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Comparison between properties of dorsal and ventral skin mucus in Senegalese sole: response to an acute stress

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ABSTRACT

Intensive culture of Senegalese sole, *Solea senegalensis,* represents an option to diversify Mediterranean aquaculture. As a flatfish species, the fish body presents marked asymmetry between the dorsal or ocular side and the ventral or blind side. Taking into account that different surroundings come into contact with each side, in the present study we aimed to compare the properties of skin mucus from both sides, which is the most external barrier and protection for the fish. Skin mucus has recently been proposed as a novel minimally invasive target for the study of physiological responses. Here, a group of ten Senegalese sole were used to measure basal mucus parameters; then four additional groups of ten specimens each were used to study a hypoxia stress challenge (post-stress groups: 15min-PS, 1h-PS, 6h-PS and 24h-PS), consisting of a 3-minute air exposure. Physicochemical parameters (viscosity, osmolality, pH and main osmotic ions) were analysed in dorsal and ventral mucus. Thereby, mucus components related to stress responses such as cortisol, glucose, lactate, soluble protein and antioxidant power, were measured and compared between the mucus types and in response to hypoxia. Rheograms revealed similar non-Newtonian behaviour of dorsal and ventral mucus viscosities, which suddenly increased in the 15min-PS group, thus providing fish with immediate insulating and mechanical protection. However, ventral side viscosity of the 24h-PS group was lower than basal values and its functionality was compromised. Whereas chemical parameters did not differ between sides or in response to hypoxia, components that are soluble in mucus showed asymmetry with soluble protein and lactate being twofold higher in ventral mucus. The classical stress response of increased plasma cortisol accompanying glucose and lactate release was observed. In parallel, mucus glucose and lactate showed rapid but transitory exudation in the 15min-PS group, while cortisol was detected at minimal levels in skin mucus, and therefore is not recommended as a mucus indicator for this species. Mucus-soluble protein also rose in the 15min-PS group, which indicates greater exudation of defensive-related proteins. Finally, the antioxidant power of

dorsal mucus increased and accumulated, without returning to basal levels. All these findings indicate that flatfish skin mucus properties depend on animal side, and further studies will be necessary to elucidate whether other mucus defensive parameters show this asymmetry and their relevance in these benthonic species.

INTRODUCTION

Senegalese sole, *Solea senegalensis*, represents a new option for south-European aquaculture, since it has a high commercial value and occupies different market areas from gilthead sea bream or European sea bass, thus it does not compete with these species. Nevertheless, production of Senegalese sole has failed to reach successful commercial development over the last two decades, mostly due to high incidences of disease (Morais et al., 2016). In addition to high vulnerability to diseases, other problems that have been reported are difficulties in weaning, a decreased growth rate at high stocking densities, pigmentation abnormalities and malformations related to eye migration (Dinis et al., 1999). As a demersal marine flatfish living on sandy or muddy bottoms, Senegalese sole presents an asymmetric body with the eyes on the right side. Thus, a classical differentiation is made between the ocular or dorsal side, with dark-brown camouflage pigmentation; and blind or ventral side, with whitish or yellowish coloration. However, little is known of the skin mucus, which is the first line of defence against the surrounding environment, or whether differences exist in its functionality when comparing the dorsal and ventral sides of flatfish.

Skin mucus acts as a dynamic and semipermeable barrier that performs a number of functions in fish, such as osmoregulation, respiration, nutrition or locomotion (Esteban, 2012; Negus, 1963; Sanahuja and Ibarz, 2015; Shephard, 1994; Subramanian et al., 2007, 2008). In common with other mucus secretions, fish skin mucus is a hydrated gel mainly of mucopolysaccharides, mucins, which are the insoluble fraction and are responsible for rheological properties. Recently, it has been demonstrated that exuded fish skin mucus contains components related to defence and metabolism (Cordero et al., 2015; Patel and Brinchmann, 2017; Pérez-Sánchez et al., 2017; Rajan et al., 2011; Sanahuja and Ibarz, 2015). In Senegalese sole, Chabrillon et al. (2005a,b) reported the capacity of different microorganisms isolated from farmed fish to adhere to sole mucus, and Mabrok et al. (2016) evaluated mucus and plasma bactericidal activities against *T. maritimum*. Guardiola et al. (2017) reported the presence of several lectin forms and immune-related enzymes providing sole mucus with bactericidal activity. With regards to the skin mucosa, several studies have reported that the sole epidermis is composed of stratified epithelium containing three cellular layers: the outermost or mucosa layer, the middle or fusiform layer and the stratum germinativum or basal layer (Sarasquete et al., 1998, 2001). In the mucosa, two mucous cell types are differentiated: type A cells containing several round vesicles of different electron density, and type B

cells containing mucosomes of uniform electron density (Arellano et al., 2004). However, there are no reports of specific ventral and dorsal characteristics for skin or skin mucus of Senegalese sole or of differences between them, which also should be strongly related to mechanical protection for the different environment of each side.

Fish in aquaculture are often exposed to husbandry-related acute and chronic stressors which induce physiological alterations, the responses to which are specific to the stress imposed. Responses to different stressors have been evaluated in Senegalese sole: acute hypoxia and netting (Costas et al., 2011), chronic handling (Aragão et al., 2008), high stocking densities (Costas et al., 2008; Salas-Leiton et al., 2010), osmotic challenge (Arjona et al., 2007, 2009; Aragão et al., 2010) and temperature acclimation (Arjona et al., 2010). Most studies report cortisol increase, as well as glucose and lactate release in plasma, as stress indicators; as reported in other teleost fish (Mommsen et al., 1999). Recently, it has been observed that the components of exuded mucus are also modified in response to stressors (Cordero et al., 2015; Patel and Brinchmann, 2017; Pérez-Sánchez et al., 2017; Rajan et al., 2011; Sanahuja and Ibarz, 2015), with some of the stress indicators, such as cortisol, glucose and lactate, being proposed as feasible non-invasive biomarkers (Guardiola et al., 2016; Fernández-Alacid et al., 2018, 2019; De Mercado et al., 2018). Other physico-chemical parameters such as viscosity, conductivity or osmolarity are also used to compare mucus properties between species. Thus, Guardiola et al. (2015, 2017) found that protein amounts, osmolality and density values correlated with skin mucus viscosity. Moreover, ion composition and gradients between the surrounding water and mucus would offer a reduced ion gradient to the plasma, thereby reducing the cost of ion transport (Handy et al., 1989). However, no data exists on the response of these parameters comparing both sides of sole skin nor on the response to stressors.

