Article

Skin Mucus as a Relevant Low-Invasive Biological Matrix for the Measurement of an Acute Stress Response in Rainbow Trout (*Oncorhynchus mykiss*)

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Abstract: Skin mucus is a non-lethal and low-invasive matrix appropriate to assess fish welfare as it contributes to their defence against external aggressions and reflects changes in fish health status. However, more information on the response of this matrix to specific stressors is needed. In this study, rainbow trout (*Oncorhynchus mykiss*) specimens were subjected to an acute stress by air exposure and sampled after 1, 6, and 24 h post-stress. Blood and skin mucus were collected, and a battery of biochemical biomarkers were measured in both matrices. Cortisol and glucose values showed the expected classical stress response in plasma, increasing after the acute stress. The same pattern was observed in skin mucus, corroborating previous data in fish, and allowing us to confirm that skin mucus can be a useful complementary matrix for stress assessment in fish. The results showed sensitivity to hypoxic stress in skin mucus for cortisol, glucose, alkaline phosphatase (ALP), aspartate transaminase (AST), alanine aminotransferase (ALT), creatinine kinase (CK), and calcium. From the 15 parameters evaluated, 12 did not show statistically significant changes between plasma and mucus; therefore, using skin mucus cannot replace the use of plasma. Finally, the principal component analysis in skin mucus revealed a complete separation between the two experimental groups, being ALP, AST, glucose, cortisol, and CK, the biomarkers that contributed the most to this separation.

Keywords: biomarkers; skin mucus; fish; acute stress; rainbow trout

1. Introduction

In daily aquaculture practice, fish can undergo different threatening situations referred to as stress, both of acute (e.g., handling) and chronic (e.g., crowding) natures. Moreover, wild fish populations can also experience stressful situations of both natures, due, for example, to sudden or persistent habitat contamination or abrupt habitat-changing events such as drought, variations in food availability, or extreme temperature changes [1–3]. Furthermore, the current climate change scenario makes these abrupt changes, which might lead to stress, more plausible. These stressful circumstances can negatively impact fish welfare and lead to an increase in the vulnerability to pathogens [4]. Stress is a process regulated by a complex neuro–immune–endocrine interaction, in which cortisol plays a key role [5,6]. For this reason, cortisol is the most broadly assessed indicator to assess quantitative stress in fish subjected to stressful stimuli.

Blood sampling is a commonly used tool to monitor animals' health status and a widespread sample of choice for systemic cortisol determinations [6]. Nevertheless, non-lethal blood sampling is still a highly invasive procedure. Cortisol, as a steroid hormone, can diffuse through cell membranes given its lipophilic nature and bind to corticosteroid receptors in most organs and tissues. Moreover, cortisol can be found and measured in multiple biological matrices, such as bile, faeces, urine, and mucus [6–8]. Skin mucus is a biological matrix that allows for the evaluation of several biochemical parameters associated with animal welfare [9]. This matrix is altered by the fish's health conditions similarly to plasma, allowing easy, low-invasive sampling. Moreover, skin and skin mucus are permanently in contact with the nearby aquatic environment, acting as the first line of defence against a wide range of stressors (e.g., microorganisms, water quality, and pollutants) [10,11]. A positive correlation among cortisol levels in plasma and skin mucus has been previously observed in stressful situations [7,12]. Moreover, cortisol levels measured in mucus have been considered a good stress indicator, as they show a high correlation with other stress biomarkers in plasma in several fish species [13]. For example, skin mucus cortisol has been used to evaluate acute stress in fish after situations such as crowding [12], exposure to anaesthetic agents [14], transport [7], changes in salinity [15], or hypoxia [16,17]. Moreover, mucus is constituted by a vast number of protective substances; among them are enzymes, such as proteases, phosphatases, and esterases, that can be analysed to assess fish health status [14]. Altogether, skin mucus is a very promising matrix to assess fish welfare, as it plays a significant role in their defence against external aggressions and reflects changes in fish health status. Using alternative methods or matrices that avoid animals is in line with current European Union demands (European directive 210/63/EU) dealing with the protection of animals that are used for scientific purposes, which aims to reduce the number of animals used in scientific research as well as to improve their health and welfare. This can be achieved by replacing the sampling of organs or tissues requiring animal sacrifice by alternative matrices providing equally valuable information. However, more information evaluating the responsiveness of skin mucus as an alternative matrix in the face of different types of stressful stimuli is still needed.

Our team has previously demonstrated that several biochemical parameters in skin mucus can be used as accurate tools in the health assessment of fish under distinct stressful situations, from aquaculture scenarios to xenobiotic exposure [18–20]. Moreover, we validated them using an automated analyser [13,20,21], which presents a clear advantage over commercially available kits that imply the measurement of each parameter individually. This provides a higher speed of sample processing and lower sample handling, resulting in less errors due to human manipulation. These advantages are of particular importance when measuring a wide array of parameters in a high number of samples. The panel of biochemical parameters includes biomarkers of stress (cortisol and glucose), hepatic function (alkaline phosphatase—ALP, aspartate transaminase—AST, alanine aminotransferase—ALT), and muscular damage (creatinine kinase—CK), metabolism (glucose, lactate dehydrogenase—LDH, creatinine, triglycerides, and cholesterol), inorganic elements (calcium and phosphorous), and oxidative stress (esterase activity—EA and total oxidative status—TOS). Moreover, it also included adenosine deaminase (ADA), a novel biomarker of the immune system in fish, allowing to obtain a complete snapshot of the animal health status and stress response.

