fish. (*) indicates significant differences between controls and survivors at day 7 (*$p < 0.05$ vs **$p < 0.01$; Student's T-test).

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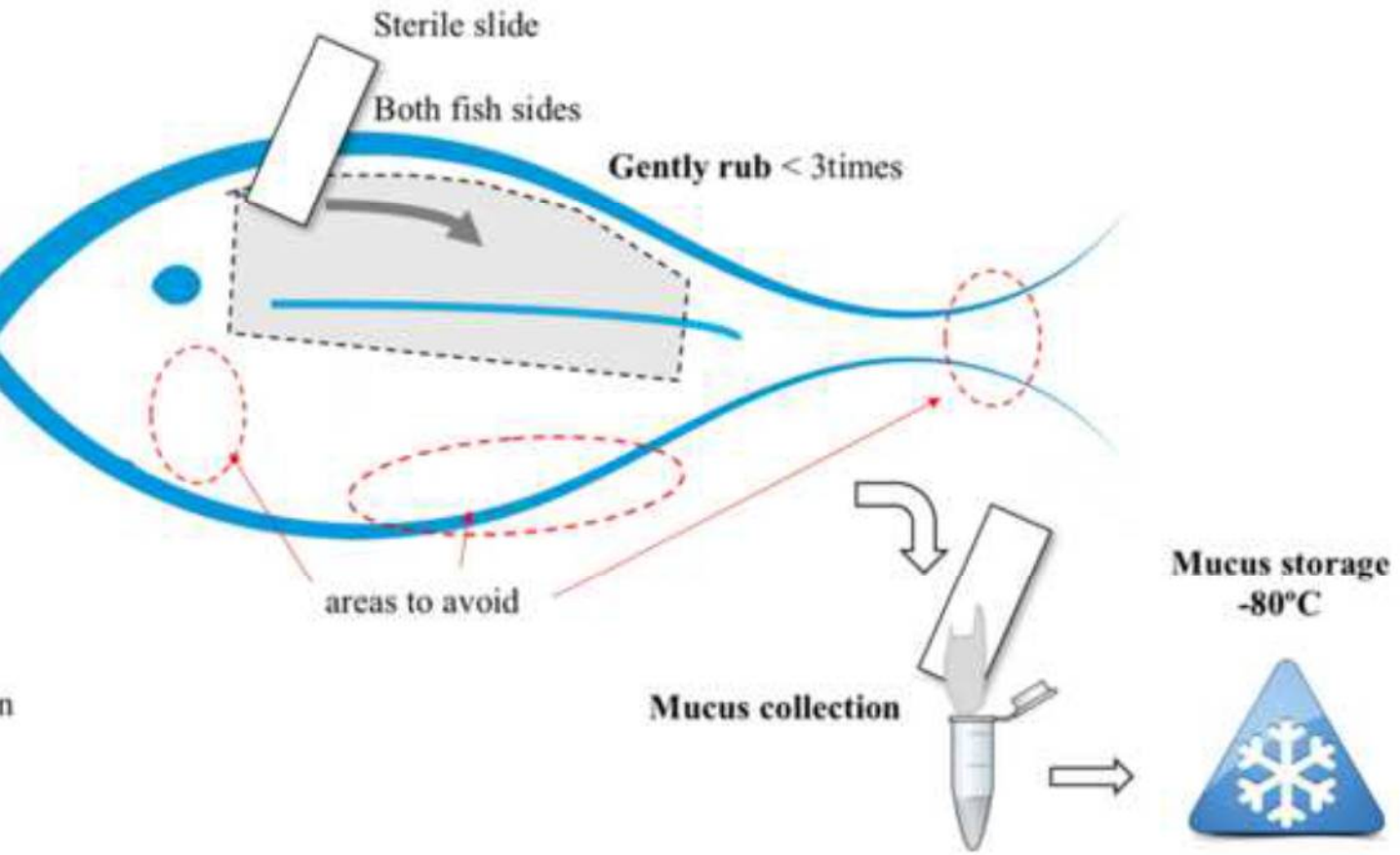
Sterile slide
Both fish sides
Gently rub < 3times
areas to avoid