In the view of the fact that Senegalese sole shows a high potential for the diversification of Mediterranean aquaculture, its ocular and blind asymmetry could be relevant for the use of skin mucus as a valuable non-invasive tool to evaluate the time-course of the mucus response to acute hypoxia stress. So, here, rheological analysis of mucus viscosity was performed to determine mechanical protection and insulation. Moreover, chemical parameters such as osmolality, pH and the main osmotic ions were analysed to determine the effects on osmoregulation. Furthermore, mucus metabolites, total soluble proteins and cortisol, together with antioxidant power, were measured as stress-related biomarkers. All the findings of this study contribute to knowledge of flatfish skin mucus which could be useful to aquaculture.

MATERIAL AND METHODS

Animals and experimental procedures

Senegalese sole (n=50**)** from the Olhão Pilot Fish Farming Station (EPPO-IPMA) were kept at the IFAPA Centro Agua del Pino facilities (Huelva, Spain). Juveniles with a body weight of 550 ± 20 g were reared in a flow-through system at 19°C ± 1ºC, at a stock density of 3 kg · m^{-3}, keeping water oxygen levels > 90% of saturation and they were fed commercial feed (Skretting L-4 Alterna). Throughout the experiment water quality was periodically checked according to Herrera et al. (2015, 2016). Briefly, several key ion concentrations were measured by Spectroquant kits (MERCK, Darmstadt, Germany) following the manufacturer's recommendations, and maintaining the following values (mg · L^{-1}) in experimental tanks: nonionized ammonia < 0.001; total ammonia < 0.1; total nitrite < 0.02; total nitrate < 0.02; microbial load values were below 20 cfu · mL^{-1}. pH (7.9 ± 0.05) and salinity (35.6 ± 0.06) were monitored through multiparametric sensors. Acute hypoxia stress was induced by exposing the animals to air for 3 min, and then returning them to their tank. Basal (non-stressed) values were sampled from 10 fish kept at rest in water. A total of 50 fish were sampled: the 10 basal animals; and then 10 fish 15 min post-stress (PS), 10 fish 1 h PS, 10 fish 6 h PS and 10 fish 24 h PS (15min-PS, 1h-PS, 6h-PS and 24h-PS groups, respectively).

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

Sample collection

Fish were anaesthetised with 2-phenoxyethanol (200 ppm, Sigma-Aldrich, Spain) to avoid the stress of manipulation. Skin mucus was immediately collected following the method described in Fernández-Alacid et al., (2018). In order to cause the least stress and harm to the animals, mucus collection was a very fast process (less than 2 min). Skin mucus was collected on sterile glass slides from the over-lateral line in a front-to-caudal direction: a sterile slide was gently wiped along both sides of the animal (dorsal and ventral) two or three times, and the epidermal mucus was carefully pushed and collected in a sterile tube (2 mL), taking care to avoid contamination with blood and/or urino-genital and intestinal excretions. Raw mucus samples of 500 mL were used for viscosity analysis and the rest of the mucus was homogenised using a sterile Teflon implement to desegregate the mucus mesh before centrifugation at 14,000 g. The resultant mucus supernatants were collected, avoiding the surface lipid layer, aliquoted and stored at -80ºC. Blood samples were collected from the caudal vein with an insulin syringe. The plasma was collected after centrifugation (13,000 g for 30 min at 4ºC) and stored at -80ºC until use.

Viscosity analysis

For the analysis of viscosity properties, five fresh samples (without homogenization) were analysed following the method described in Fernández-Alacid et al. (2018). Viscosity was measured in 500 µL aliquots with a cone-plate CP-52 viscometer (cone angle 0.8°, model DV-III programmable rheometer, Brookfield Ametek, USA). To obtain a characteristic profile, viscosity was measured over a range of five different shear rates (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 taken after 1 min of shear stress application. Thus, the relative viscosity of the 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 against the square root of the 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 fitting program (CurveExpert 1.3, Copyright Daniel Hyams). The best-fit model was based on the squared correlation coefficient (R^2).

Osmolality, pH and ion concentrations

Osmolality was measured using a freezing-point osmometer (Advanced Micro Osmometer model 3300, Advanced Instruments Inc, USA). Briefly, 20 µL of dilute mucus sample was tested using the osmometer. Osmolality was expressed in mOsm · kg^{-1}. Ion concentrations and pH were measured using an electrolyte analyser (ISElyte-X9, Tecil, Spain). Briefly, 100 µL of the dilute mucus sample was absorbed by the machine and potassium (K^+), sodium (Na^+), chloride (Cl^-), and total calcium (Ca^{2+}) contents were analysed. Ion concentrations were expressed in mmol · L^{-1}.

Ferric Reducing Antioxidant Power (FRAP)

Ferric antioxidant status detection measures antioxidant power by gauging the capacity of antioxidants to convert ferric ions to ferrous ions. The FRAP was determined by an enzymatic colorimetric test (Ferric Antioxidant Status Detection kit, Invitrogen, Spain). Following the manufacturer's instructions for plasma determinations but with slight modifications, 20 µL of mucus extract or standard solutions (from 0 to 1000 µM · μL^{-1} of $FeCl^2$) was mixed, in triplicate, with 75 µL of FRAP colour solution and incubated for 30 min at room temperature. The OD was determined at λ = 560 nm with a microplate reader (Infinity Pro200 spectrophotometer, Tecan, Spain). Antioxidant values were expressed as nmol of FRAP · mL^{-1} of skin mucus, and nmol of FRAP· mg^{-1} of mucus protein.