This study aims to contribute to the use of low-invasive sampling methods in fish health, with the following specific aims: first, to measure an array of biochemical endpoints in the plasma and skin mucus of fish after an acute stress by air exposure in order to evaluate the animals' stress responses in both matrices and, second, to compare the results between the two biological matrices, i.e., plasma and skin mucus. By assessing the correlation between them, we can provide valuable information suggesting some biomarkers that can be measured in skin mucus, avoiding the more invasive sampling of fish blood. Our main hypothesis is that, like cortisol, additional biomarkers in the biochemical panel evaluated will present a positive correlation between both matrices.

2. Materials and Methods

2.1. Animals and Experimental Set-Up

Rainbow trout (*Oncorhynchus mykiss*, N = 36) (185 ± 5 g mean weight) were obtained from a local fish farm (Oliana, Spain). The fish were initially kept in 14 °C recirculating freshwater under a 12 h light–dark photoperiod regime for two weeks. The fish density in the aquaria was 8.4 kg/m^3. During acclimation, fish were daily fed with a commercial diet (Trouw T6 Classics 3P, Trouw España, Madrid, Spain). Dissolved oxygen was kept above 90% as well as a pH within the range of 6.0–8.5. Nitrate, nitrite, and ammonia levels were less than 1.0 mg/L, 0.05 mg/L, and 0.07 mg/L, respectively. All experimental procedures involving fish were carried out according to the 3 R's principles of Animal Experimentation (Replacement, Reduction, and Refinement) following Spanish legislation (Law 32/2007 and RD53/2013), which agrees with the International Guiding Principles for Biomedical Research Involving Animals (EU 2010/63). After the acclimation period, a group of nine fish was maintained under controlled conditions (control—non-stressed fish), and the remaining fish were quickly captured with a net, maintained in an air exposed net for 3 min, and then released back into the holding tanks (250 L tanks). The fish were maintained in resting conditions in their respective holding tanks and sampled at 1, 6, and 24 h post-stressor. At the end of the post-stress period, nine fish per sampling time were sacrificed by anaesthetic overdose with MS222, and skin mucus and blood were sampled. In order to avoid stress effects due to sampling, all nine fish of the same sampling time were removed and anesthetized at the same time. Each experimental condition consisted of two replicate tanks (i.e., two tanks for every sampling time).

2.2. Blood and Skin Mucus Collection and Sample Preparation

Blood was extracted from the caudal vein using heparinized syringes and processed for plasma isolation (1500 rpm for 10 min). Skin mucus was collected by carefully rasping the dorsolateral surface of the fish, avoiding contamination by other fluids, as previously described [22]. Skin mucus samples were homogenized with 1 volume of Tris-buffered saline (TBS, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl), vigorously shaken, and centrifuged (3000 rpm, 10 min, 4 °C). Skin mucus and plasma samples were immediately stored at −80 °C until analysis.

All biomarkers were previously assessed for linearity and intra-assay precision in fish plasma and skin mucus, as described elsewhere [19]. For all biomarkers, intra-assay coefficients of variation were below the 15%, and values from 1/2 dilution with ultrapure (MilliPore, Burlington, MA, USA) water showed less than 15% variation in comparison to the expected results. We also added information on Page 4, line 152, indicating that all the samples were measured in one batch to avoid possible inter-day variations. All the measurements were performed within a month after sample obtaining.

2.3. Biochemical Measurements

Biochemical parameters were determined using commercial kits and following the manufacturer's indications (Biomérieux, Marcy-l'Étoile, France; Olympus Systems Reagents; Olympus life and Material Science Europe GmbH, Hamburg, Germany; Adenosine Deaminase assay kit, Diazyme Laboratories, Poway, CA, USA for ADA). Cortisol was measured both by radioimmunoassay (RIA) and chemiluminescence enzyme immunoassay (CIA), as described previously [13]. Analytes were measured using automatic analysers (Olympus Diagnostica AU600, GmbH, Freiburg, Germany) in all cases except for cortisol, measured using Immulite® 1000 (Immulite System; Siemens Health Diagnostics, Deerfield, IL, USA).