Mucus collection
Mucus storage
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Figure

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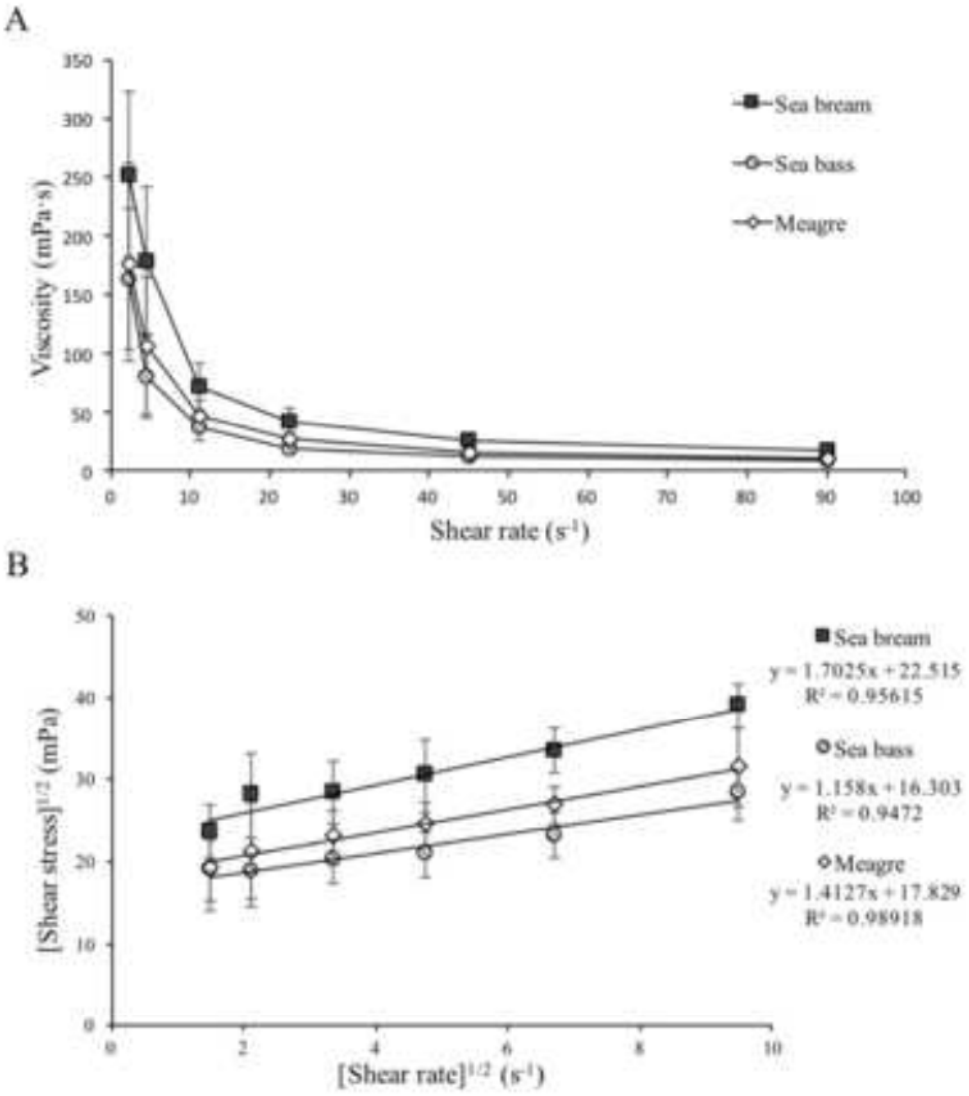

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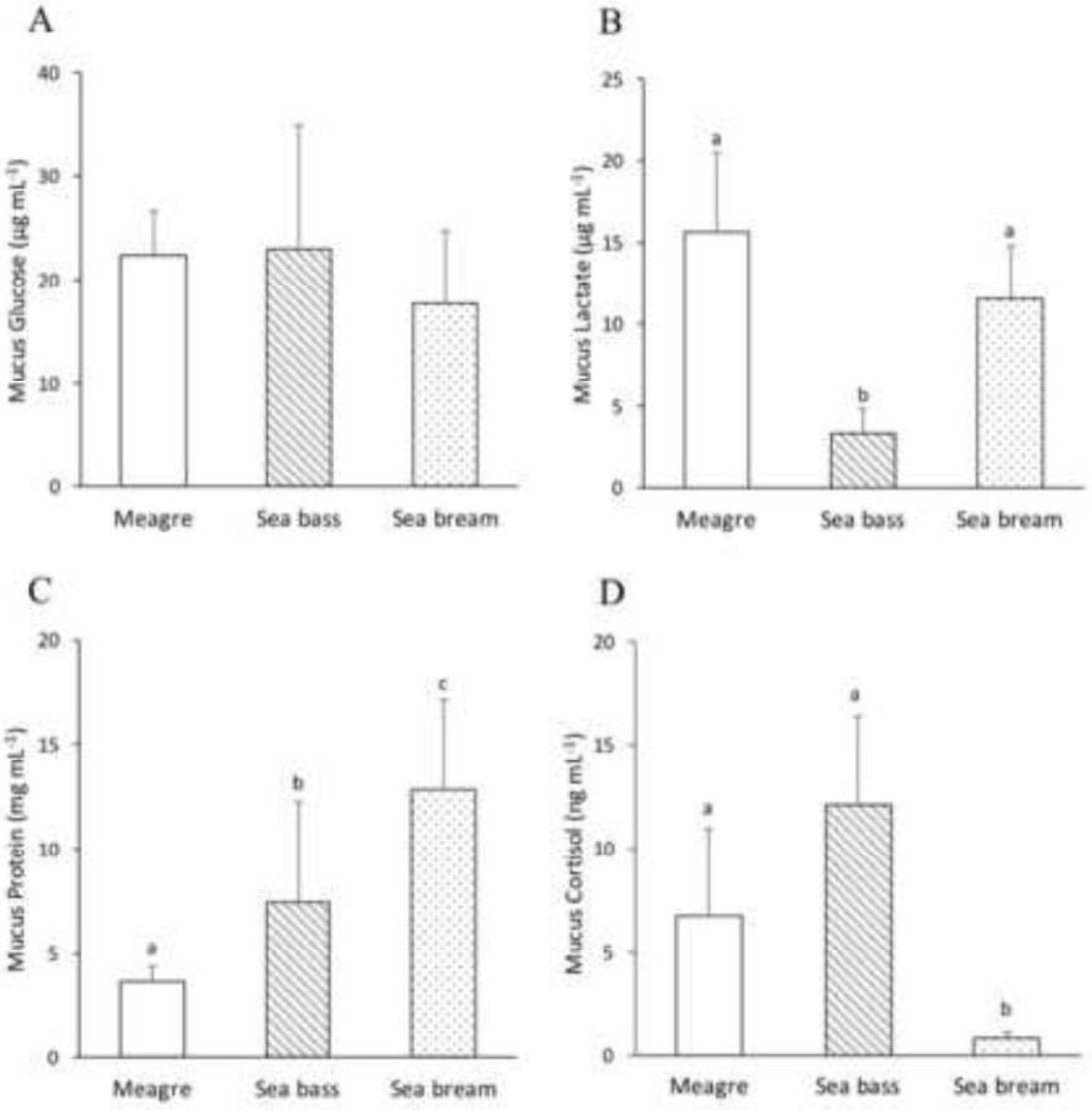