Metabolites, total soluble proteins and cortisol analysis

Plasma glucose and lactate concentrations were measured using commercial kits from Applied Analytical Chemistry S.A. (QCA Liquid Glucose) and Spinreact (Lactate Ref. 1001330) adapted to 96-well microplates. The protein concentration was determined using the Bradford assay (Bradford, 1976) with bovine serum albumin (BSA; Sigma) as the standard. Cortisol concentration in plasma was quantified using an ELISA kit (EA65, Oxford Biomedical Research, USA) modified and adapted for fish (Herrera et al., 2016). For extractions, the plasma was diluted with diethyl ether (1:10). After decanting, the supernatant was transferred to another tube and the diethyl ether was evaporated using nitrogen gas. Then, the remaining substance was diluted (1:6) with an extraction buffer supplied by the manufacturer and the resultant dilution constituted the sample to be analysed. The lower limit of detection for this ELISA assay is 0.1 ng · mL^{-1} (81% binding). The inter- and intra-assay coefficients of variation are 9.8% and 4.6% respectively, with an average recovery of 90%.

Mucus-soluble metabolites, total soluble proteins and cortisol were determined from the mucus extracts. To analyse the total amount of soluble protein, the samples were diluted 1:10 (v:v) in BCA (ThermoFisher) and mixed with $CuSO^4$ (50:1, v:v); to avoid aggregation, the samples were warmed for 3 min at 55ºC. The OD was determined at λ = 562 nm. Glucose and lactate concentrations were determined by the respective enzymatic colorimetric tests (SPINREACT®, Barcelona, Spain) following the manufacturer's instructions for plasma determinations, but with slight modifications. The OD was determined at λ = 505 nm with a microplate reader (Infinity Pro200 spectrophotometer, Tecan, Spain). The glucose and lactate values were expressed as µg · mL^{-1} of skin mucus and µg · mg^{-1} of mucus protein. Cortisol levels were measured using an ELISA kit (IBL International, Germany) as previously described for fish mucus (Fernández-Alacid et al., 2018, 2019). Briefly, following the manufacturer's instructions, 50 µL of mucus extract or standard solution was mixed with enzyme conjugate (100 µL) and incubated for 2 h at room temperature. The substrate solution (100 µL) was added after rinsing the wells with a washing solution, and incubated for 30 min. The reaction was stopped by adding 100 µL of stop solution

and the OD was determined at $\lambda = 450$ nm with a microplate reader (Infinity Pro200 spectrophotometer, Tecan, Spain). The cortisol values were expressed as ng cortisol · mL^{-1} of skin mucus and ng · g^{-1} of mucus protein.

Statistical analysis

Viscosity data from Casson's model equations were compared at each shear rate between basal, and 15 min, 1 h, 6 h and 24 h PS using one-way ANOVA. Data for all the compounds are presented as mean values ± standard error of mean (SEM) and the differences during the time-course were analysed by one-way ANOVA (with Bonferroni's test). Comparisons between dorsal and ventral mucus were also performed at each time point by Student's t-tests. Differences were considered statistically significant at $p < 0.05$. All statistical analysis was performed using SPSS Statistics for Windows, Version 22.0 (IBM Corp.; Armonk, USA).

RESULTS

Comparison between dorsal and ventral mucus

Raw skin mucus samples from dorsal and ventral sides were analysed for viscosity; whereas soluble mucus extracts were used to determine osmotic parameters and components (glucose, lactate, protein, cortisol and antioxidant power). Rheograms revealed similar non-Newtonian behaviour for dorsal and ventral mucus (Figure 1), meaning that it was shear-dependent: the viscosity decreased as the shear rate increased, exhibiting markedly pseudoplastic behaviour (Fig. 1A). No significant differences were observed between the viscosity of dorsal and ventral mucus at any of the shear rates used in this analysis (4.50 s^{-1}, 11.25 s^{-1}, 22.5 s^{-1}, 45 s^{-1} and 90 s^{-1}). However, ventral mucus showed higher viscosity at all shear rates. To improve the comparison of viscosity parameters, the creep threshold was evaluated by adjusting the experimental data to Casson's model equation (Fig. 1B). 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. Whereas the intercept point was similar for dorsal and ventral mucus, a clearly steeper slope (2.9 vs 4.8) was evident for ventral mucus, indicating its greater resistance to deformation (or movement) by friction.

Comparisons of the chemical properties of Senegalese sole mucus, such as osmolality, pH and the main ion concentrations (chloride, sodium, total calcium and potassium), are provided in Table 1. Osmolality values were around 1100 mOsm · kg^{-1}, with no differences between animal sides; and the sum of the main ions precisely approached the mucus osmolality values. Meanwhile, the pH values strictly ranged between 7.30 and 7.31. The most important compounds in fish skin mucus, soluble protein determinations, and the amounts of glucose and lactate, are also shown in Table 1. Levels of soluble

protein in ventral mucus were double those of the dorsal side (451 ± 41 vs 200 ± 33 μg · mL^{-1}, respectively, $p < 0.05$), in agreement with the higher viscosity of ventral mucus. Similarly, glucose and lactate amounts per mL of skin mucus were higher in the ventral side; although when expressed per mg of mucus protein, the differences between types of mucus were attenuated. Mucus cortisol levels, another interesting parameter in fish skin mucus, were at the limit of analytical detection, and did not show any differences with respect to the two sides of the animal. Finally, antioxidant power, measured as FRAP, was also determined. Although no differences were obtained per mL of mucus between sides, when expressed per mg of protein, the antioxidant power of the ventral side was significantly lower than for the dorsal side.

Response of plasma parameters to acute hypoxia stress

Table 2 shows the time-course response of plasma glucose, lactate and proteins, as well as the cortisol levels. The stress response to the induced 3 min hypoxia was evaluated 15min-PS and 1 h-PS, as the immediate stress response, and 6h-PS and 24h-PS, to determine the recovery capacity of these parameters. The immediate response to the hypoxia was a significant increase of plasma glucose and lactate, which doubled their circulating levels ($p < 0.05$) just 15min-PS. Whereas plasma glucose levels remained high 6h-PS, lactate levels diminished to basal levels, indicating the transient effect of hypoxia. Surprisingly, plasma cortisol showed basal levels that were more elevated than expected, and did not correspond to the lower 24h-PS levels. Excluding the initial values, there was a clear post-stress response of increased plasma cortisol 15min-PS and 1h-PS (with the highest values at this latter time point, $p < 0.05$) and cortisol amounts reverting 6h-PS to the lower 24h-PS values ($p < 0.05$). With regard to plasma proteins, no changes were recorded during the post-stress period.