2.4. Data and Statistical Analysis

Statistical data analysis was performed using GraphPad Prism 7.0. A one-way analysis of variance (one-way ANOVA) was performed to assess the significant effects of the stress response. Before the analysis, data were checked for normality (Shapiro–Wilkins test) and equal variance (Spearman's test) and transformed when necessary [23]. The analysis

was followed by the Dunnet test (α = 0.05) to detect differences from the control group. Differences were considered significant when $p < 0.05$. An analysis of correlations between biomarkers in the two matrices and different biomarkers in the same matrix was conducted using the Spearman correlation coefficient. SPSS version 22 statistical software (IBM) was used to apply the syntaxis model for the false discovery rate, as described by the Benjamini and Hochberg method [24]. Results are expressed as median ±25–75th percentile. Given that several analytes evaluated in this study were found to be affected by stress in fish in a direct (comparing their values at different time points) or indirect (showing correlation with cortisol) way, a PCA analysis was performed in order to better characterize the effect of acute stress on the biochemical parameters and, thus, obtain a more general view on the alterations suffered by fish. For this, Metaboanalyst 5.0 (MetaboAnalyst) was used.

3. Results and Discussion

The results for biochemical parameters measured in the plasma and skin mucus of rainbow trout after acute stress are shown in Figure 1. In this study, cortisol was measured using two previously validated fish plasma and skin mucus samples [13]. Although the CIA offers a series of advances compared to RIA, the latter was also used with comparative purposes, since it is considered a "golden standard" for cortisol determination in fish [25,26]. Results obtained using both methods were correlated in both matrices (Table 1), confirming our previous observations [13]. Cortisol determined by both RIA and CIA showed a significant increase 1 h post-stressor in both plasma (4.8- and 10.4-fold higher values, respectively) and skin mucus (5.3- and 4.2-fold, respectively) compared to the control group. At 6 h post-stressor, cortisol levels decreased in both matrices and were still higher than in the control group for plasma (3.1- and 6.8-fold higher for RIA and CIA, respectively) but lower to control in skin mucus (0.9- and 0.8-fold lower for RIA and CIA). At 24 h post-stressor, cortisol levels had returned to control levels in both plasma and skin mucus. These results confirm the data from our previous study in which cortisol increased significantly in plasma and skin mucus 1 and 6 h after stress compared to controls, decreasing then to control levels after 24 h [13]. In agreement with previously reported results [27], the return to basal levels after 24 h may indicate an adaptation to the stressful situation and the activation of feedback mechanisms at hypothalamic and pituitary levels. Therefore, the cortisol-related data obtained in this study confirm the validity of the experimental set-up [25,26]. Glucose showed similar dynamics as observed for cortisol. This finding was expected since cortisol induces gluconeogenesis in the liver [28]. Glucose levels increased at 1 h post-stressor in both matrices compared to the control (1.9- and 2.1-fold higher for plasma and skin mucus, respectively). In plasma, glucose levels remained higher than in the control group 6 h post-stressor (1.3-fold) and decreased 0.6-fold at 24 h. The similarity of dynamics with cortisol resulted in a positive correlation between both parameters within each biofluid (Table 1). These data agree with different studies that reported increased plasma glucose concentrations in rainbow trout at 1, 3, and 24 h after 3 min of air exposure [29] and then returning to basal levels at 24 h [30]. Similarly, in a study with meagre (*Argyrosomus regius*), Fernández-Alacid et al. found that both cortisol and glucose increased in skin mucus after acute air exposure stress [17]. Nevertheless, the same authors conducted a study using the same air exposure stressor on another flatfish species (*Solea senegalensis*) and found increases in skin mucus glucose and lactate levels, but not in cortisol [31]. Therefore, although the classical response after acute stress is generally corroborated for fish, species-specific studies add valuable information concerning the adequacy of fish mucus as well as the most relevant parameters to be assessed for each fish species. Importantly, in the present study, some of the biochemistry parameters evaluated were significantly correlated with cortisol (Table 1). This data is especially relevant considering that cortisol is the most widely used stress biomarker and is currently considered one of the gold standards for stress studies.