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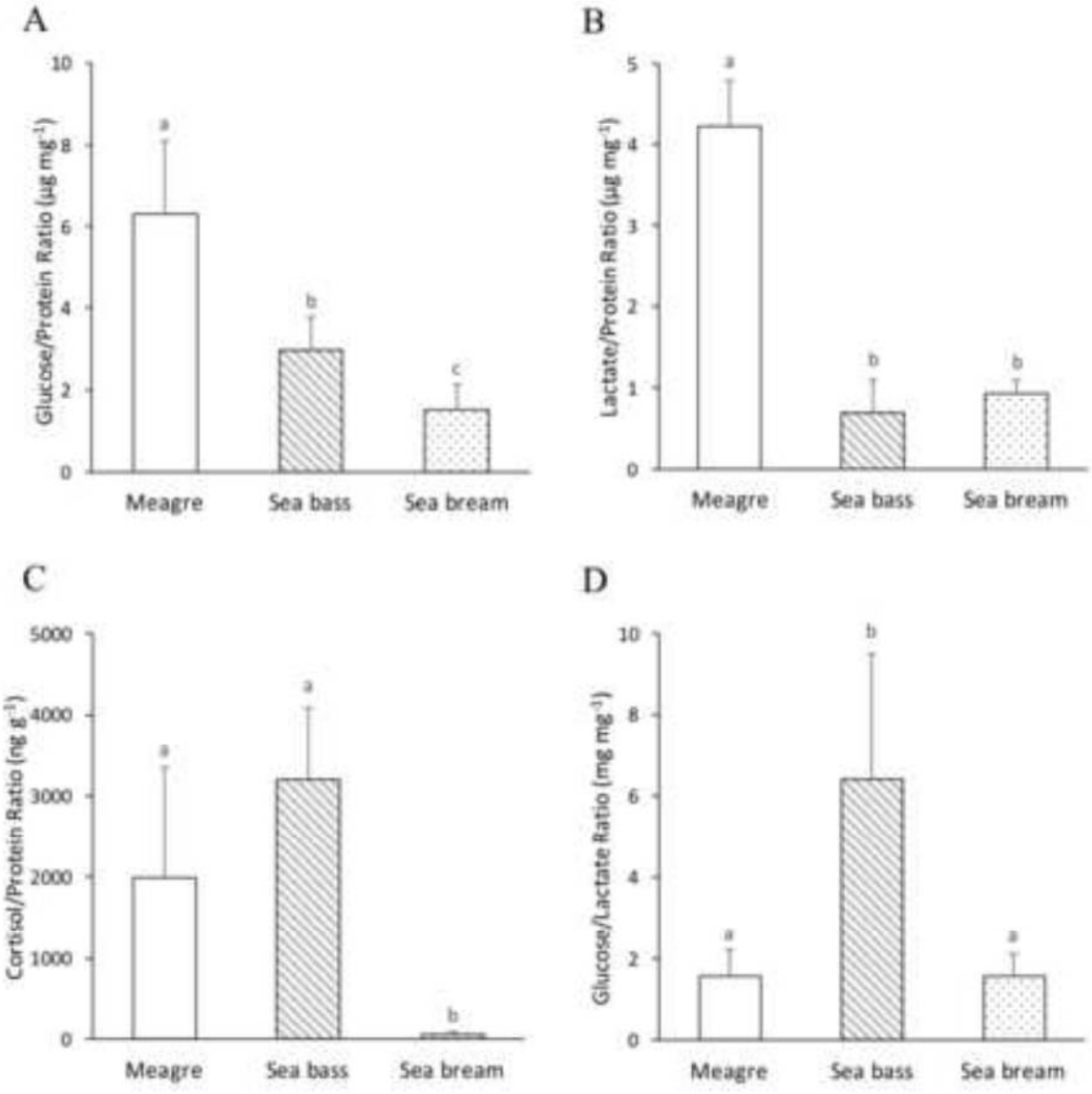

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