Response to acute hypoxia stress of dorsal and ventral mucus properties and composition

At each post-stress time point, mucus from the dorsal and ventral sides was obtained to determine the effects of the induced stress, and to evaluate differences in physical properties (viscosity), chemical properties (osmolality, pH and ions) and components (antioxidant power, metabolites, total soluble proteins and cortisol) between the responses of the two types of mucus.

The immediate effect of hypoxia on skin mucus was an increase of the volume of mucus collected with respect to basal values (visual observation). The viscosity study, rheograms and Casson's linearization and equations, showed a significant increase of physical properties of both types of exuded mucus 15min-PS with respect to basal values (Figure 2). The dorsal mucus viscosity increased for all shear rates; although it was only significant for higher shear rates, resulting in an increase in both intercept point and slope of Casson's regression equation, compared with basal values. The ventral mucus viscosity also increased, and although 15min-PS it presented the highest values recorded for any mucus, differences with basal values were only significant for the 4.5 s^{-1} shear rate. These differences in physical

properties only 15min-PS evidence the immediate changes on mechanical and insulating protection provided by mucus on both sides of the animal. The time-course study also demonstrated that the skin mucus viscosity rapidly reverted to basal values 1h-PS; it even diminished gradually at later post-stress times, with the ventral mucus viscosity 24h-PS being significant lower at lower shear rates. These data indicate the rapid renewal of mucus, between 15min-PS and 1h-PS, offering lower mechanical protection 24h-PS with the lowest intercept points (the yield stress) for mucus from both sides of the animal.

Figure 3 summarises the time-course response of mucus osmolality and pH values. Neither chemical parameter showed effects of the induced hypoxia, nor did the composition of the major ions (data not shown due the lack of changes with respect the basal concentrations). Despite the great changes in skin mucus physical properties, our data on osmolality and pH indicate that the capacity for ion adsorption to mucus was not modified by this stressor condition. Skin mucus soluble glucose and lactate responded to hypoxia as in plasma: with an immediate significant increase of their amounts 15min-PS (Fig. 3C and 3D, respectively). These increments were only transient and 6h-PS both metabolite amounts reverted to basal levels in both mucus sides. Mucus soluble protein (Fig. 3E) also experienced an acute response 15min-PS, being significantly increased for dorsal mucus, where its concentration was doubled ($p < 0.05$) with respect to the previous basal levels. Again, these immediate effects reverted just 1h-PS. These results match those observed for the physical properties. Contrary to the plasma level results, mucus cortisol did not change throughout the post-stress time-course (Fig. 3F). The mucus antioxidant power (Fig. 3G) acutely increased 1h-PS for dorsal mucus, increasing its concentration threefold; this was partially reverted 6h-PS and 24h-PS. Because of the changes in soluble protein observed in both types of mucus, when referring metabolites, cortisol and antioxidant power per mg of mucus protein (Fig. 4), some of the observed changes in glucose and lactate were attenuated. In contrast, mucus antioxidant power gradually increased until 6h-PS, and did not revert 24h-PS.

DISCUSSION

As a flatfish with an asymmetric body, Senegalese sole shows anatomical and functional differences between its blind side (ventral) and ocular side (dorsal). Recently, fish skin mucus has been proposed as a novel target for physiological studies and a means to conduct minimally invasive monitoring of several fish species (Rajan et al., 2011; Cordero et al., 2015; Sanahuja and Ibarz, 2015; Guardiola et al., 2014, 2016; Patel and Brinchmann, 2017; Pérez-Sánchez et al., 2017; Fernández-Alacid et al., 2018, 2019), including Senegalese sole (Guardiola et al., 2017). The current study aimed to determine if differences exist in physicochemical properties and the composition of mucus from both body sides of flatfish species due to their asymmetry. Additionally, we studied the time-course response of both mucus sides to an induced acute stress, air exposure for 3 min (hypoxia), at 15min-PS, 1h-PS, 6h-PS and 24h-PS. The

plasma parameters studied as indicators of primary (cortisol) and secondary (glucose and lactate) stress responses confirmed the classical stress response of an acute increase in plasma cortisol and concomitant glucose and lactate release. The time-course dynamics were in agreement with the previous observations of Costas et al. (2011) in this species, and in the most fish, where cortisol and glucose reach their highest concentrations after 0.5–1 h; although plasma levels are stressor dependent and species specific (Martinez-Porchas et al., 2009; Pankhurst, 2011).

Comparison of physicochemical mucus properties and response to acute stress

The physicochemical characteristics of fish skin mucus define it as a viscous fluid with adhesive, viscoelastic and rheological properties, functions which are mainly attributed to its mucin contents and hydration (Shephard, 1994). Recently, we reported the viscosity of the raw skin mucus of three pelagic species: gilthead sea bream, European sea bass and meagre (Fernández-Alacid et al., 2018). The mucus showed clear non-Newtonian viscous behaviour, whereby it exhibited greater viscosity at lower shear rates than at higher ones, irrespective of species. Here, we measured the viscosity of both mucus sides of Senegalese sole, also showing this non-Newtonian behaviour. However, Casson's equations obtained for the dorsal and ventral mucus resulted in steeper slopes (K_i, plastic viscosity coefficient) of 2.8 and 4.7, respectively, than for those pelagic species, whose values ranged between 1.2 and 1.7. Changes in mucus physical properties, such as viscosity, were associated with swimming capacity in pelagic species. It has been 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 (Rosen and Cornford, 1971; Lebedeva, 1999; Roberts and Powell, 2005). This should not be observable in benthonic species, where mucus physical parameters would be strongly related to greater mechanical protection from the substrate. This is consistent with the apparent higher viscosity of ventral mucus observed here.