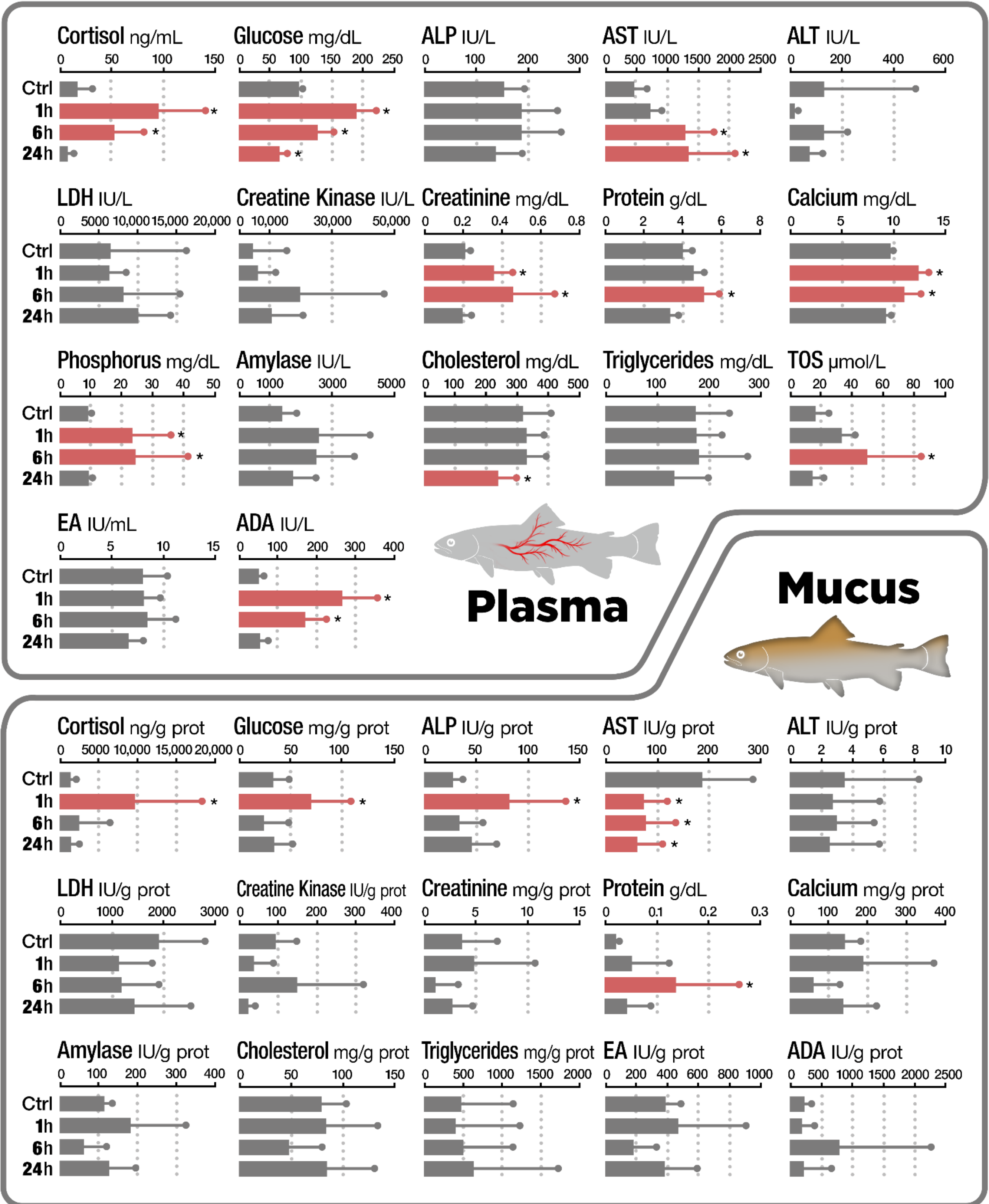

Figure 1. Biochemical parameters measured in plasma and skin mucus of rainbow trout after an acute stress, sampled 1, 6, and 24 h post-stressor. Values are presented as mean ± standard deviation (n = 9). Data were analysed using a one-way ANOVA test, and all groups were compared with the control group ($p < 0.05$). Significant differences are marked with an asterisk (*). Alkaline phosphatase (ALP), aspartate transaminase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), total oxidative status (TOS), esterase activity (EA), and adenosine deaminase (ADA).

Table 1. Spearman correlation between cortisol (measured by RIA) and plasma and mucus analytes. Cortisol (Cort), glucose (GLUC), alkaline phosphatase (ALP), aspartate transaminase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), creatinine kinase (CK), creatinine (CREA), proteins (PROT), amylase (AMYL), cholesterol (CHOL), triglycerides (TRIGL), calcium (CA), phosphorous (PHOS), total oxidative status (TOS), esterase activity (EA), and adenosine deaminase (ADA). * In bold, statistically significant differences after applying the False Discovery Rate as per the Benjamini and Hochberg method ($p < 0.05$).