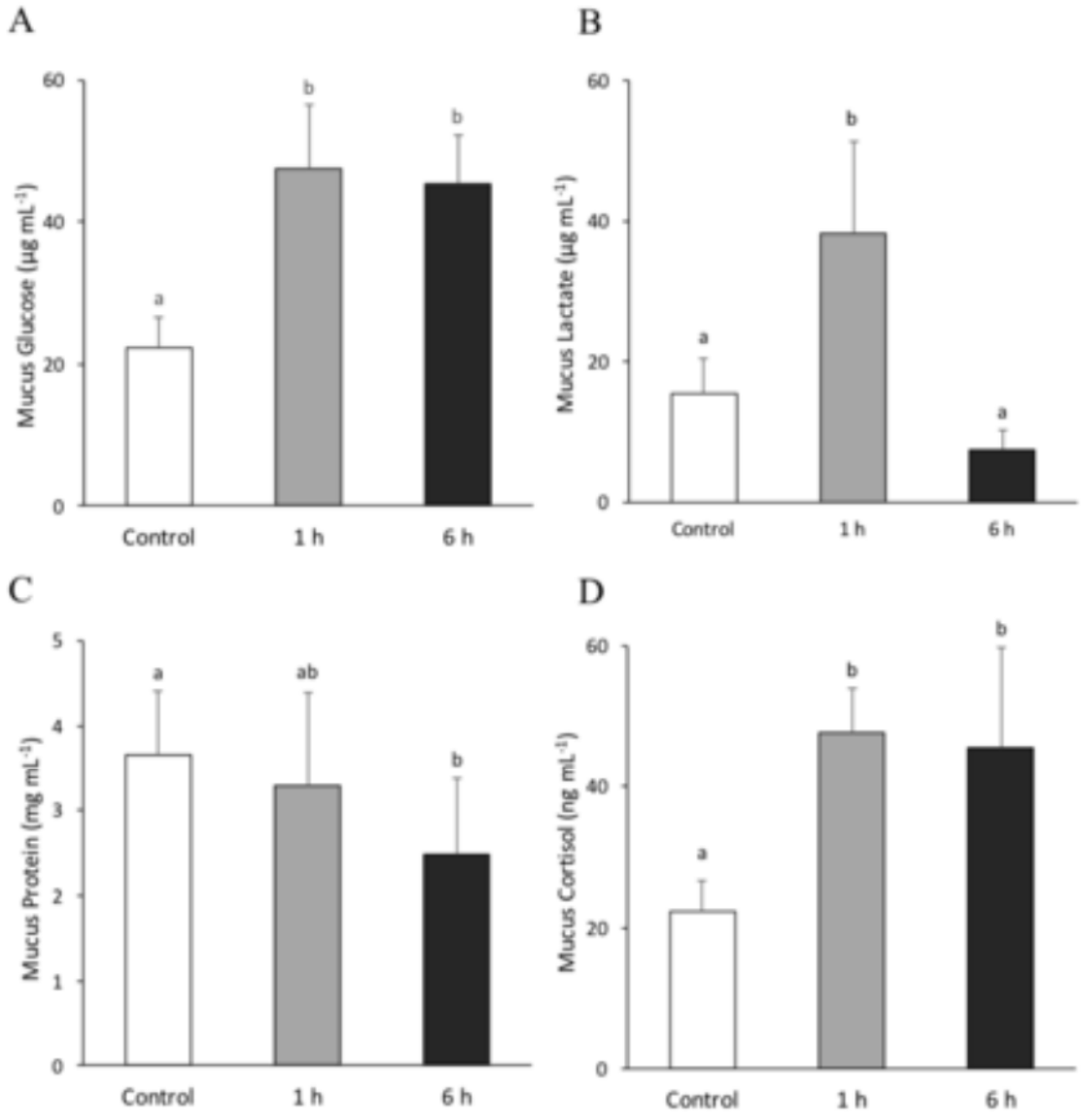

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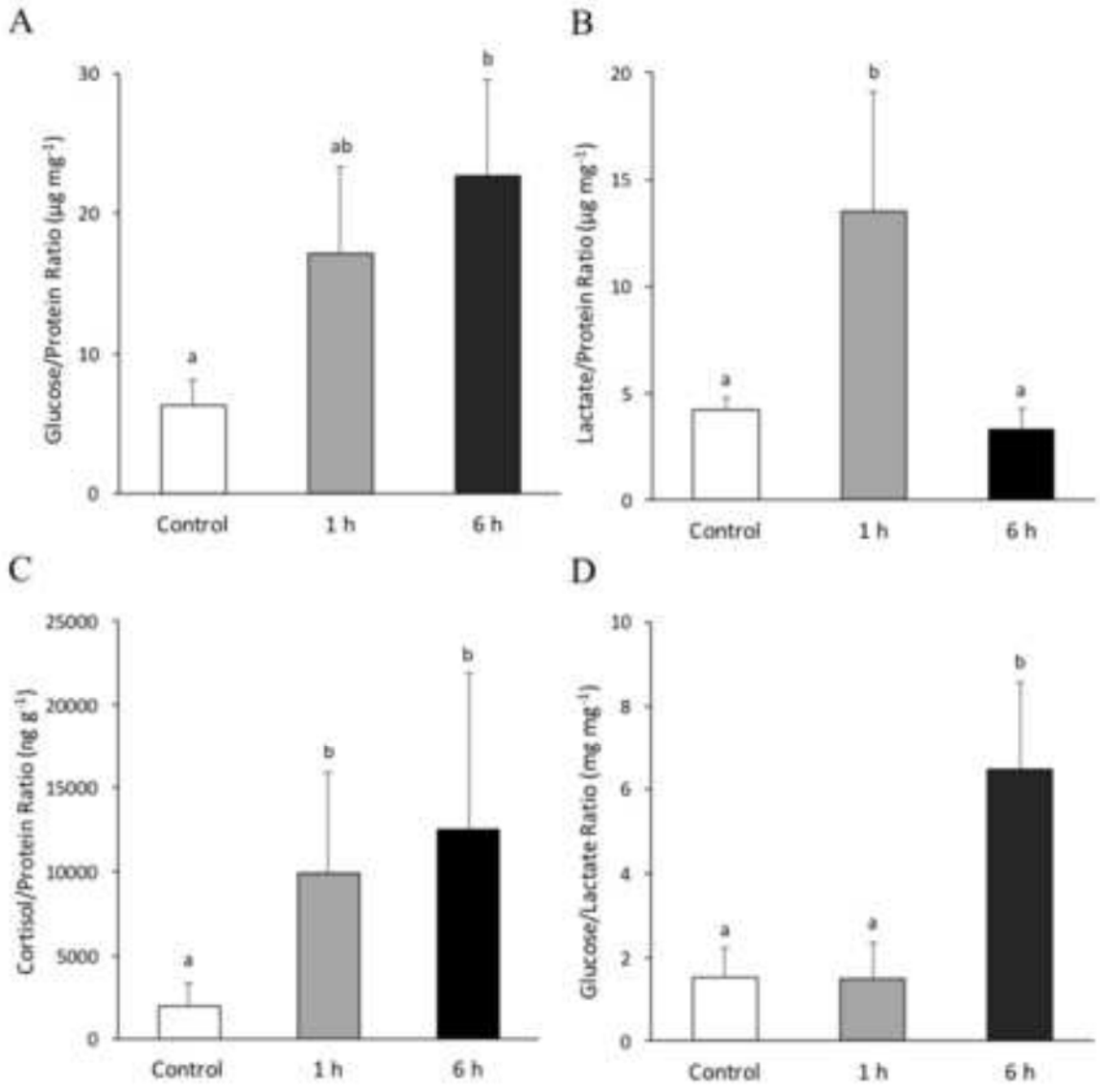