Under stressful situations, one of the most evident fish responses is the increase of skin mucus exudation (Shephard, 1994; Vatsos et al., 2010; Fernández-Alacid et al., 2018, 2019). In response to a 3 min hypoxia challenge, the skin mucus collected did increase, mainly 15min-PS and 1h-PS (visual observation). However, changes in skin mucus physical properties after an acute stress have not been reported before in fish. Rheograms obtained 15min-PS showed a sudden increase in viscosity at all shear rates, with a similar response in dorsal and ventral sides. Moreover, Casson's model provided a more objective approach, evidencing higher intercept points and steeper slopes for 15min-PS mucus than for basal mucus. Both parameters would indicate a higher yield stress (intercept point) and resistance to deformity (slope) for the immediately exuded mucus 15min-PS. Although no data existed on this phenomenon, it seems similar to the response observed in lungfish which survive drought by secreting

mucus that hardens into a protective shell around them (Fishman et al., 1986; Sturla et al., 2002). Interestingly, the response in sole was only transient and mucus viscosity reverted to basal levels rapidly, 1h-PS. Viscosity values even continued to decline with the 24h-PS value being the lowest; and considering its protection function, this could be harmful and would need to be considered in repetitive aquaculture acute stressors such as excessive handlings. We attributed the changes in viscosity to a sustained stimulus of skin mucus exudation, and the possible differences in the newly formed mucus replacing previously secreted mucus. In view of the current results in sole, it will be crucial to study mucus renewal rates under basal condition and in response to stress; as well as to analyse the effects of the distribution of secretory cells in the epidermis, in particular on mucus composition and renewal rate.

Mucus chemical properties were measured as the osmolality and main ion concentrations for mucus from both sides of the animal. Mucus osmolality values in the present study were around 1,100 mOsm · kg^{-1}, irrespective of the mucus side, similarly to those of other marine species (Guardiola et al., 2015). However, these results do not agree with a previous study of Senegalese sole (Guardiola et al., 2017) where half this osmolality was reported. Although there are no data on ionic composition in other species, the major ions measured here, chloride, sodium, total calcium and potassium, explained 95% of measured osmolality. Taking into account that typical seawater osmolality is around 1,000 mOsm · kg^{-1}, ion adsorption to sole mucus corresponded to that of the surrounding environment. However, chemical properties also seem to be temperature dependent and this would condition parameters such as osmolality and mucus density (Guardiola et al., 2015). In response to stress, no chemical parameters changed, thereby ruling out their dependence on hypoxia and the use of these parameters as hypoxia-related indicators.

Comparison of mucus metabolites, total soluble proteins, cortisol and antioxidant power in response to acute stress

Recent studies in fish skin mucus have demonstrated that mucus metabolites, such as glucose and lactate, and total soluble proteins, as well as mucus cortisol and antioxidant power, can be considered as good non-invasive biomarkers for evaluating fish physiological responses (Guardiola et al., 2016; De Mercado et al., 2018; Fernández-Alacid et al., 2018, 2019). Therefore, these components were used here to compare dorsal and ventral mucus and their response to hypoxia stress. Soluble protein, glucose and lactate showed higher concentrations in ventral mucus under basal condition. In sole mucus, Guardiola et al. (2017) reported soluble protein levels in the same range (0.2-0.8 mg · mL^{-1}), but lower than for sea bream, sea bass and meagre (around 1, 8 and 4 mg · mL^{-1}, respectively) reported in Fernández-Alacid, et al. (2018). The discrepancy between lower levels of soluble protein in mucus and higher viscosity among

these species would indicate that viscosity properties depend on heavy proteins such as mucopolysaccharides (mucins), which are mainly insoluble. Interestingly, twice the amount of protein in ventral mucus with respect dorsal mucus could be related with a higher defensive capacity, as the main innate defence components of skin mucus are proteins (Esteban, 2012; Jurado et al., 2015; Sanahuja and Ibarz, 2015).

With regard to glucose and lactate levels, no previous data exist on sole skin mucus. However, glucose was 4-fold lower than in pelagic species, whereas lactate seems to be more species specific (Fernández-Alacid, et al., 2018). Mucus glucose is related to blocking bacterial adhesion, which correlates negatively with carbohydrate-rich mucus constituents and positively with lipid- and protein-rich mucus constituents (Tkachenko et al., 2013). The carbohydrate residues in the several lectin forms have been reported to be related with the progress of infection in sole, as in other fish species, (Al-Banaw et al., 2010; Guardiola et al., 2014, 2017) and their lower levels in both types of skin mucus would imply a higher sensibility to pathogens of this species. Mucus lactate may be produced at the level of epidermal cells as a consequence of the anaerobic cellular metabolism provoked by hypoxia (Omlin and Weber, 2010). Thus, higher lactate levels in ventral mucus than in dorsal mucus would match their different environments: bottom vs water. For the first time, measurements of mucus cortisol are provided for Senegalese sole: 0.06-0.1 ng · mL^{-1}. These are very low levels compared with reported values of 1-15 ng · mL^{-1} for other marine species (Guardiola et al., 2016; Fernández-Alacid et al., 2018, 2019), and here were at the limit of detection. The last parameter studied was the antioxidant power, as a key mechanism to deal with a state of pernicious oxidative stress at the mucus level. Scarce data exist on mucus antioxidant power, but De Mercado et al. (2018) proposed oscillations of antioxidant levels around the basal level to maintain redox homeostasis in the skin mucus of rainbow trout. In sole, mucus antioxidant power was higher than in trout, but the measurements agree with the levels in marine pelagic species (unpublished data). However, dorsal levels, expressed as per mg of protein, were significantly higher than ventral, levels, possibly due to its putatively higher exposure to oxidation from the surrounding water than the ventral side.