		Plasma Cortisol (RIA)			Skin Mucus Cortisol (RIA)		
		r	95% Confidence Interval	P (Two-Tailed)	r	95% Confidence Interval	P (Two-Tailed) *
Plasma biomarkers	**Cort RIA (ng/mL)**	-	-	-	**0.441**	**0.1166 to 0.6808**	**0.008**
	Cort CIA (µg/dL)	**0.927**	**0.8578 to 0.9631**	**<0.0001**	**0.373**	**0.03497 to 0.6342**	**0.027**
	GLUC (mg/dL)	**0.697**	**0.4708 to 0.8378**	**<0.0001**	0.19	−0.1635 to 0.4994	0.276
	ALP (UI/L)	0.172	−0.1760 to 0.4813	0.317	0.191	−0.1624 to 0.5002	0.273
	AST (UI/L)	−0.04	−0.3722 to 0.3019	0.818	0.021	−0.3236 to 0.3607	0.905
	ALT (UI/L)	−0.056	−0.3860 to 0.2872	0.747	−0.323	−0.5991 to 0.02176	0.058
	LDH (U/l)	−0.118	−0.4378 to 0.2289	0.494	0.113	−0.2387 to 0.4383	0.519
	CK (UI/L)	−0.044	−0.3756 to 0.2982	0.800	0.208	−0.1444 to 0.5139	0.230
	CREA (mg/dL)	**0.72**	**0.5048 to 0.8506**	**<0.0001**	**0.361**	**0.02090 to 0.6257**	**0.033**
	PROT (g/dL)	**0.635**	**0.3790 to 0.8010**	**<0.0001**	0.322	−0.02296 to 0.5983	0.059
	AMYL (UI/L)	0.242	−0.1044 to 0.5355	0.156			
	CHOL (mg/dL)	0.327	−0.01197 to 0.5984	0.052	**0.437**	**0.1112 to 0.6778**	**0.009**
	TRIGL (mg/dL)	0.092	−0.2532 to 0.4167	0.593	0.175	−0.1782 to 0.4879	0.315
	CA (mg/dL)	**0.818**	**0.6629 to 0.9052**	**<0.0001**	**0.486**	**0.1720 to 0.7100**	**0.003**
	PHOS (mg/dL)	**0.609**	**0.3422 to 0.7853**	**<0.0001**	0.294	−0.05358 to 0.5782	0.086
	TOS (µmol/L)	**0.615**	**0.3450 to 0.7906**	**<0.0001**	0.322	−0.02897 to 0.6017	0.064
	EA (IU/mL)	0.249	−0.1016 to 0.5452	0.149	0.262	−0.09400 to 0.5584	0.135
	ADA(UI/L)	**0.696**	**0.4557 to 0.8422**	**<0.0001**	**0.465**	**0.1280 to 0.7055**	**0.007**
Skin mucus biomarkers	**Cort RIA (ng/g prot)**	**0.441**	**0.1166 to 0.6808**	**0.008**	-	-	-
	Cort CIA (µg/g prot)	**0.528**	**0.2266 to 0.7371**	**0.001**	**0.909**	**0.8229 to 0.9544**	**<0.0001**
	GLUC (mg/g prot)	0.142	−0.2109 to 0.4616	0.417	**0.814**	**0.6534 to 0.9042**	**<0.0001**
	ALP (UI/g prot)	0.222	−0.1300 to 0.5246	0.200	**0.659**	**0.4085 to 0.8169**	**<0.0001**
	AST (UI/g prot)	−0.122	−0.4458 to 0.2298	0.485	**−0.336**	**−0.6084 to 0.0071**	0.048
	ALT (UI/g prot)	0.017	−0.3276 to 0.3569	0.925	−0.268	−0.5592 to 0.08160	0.119
	LDH (UI/g prot)	−0.254	−0.5487 to 0.09661	0.141	−0.158	−0.4744 to 0.1952	0.366
	CK (UI/g prot)	0.053	−0.2951 to 0.3879	0.764	**−0.377**	**−0.6372 to −0.04**	**0.026**
	CREA (mg/g prot)	−0.135	−0.4560 to 0.2176	0.440	**0.526**	**0.2244 to 0.7360**	**0.001**
	PROT (mg/dl)	0.214	−0.1384 to 0.5184	0.217	**−0.538**	**−0.7431 to −0.2393**	**0.001**
	AMYL (UI/g prot)	−0.112	−0.4373 to 0.2398	0.523			
	CHOL (mg/g prot)	−0.142	−0.4615 to 0.2110	0.418	**0.591**	**0.3121 to 0.7764**	**<0.0001**
	TRIGL (mg/g prot)	−0.115	−0.4399 to 0.2368	0.511	**0.503**	**0.1935 to 0.7208**	**0.002**
	CA (mg/g prot)	−0.181	−0.4927 to 0.1721	0.299	**0.586**	**0.3044 to 0.7730**	**<0.0001**
	EA (UI/g prot)	−0.223	−0.5255 to 0.1289	0.197	**0.563**	**0.2730 to 0.7589**	**<0.0001**
	ADA (UI/g prot)	−0.115	−0.4399 to 0.2368	0.511	−0.087	−0.4170 to 0.2631	0.619

Protein levels showed a significant increase compared to control only at the 6 h post-stressor in both matrices (1.2- and 6.4-fold for plasma and skin mucus, respectively), presenting control levels at the other time-points. A well-known response mechanism of fish to stress is the increase in the production of skin mucus [32]. Although many different proteins can be found in skin mucus, they are mainly high molecular weight glycoproteins called mucins [33], which can be disrupted in response to a wide range of injuries or challenges [34]. The increased protein content in skin mucus could be due to an increase in mucus and mucins production as a stress response or defence mechanism. Moreover, other proteins present in skin mucus could be involved in this increase, as the main defence components in skin mucus are proteins [35,36], and this could lead to an interesting continuity of research in this direction. Previous research has pointed out that specific stresses can be associated with the increase of specific proteins in different fish

species. For instance, an increase in lectins and cytokeratins has been related to thermal stress in turbot [37], transportation stress is associated with an increase in the production of sulphated mucins in channel catfish (*Ictalurus punctatus*) [38], and sea lice infestation increases the abundance of lectins in Atlantic salmon (*Salmo salar*) [39]. The increase in plasma proteins could be attributed to cell damage caused to different tissues during air exposure, resulting in the release of specific proteins to the bloodstream.