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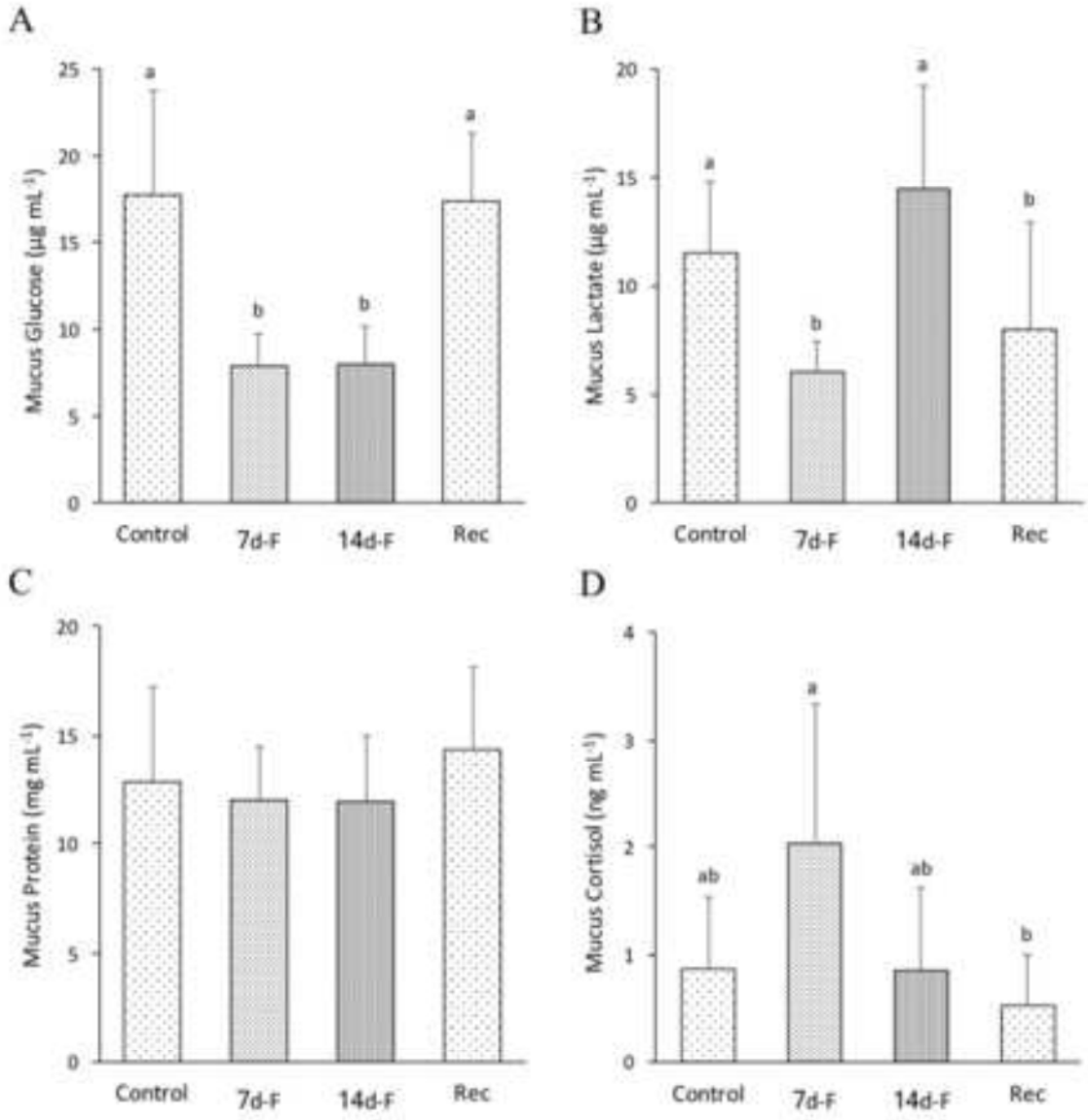

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