In response to hypoxia stress, mucus glucose and lactate increased acutely 15min-PS, and gradually returned to basal levels 6h-PS. The response of lactate was similar to that reported in skin mucus of rainbow trout (De Mercado et al., 2018) and meagre (Fernández-Alacid et al., 2019), and also matched the plasma lactate dynamics. It has been suggested that increasing lactate oxidation through a change in metabolic fuel preference is the immediate response to lower oxygen availability (Omlin and Weber, 2010), and this can also be detected in fish mucus. In contrast, plasma glucose was still higher 6h-PS; whereas mucus glucose levels reverted to basal values. Fernández-Alacid et al. (2019) reported a strong correlation between plasma and skin mucus glucose levels in meagre responding to hypoxia 1h-PS

and 6h-PS. As we indicated before from the viscosity changes, the mucus renewal rate should be the basis of the time-course performance of mucus components. However, although no data exists in the literature, daily rhythms on the mucus composition cannot be discarded and further studies should tackle on both renewal rates and daily rhythms of mucus components. In agreement with this, soluble mucus protein increased transitorily 15min-PS in mucus from both sides of the animal. It seems that mucus protein could be affected by the kind of stressor (Fernández-Alacid et al., 2019), and mainly in response to infection (Fernández-Alacid et al., 2018); again related to mucus renewal and exudation rates (Azeredo et al., 2015). Contrary to the case of plasma cortisol, exuded cortisol did not show any dynamics in the PS time-course, thereby invalidating it as a non-invasive marker for this species. Finally, the mucus antioxidant power showed different dynamics with respect to the dorsal and ventral sides. In response to stress, dorsal exudation of antioxidant power increased 1h-PS; while the ventral exudation did not change. When antioxidant power was expressed per mg of protein, the antioxidant defence of dorsal mucus did not decrease over the 24h-PS period. The only antioxidant power findings was reported in rainbow trout skin mucus after hypoxia stress (De Mercado et al., 2018) with a transient pick 2h-PS. We hypothesise that as the main antioxidant power is provided by protein components (antioxidant enzymes and glutathione) and since soluble protein reverted to basal values, the high values sustained for 24h-PS would suggest the relevance of this defence at the dorsal side in response to hypoxia.

CONCLUSION

In conclusion, we have reported and compared the ventral and dorsal skin mucus properties of Senegalese sole and their responses to acute hypoxia stress. The viscosity of the raw mucus indicates that mechanical and viscoelastic protection are different for the two sides: higher in ventral mucus in accordance with its higher mechanical friction. In response to hypoxia, an immediate and transient increase of viscosity apparently wraps and protects the stressed fish. However, the effects of these acute secretions would be harmful after 24 h, when fish present ventral mucus with lower viscosity and protection. Chemical parameters do not show differences between dorsal and ventral sides, and neither side responds to hypoxia. Ventral metabolites, lactate and glucose, together with soluble proteins are higher than in dorsal mucus, showing that mucus metabolite exudation is side dependent and thus the secretory mechanisms underlying skin cells and the origin of mucus components require further studies. In response to stress, mucus glucose and lactate matches plasma increases; whereas mucus cortisol does not seem an adequate stress indicator for this species. Interestingly, the antioxidant power of dorsal mucus increases to cope with hypoxia, but this response is not transitory, indicating putative differences of protein exudation (with a maximum 15min-PS) and antioxidant defence exudation. The identification in flatfish skin mucus of

specific physicochemical properties and defences depending on the animal side will be very useful to study the welfare of benthonic species of fish in a non-invasive way.

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Figure 1. Physical properties of skin mucus in Senegalese sole. Rheograms (A) and Casson's model transformation plots (B). Values are mean ± standard error of mean from individual fish analyses (n=5).

Figure 2. Analyses of skin mucus viscosity response to acute hypoxia stress in Senegalese sole; dorsal rheograms (A) and Casson's model transformation plots (C); and ventral rheograms (B) and Casson's model transformation plots (D). Values are mean ± standard error of mean from individual fish analyses (n=5). Lowercase letters (a, b, c) indicate groups of significance for post-stress times and each shear rate or between slope and intercept point of Casson´s equations ($p < 0.05$, one-way ANOVA).

Figure 3. Response of skin mucus osmolality, pH, metabolites, total soluble proteins, cortisol and antioxidant power to acute hypoxia stress: skin mucus osmolality (A), pH (B), glucose (C), lactate (D), protein (E), cortisol (F) and FRAP (G). Values are mean ± standard error of the mean from individual fish (n=10). A capital letter (A, B) indicates significant dorsal differences between basal, 15min-PS, 1h-PS, 6h-PS and 24h-PS values ($p < 0.05$, one-way ANOVA). A lowercase letter (a, b) indicates significant ventral differences between basal, 15min-PS, 1h-PS, 6h-PS and 24h-PS values ($p < 0.05$, one-way ANOVA). An asterisk (*) indicates significant differences between dorsal and ventral sides (*$p < 0.05$, Student's t-test).

Figure 4. Response of skin mucus indicators to acute hypoxia stress: glucose per mg of protein (A), lactate per mg of protein (B), cortisol per mg of protein (C) and antioxidant power per mg of protein (D). Values are mean ± standard error of the mean from individual fish (n=10). A capital letter (A, B) indicates significant dorsal differences between basal, and 15min-PS, 1h-PS, 6h-PS and 24h-PS values ($p < 0.05$, one-way ANOVA). A lowercase letter (a, b, c) indicates significant ventral differences between basal, and 15min-PS, 1h-PS, 6h-PS and 24h-PS values ($p < 0.05$, one-way ANOVA).

Table 1. Comparison between dorsal and ventral sides for basal values.

	DORSAL	VENTRAL	p-value
Chemical properties			
Osmolality (mOsm/Kg)	1108 ± 32.2	1095 ± 32.3	ns
pH	7.30 ± 0.00	7.31 ± 0.00	ns
Cl^- (mmol/L)	571 ± 2.92	540 ± 15.6	ns
Na^+ (mmol/L)	478 ± 2.85	463 ± 11.0	ns
Ca^{2+} (mmol/L)	18.4 ± 0.07	18.3 ± 0.33	ns
K^+ (mmol/L)	10.4 ± 0.09	10.5 ± 0.13	ns
Compounds			
Protein (μg/mL)	200 ± 32.6	451 ± 40.6	p = 0.021
Glucose (μg/mL)	4.12 ± 0.62	7.05 ± 2.40	ns
Lactate (μg/mL)	0.85 ± 0.20	1.93 ± 0.61	p = 0.041
Gluc/Pr ratio (μg/mg)	22.1 ± 2.20	20.6 ± 1.10	ns
Lact/Pr ratio (μg/mg)	3.98 ± 0.86	5.61 ± 1.29	ns
Cortisol (ng/mL)	0.06 ± 0.01	0.10 ± 0.03	ns
Cort/Pr ratio (ng/g)	395 ± 67.9	316 ± 30.5	ns
Antioxidant power			
FRAP (nmol/mL)	17.2 ± 1.20	30.2 ± 6.40	ns
FRAP/Protein (nmol/mg)	100 ± 8.97	64.7 ± 9.24	p = 0.047

Values are mean ± standard error (SEM) from individual fish (n=10).
P-values indicates significant differences between dorsal and ventral sides (Student's t-test).