LDH and CK levels did not change significantly, and CK was slightly correlated with cortisol in skin mucus. Regarding hepatic enzymes, ALP remained unaltered in both matrices except for T = 1 h in skin mucus, which was 3.1-fold higher in comparison to in the controls. When compared to initial values, AST in plasma reported a significant increase at 6 and 24 h post-stressor (2.5- and 2.3-fold higher, respectively). On the contrary, AST in skin mucus showed a decrease compared to controls at all post-stress sampling time-points (0.4-, 0.5-, and 0.2-fold lower values, for 1, 6, and 24 h post-stress), needing further research to clarify the reasons for this decrease. Both in plasma and skin mucus, ALT levels remained unaltered in all sampling conditions compared to the control. These biomarkers are present in hepatic cells, and their levels increase in cases of liver damage. However, ALP and AST are present in high concentrations in other tissues different than the liver, such as skeletal muscle and may therefore point out damage at different levels. The lack of change in CK (more specific of muscular damage) suggests no damage in skeletal or cardiac muscle. Therefore, the modulation observed in AST and ALP may suggest that the exposure to air caused alterations in fish, although it cannot determine the specific tissue damage. Previous studies have reported changes in hepatic *avtrs* and *itr* gene expression were found after 3 min of air exposure [40]. None of these parameters correlated significantly with plasma cortisol, although skin mucus ALP and AST correlated significantly with cortisol measured in skin mucus, although the correlations were low to very low (Table 1).

When compared to the control, creatinine values showed a statistically significant 1.2-fold increase in plasma at 1 and 6 h post-stressor, regaining basal levels at 24 h post-stressor. By contrast, in skin mucus creatinine remained unaltered. Creatinine was positively correlated with cortisol in plasma, and creatinine measured in both matrices was positively correlated with cortisol in skin mucus, although these correlations were low. Similar to in mammals, creatinine in fish is produced in muscle. It is also employed as a marker of renal damage since it is excreted via glomerular filtration [41]. Therefore, the increase in creatinine could result from muscular damage or a reduced glomerular filtration rate. By contrast, the other metabolic biomarkers analysed (amylase, cholesterol, and triglycerides) did not show statistically significant changes compared to controls in either plasma or skin mucus. However, cholesterol measured in both matrices and triglycerides in skin mucus were positively correlated (low correlation) with cortisol levels in skin mucus. As commonly performed in literature, our observations stopped 24 h after exposure to the stressor. Thus, we did not evaluate if there was a delay in biomarker recovery in skin mucus with respect to plasma but focused on the possible correlations presented between both matrices at the same time point. Therefore, further studies are desirable to discern the possible biological significance of our findings.

Calcium levels increased in plasma at both 1 (1.2-fold) and 6 h (1.1-fold) post-stress and in skin mucus 6 h post-stressor. When compared to control levels, phosphorus increased in plasma at 1 and 6 h post-stressor (2.0-fold higher in both cases), while in skin mucus, phosphorus levels were below the limit of detection of the assay. A previous study reported an increase of calcium, chloride, and sodium in the plasma of bonefish *Albula vulpes* 1-, 2-, and 4 h after air exposure [42], confirming the alteration of some plasma inorganic elements after air exposure; however, phosphorus was not measured in the study. On the other side, no changes in calcium were reported after 3 and 5 min air exposure in pacu (*Piaractus mesopotamicus*) [43]. Both calcium and phosphorous in plasma were positively correlated with plasma cortisol. Moreover, calcium in both matrices positively correlated with skin mucus cortisol as well. Therefore, these results suggest that air exposure for

3 min causes an increase in the measured inorganic elements in plasma, returning to basal levels after 24 h, and these increments were correlated with cortisol in both matrices.

Regarding oxidative stress, TOS in plasma significantly increased (2.3-fold) at 6 h post-stressor compared to control and was correlated with plasma cortisol. The TOS in skin mucus provided results below the lower limit of detection of the assay. EA appeared unaltered for all conditions, both in plasma and skin mucus, although in skin mucus EA was positively correlated with cortisol. These results may suggest an increase in oxidants after 6 h and that the EA enzyme is probably not involved in the protection against the oxidative stress induced by the air exposure. Similarly, increases in TOS with no changes in EA were observed in *Sparus aurata* challenged with pharmaceutical gemfibrozil [44].

Circulating ADA levels were statistically significantly higher at 1 and 6 h post-stressor (4.6- and 3.0-fold, respectively) compared to the control group, regaining basal levels at 24 h. Furthermore, ADA in plasma was positively correlated with plasma and skin mucus cortisol levels. Meanwhile, ADA remained unaltered in skin mucus for all sampling time-points. In humans, ADA increases after inflammation [45] or diseases that cause increases in T lymphocytes [46,47], being suggested as a biomarker of cell-mediated immunity [48,49] and inflammation [50]. In silver catfish naturally infected with *Ichthyophthirius multifiliis*, a downregulation of serum ADA activity was observed, contributing to restricting the inflammatory process [51]. In zebrafish (*Danio rerio*) brain, deaminase activity levels and gene expression patterns have shown alterations after chronic ethanol exposure [52] and when exposed to unpredictable chronic stress [53]. In fish exposed to 90 min of restraint stress, the increase in adenosine-mediated signalling was proposed as a possible strategy to re-establish homeostasis and normal behaviour after a stressful event [54].