Table 2. Response of plasma metabolites, protein and cortisol to an acute hypoxia stress.

			Post-Stress							
	Basal		**15min-PS**		**1h-PS**		**6h-PS**		**24h-PS**	
Glucose (mg/dL)	35.9 ± 1.7	a	66.3 ± 3.9	b	62.1 ± 5.1	b	61.3 ± 6.4	bc	48.4 ± 6.8	ac
Lactate (mg/dL)	4.5 ± 0.9	a	9.7 ± 1.1	b	10.8 ± 2.6	b	3.7 ± 0.8	a	7.4 ± 3.3	a
Cortisol (ng/mL)	67.4 ± 23.6	ab	50.0 ± 13.2	ab	101.7 ± 26.0	b	16.7 ± 10.1	ac	10.1 ± 3.7	c
Protein (g/dL)	3.3 ± 0.1		2.9 ± 0.1		3.4 ± 0.1		3.3 ± 0.2		3.1 ± 0.2	

Values are mean ± standard error of mean from individual fish (n=10).
Different letters indicate different groups of significance during post-stress time-course ($p < 0.05$, One-Way ANOVA).

Figure 1

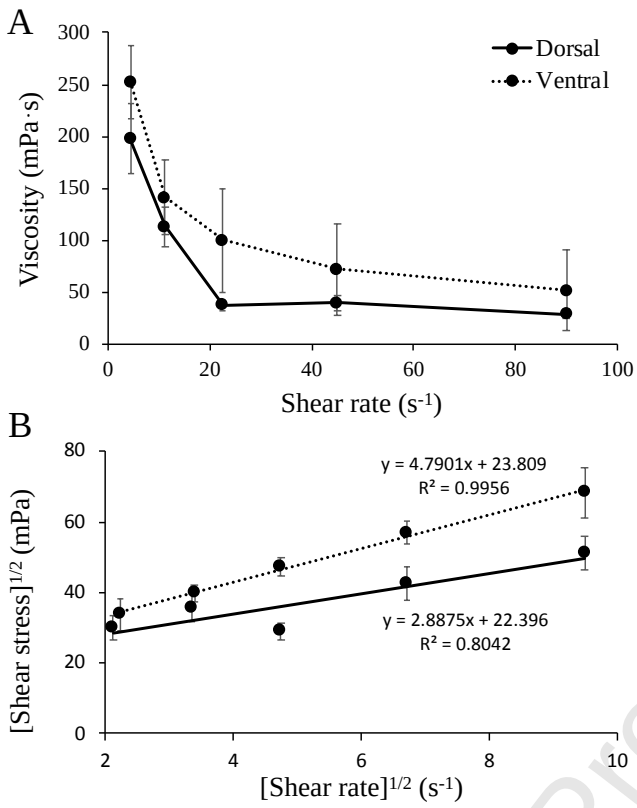
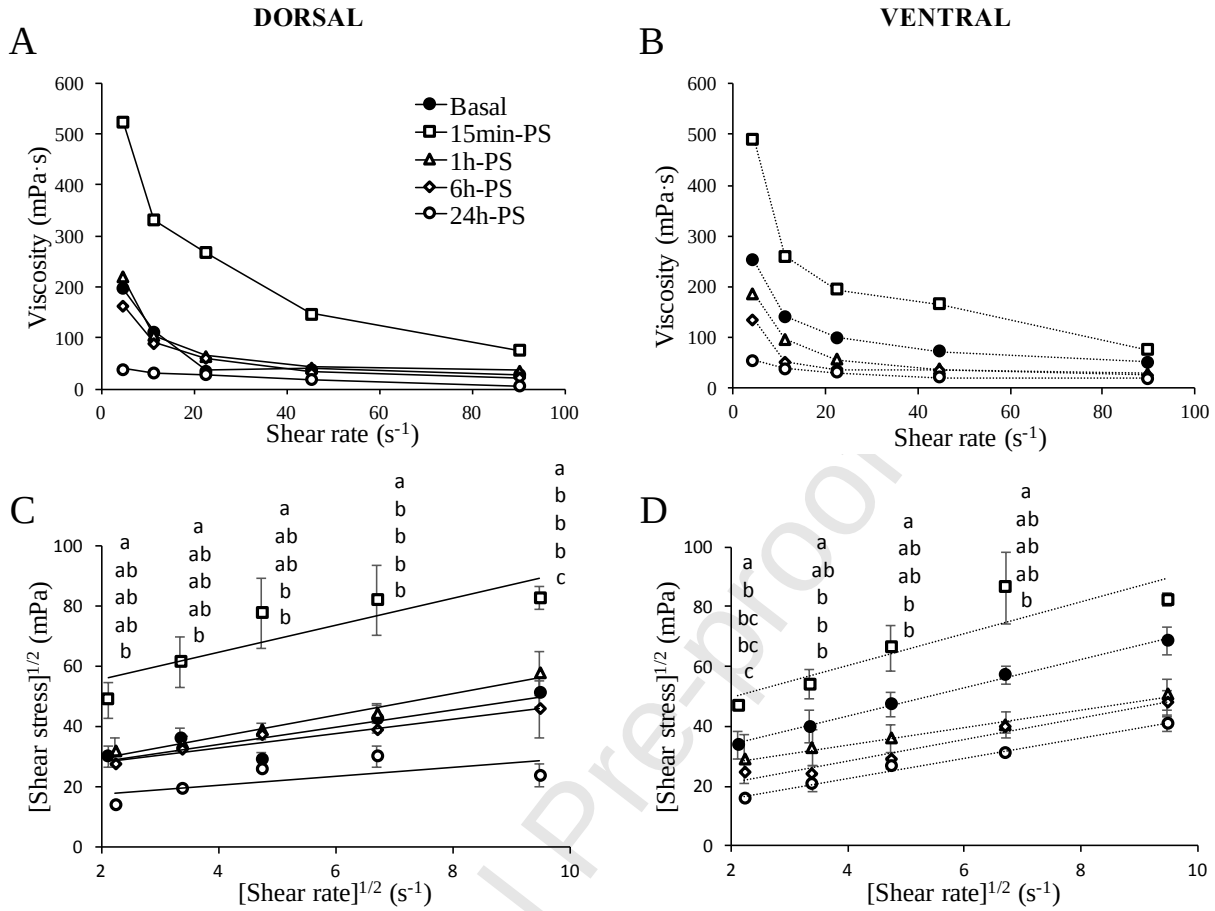


Figure 2



	Dorsal					Ventral				
	Slope (K_i)		Intercept point (σ_0)		Equation	Slope (K_i)		Intercept point (σ_0)		Equation
Basal	2.88±0.10	ab	22.4±1.00	ab	y=2.88x+22.4	4.79±1.45	a	23.8±2.48	a	y=4.79x+23.8
15min-PS	4.47±0.41	a	46.8±2.87	a	y=4.47x+46.8	5.35±1.21	a	38.9±3.45	a	y=5.35x+38.9
1h-PS	3.56±0.49	ab	22.3±2.94	ab	y=3.56x+22.3	2.92±0.35	b	22.2±5.19	ab	y=2.92x+22.2
6h-PS	2.36±0.01	ab	23.5±0.12	ab	y=2.36x+23.5	3.57±0.11	ab	14.1±0.55	b	y=3.57x+14.1
24h-PS	1.45±0.01	b	14.6±0.03	b	y=1.45x+14.6	3.35±0.28	ab	9.10±0.18	b	y=3.35x+9.1

Figure 3

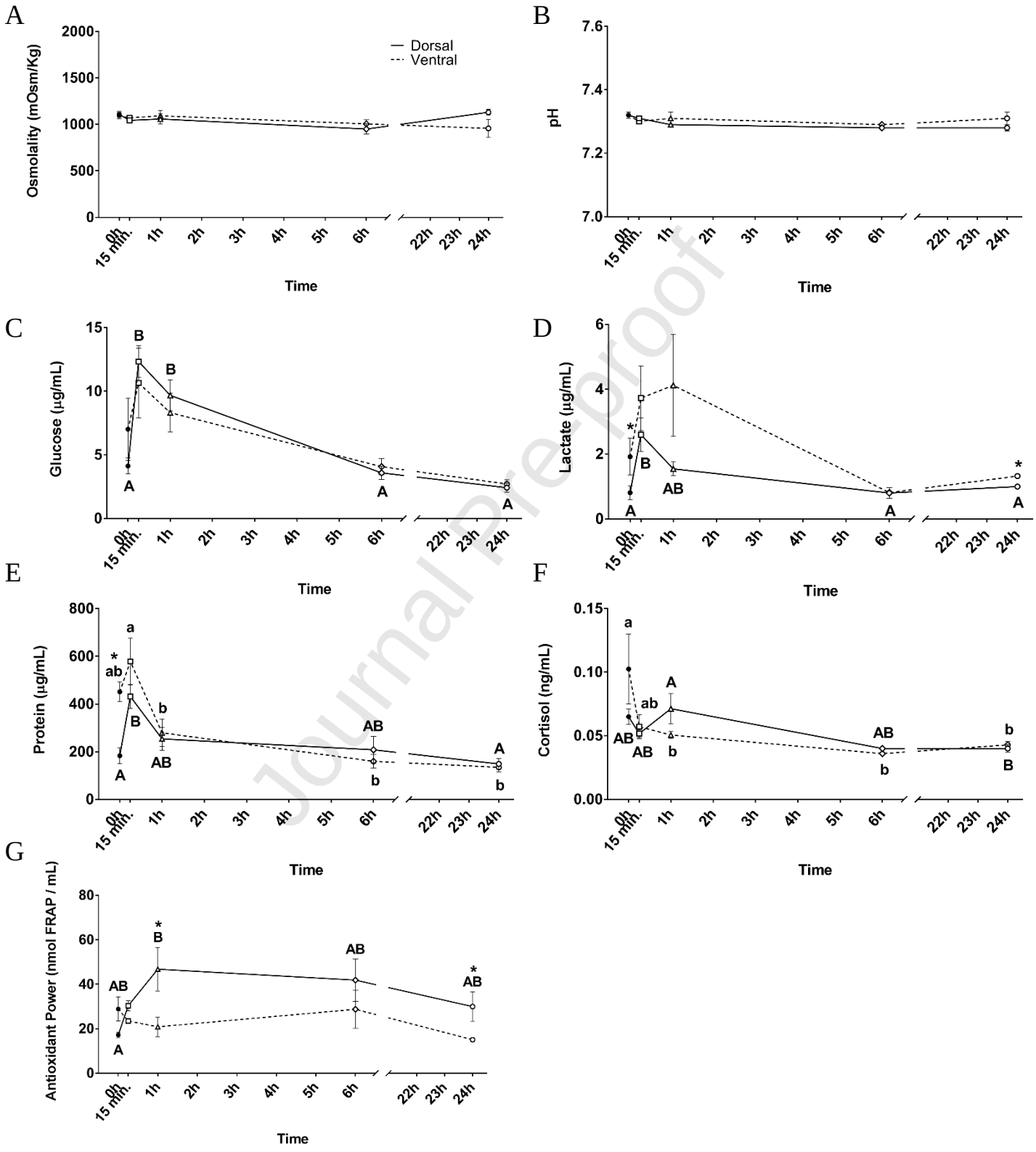


Figure 4

Highlights

- Senegalese sole display a classic plasma response to acute hypoxia: increased glucose, lactate and cortisol levels
- We compare mucus properties of dorsal-ocular and ventral-blind sides, for the first time in a flatfish species
- We provide skin mucus viscosity and metabolite stress marker time-course data in response to hypoxia
- Our study reveals asymmetry in mucus properties, possibly due to the surrounding environment