When the correlations between plasma and skin mucus were determined for each biomarker (Table 2), three of them showed a correlation with statistical significance. Cortisol measured with RIA and CIA showed a positive correlation (r = 0.44 and r = 0.48, respectively) between the two matrices, and so did cholesterol (r = 0.405). In contrast, the correlation found for CK between plasma and skin mucus was negative (r = −0.52). In addition, p values were <0.1 in three additional biomarkers (ALP r = 0.28, p = 0.098; AST r = −0.294, p = 0.087; and LDH r = −0.315, p = 0.065), and further studies with a higher number of samples would be convenient to confirm or deny the existence of a correlation of significative relevance between the two matrices. In a study with meagre, Fernández-Alacid et al. also found positive correlations between plasma and skin mucus parameters in meagre after hypoxia stress, reporting correlations in glucose, lactate, cortisol and proteins [54]. Altogether, the results suggest that skin mucus can be sampled as an alternative to plasma, since most biomarkers showed a similar behaviour in both matrices.

Table 2. Spearman correlation for each biomarker, between plasma and skin mucus, of glucose (GLUC), alkaline phosphatase (ALP), aspartate transaminase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), creatinine kinase (CK), creatinine (CREA), amylase (AMYL), cholesterol (CHOL), triglycerides (TRIGL), calcium (CA), esterase activity (EA), and adenosine deaminase (ADA). * In bold statistically significant differences after applying the False Discovery Rate following the Benjamini and Hochberg method ($p < 0.05$).

	Plasma			**Skin Mucus**			**Spearman**		
	Median	**25% Percentile**	**75% Percentile**	**Median**	**25% Percentile**	**75% Percentile**	**r**	**95% Confidence Interval**	***p* ***
Cortisol RIA (ng/g)	30	7.775	74.48	1538	929.4	2963	**0.441**	**0.1166 to 0.6808**	**0.008**
Cortisol CIA (ug/g)	3.285	0.6873	20.58	3.7	2.3	6	**0.476**	**0.1593 to 0.7034**	**0.004**
GLUC (mg/g)	115.3	85.43	190.5	34.8	17.6	55.7	−0.013	−0.3537 to 0.3309	0.941
ALP (UI/g)	151	122.8	196.7	36.6	23.4	50	0.284	−0.06452 to 0.5709	0.098
AST (UI/g)	732.8	570.5	1261	89.14	35.98	139.4	−0.294	−0.5780 to 0.05388	0.087
ALT (UI/g)	29.85	14.95	106.1	1.7	0	4.7	0.132	−0.2199 to 0.4541	0.448
LDH (UI/g)	5935	3716	10398	1416	670.6	1943	−0.315	−0.5934 to 0.03048	0.065
CK (UI/g)	3693	1200	13200	34.6	17.7	86	**−0.520**	**−0.7318 to −0.2157**	**0.001**
CREA (mg/g)	0.25	0.19	0.4	2.19	0.36	4.56	0.005	−0.3375 to 0.3471	0.975
AMYL (UI/g)	1764	1325	2314	119.1	53.9	152.6	−0.147	−0.4659 to 0.2056	0.399
CHOL (mg/g)	307	260.9	356.2	72.2	33.9	97.3	**0.405**	**0.07322 to 0.6566**	**0.016**
TRIGL (mg/g)	148	115.9	220.3	131.1	60.7	478.8	−0.242	−0.5398 to 0.1091	0.161
CA (mg/g)	9.84	9.575	11.87	120.6	44.3	175.1	−0.096	−0.4246 to 0.2545	0.582
EA(UI/g)	7.18	6.47	9.05	322.6	152.8	470	0.039	−0.3123 to 0.3814	0.826
ADA (UI/g)	72.2	47.15	196.4	135.2	39.2	323.4	0.070	−0.2952 to 0.4180	0.702

The PCA analysis (Figure 2), performed using all plasma and skin mucus data, revealed that TOS, glucose, and cortisol in plasma and ALP, AST, and glucose in skin mucus were the biomarkers that explained the major amount of total variance and contributed the most to group differentiation. When only plasma data were evaluated, there was a slight overlapping among stressed and non-stressed animals, being again TOS, glucose, and cortisol the most contributing biomarkers to group separation. Finally, when skin mucus data were studied, ALP, AST, glucose, cortisol, and CK were the most contributing biomarkers, allowing again a complete separation between the two experimental fish groups. These data show a generalised effect of stress in fish affecting multiple systems and spot the drawbacks of just determining plasma analytes. In addition, they highlight the usefulness of skin mucus as a complementary matrix.

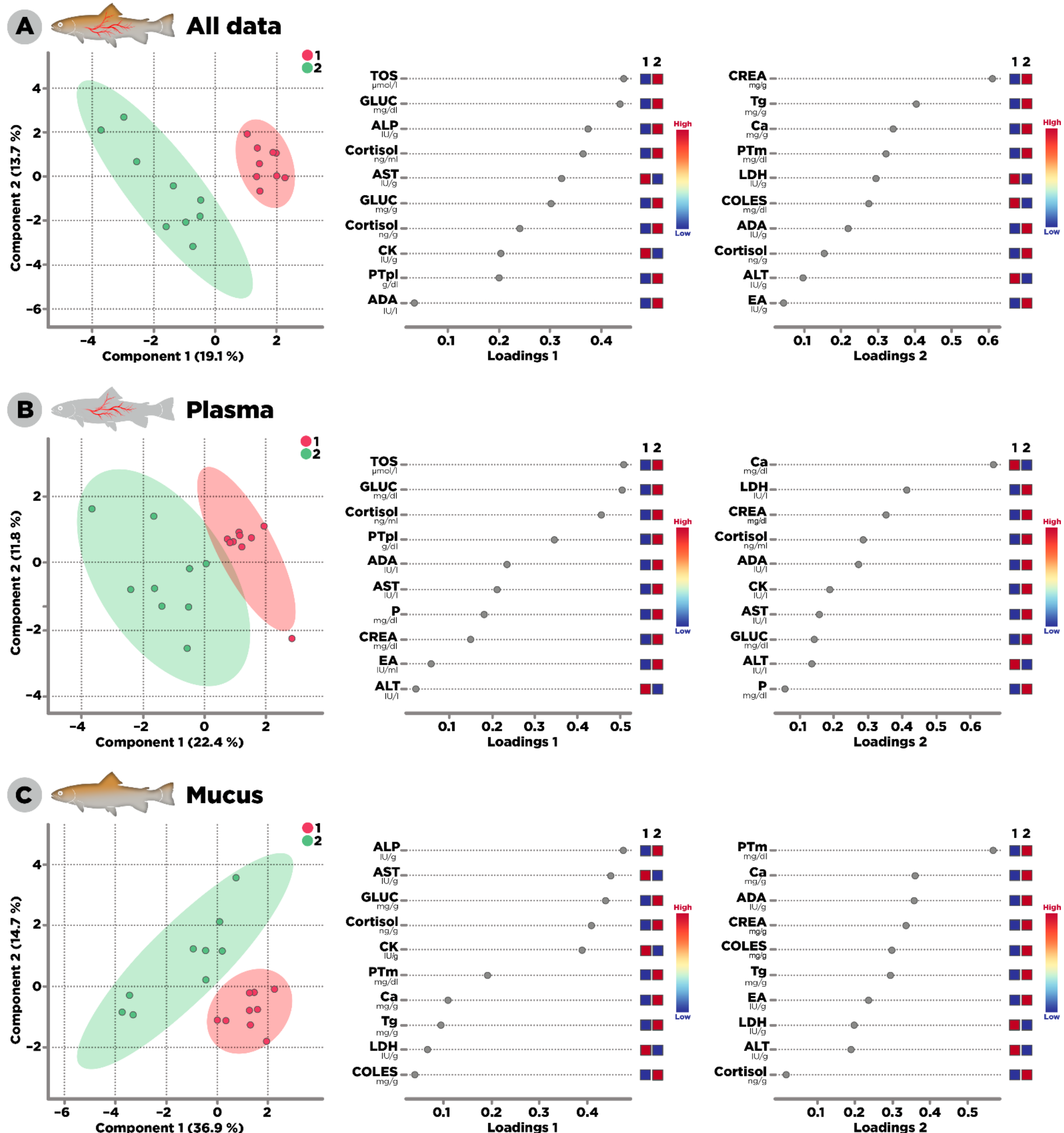

Figure 2. Sparce principal component analysis (sPCA) based on the biochemical parameters analysed. (**A**) sPCA analysis including all data. (**B**) sPCA analysis for data obtained from plasma. (**C**) sPCA for data obtained from mucus. The graphs on the left column represent Variable Importance in Projection (VIP) in two loadings (loading 1 is for component 1; loading 2 is for component 2) with 10 variables per component and 5-fold CV validation. Green area: control animals; and red area: stressed animals. For the graphs represented on the central and right column, the blue square represents low values, meanwhile the red square denotes high values, as indicated on the grade scale colour on the right of each graph. The array of parameters measured in plasma and skin mucus were as follows: glucose (GLUC), alkaline phosphatase (ALP), aspartate transaminase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), creatinine kinase (CK), creatinine (CREA), calcium (CA), phosphorus (P), proteins plasma (PTpl), proteins plasma (PTm), cholesterol (COLES), triglycerides (Tg), esterase activity (EA), total oxidative status (TOS), and adenosine deaminase (ADA).

Altogether, the usefulness of skin mucus as a low-invasive matrix for the assessment of stress in rainbow trout was evaluated in this study. After measuring some biomarkers in skin mucus, differences were observed between control (unstressed) and fish subjected to an acute stress by air exposure. In conclusion, our results show that, together with plasma, skin mucus can be used an additional matrix for stress assessment in fish, since several biomarkers present a different pattern of response in both assessed matrices.

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