Fish and Shellfish Immunology 89 (2019) 428–436

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Comparison of several non-specific skin mucus immune defences in three piscine species of aquaculture interest

Ignasi Sanahuja[a], Laura Fernández-Alacid[a], Borja Ordóñez-Grande[a], Sergio Sánchez-Nuño[a], Arantxa Ramos[b], Rosa M[a] Araujo[b], Antoni Ibarz[a,*]

[a] Departament Biologia Cellular, Fisiologia i Immunologia, Facultat de Biologia, Universitat de Barcelona, Avda Diagonal 643, E-08028, Barcelona, Spain
[b] Departament de Genètica, Microbiologia i Estadística, Facultat de Biologia, Universitat de Barcelona, Avda Diagonal 643, E-08028, Barcelona, Spain

ARTICLE INFO

Keywords:
Epidermal mucus
Antioxidant power
Carboxylesterases
Zymograms
Pathogen
Antimicrobial activity

ABSTRACT

Fish skin mucus is a viscous and semipermeable barrier made mainly of water, glycoproteins and soluble proteins. It represents an important defence against the environment and previous studies have reported the presence of different substances involved in immune defence responses in it. The aim of the present work was to characterize skin mucus protease activity by zymography and esterase activity of the subfamily of carboxylesterases in three species of interest for aquaculture: gilthead sea bream, sea bass and meagre. Mucus antioxidant power was also determined by adapting ferric reducing antioxidant power (FRAP) analysis. As a result of these non-specific immune defence parameters, we compared the antibacterial capacity of skin mucus in these species via *in vitro* dual bacteria strains–skin mucus co-culture growths. We used *Pseudomonas anguilliseptica* and *Vibrio anguillarum* as marine pathogenic bacteria and *Escherichia coli* as non-pathogenic. For each fish species, in the respective zymograms, we determined a pattern of proteolytic digestion bands. A high-molecular-weight band (around 200 kDa; H-band) was evident in sea bream and sea bass, and showed chymotrypsin activity. One or two intermediate-molecular-weight bands (around 75 kDa; I-bands) with non-trypsin and non-chymotrypsin activity, and putatively with metalloprotease activity, were evident in all species. Finally, low-molecular-weight bands (between 14 and 30 kDa; L-bands) showed distinct patterns for each species and matched trypsin activity. Despite the conservative pattern of digestion bands, the levels of total proteolytic activity (TPA) were 5 and 10 times higher in meagre than in sea bass and sea bream, respectively. In parallel, three carboxylesterase activities were detected in the mucus of the three fish species, using myristate (pNPM-CE activity), butyrate (pNPB-CE activity) and acetate (pNPA-CE activity) as substrates. Both pNPB-CE and pNPA-CE were the most abundant in fish mucus, and meagre was again the species with the highest levels. In contrast, the antioxidant power of meagre skin mucus was the lowest. We established the capacity of skin mucus to block or limit bacterial growth (lytic activity) using 24 h growth curves. The log-growth phase of *V. anguillarum* was strongly blocked by sea bream and meagre mucus for a few hours; but not by sea bass mucus. However, if mucus was not renewed, log-growth was at the end of 24 h studied period. For its part, *P. anguilliseptica* growth curve was delayed by the three mucus types during the entire growth period. Only meagre achieved lytic activity against *E. coli* growth. All parameters studied here will be of a great interest as non-invasive bioindicators of non-specific immune defences in fish skin mucus.

1. Introduction

To meet the growing demand for aquatic food, nowadays fish is increasingly farmed, and in the medium term farmed fish will be the most important source of income from aquatic food production [1]. The domestication of fish is still in its infancy, compared with terrestrial animals, so fish farmers have undertaken to expand the range of fish species farmed and also to improve fish production [2]. Fish culture faces several problems associated with natural behaviour (e.g., confinement and overpopulation) that can result in stress, growth arrest, immune suppression and ultimately, a loss of production [3]. Nowadays, classic diagnoses of the physiological and health status of fish are provided by haematological analyses and clinical chemistry [4,5]. Recently, however, there has been special interest in the study of physiological status via non-invasive methods in order to prevent additional stressors in fishes. As the first line of defence in fish [6], the

* Corresponding author.
E-mail address: tibarz@ub.edu (A. Ibarz).

https://doi.org/10.1016/j.fsi.2019.04.008
Received 21 December 2018; Received in revised form 25 March 2019; Accepted 4 April 2019
Available online 09 April 2019
1050-4648/

epidermal mucus has been suggested as a valuable tool to study fish physiological status without causing damage or having adverse effects on fish [7–11].

Skin mucus is secreted by epidermal goblet cells present in the epithelia which, unlike the case in humans, constitute a living cell stratum. Being alive, their cells are constantly secreting and absorbing products such as ions, hormones and waste compounds; but they can also represent a source of external pathogens and chemicals [6,12]. Mucus, due to being situated between epithelia and the environment, acts as a semipermeable barrier which ultimately interacts with the environment and all the products that it brings into contact with the fish. Another characteristic that makes mucus an interesting tool is its range of components and functions. It is mainly composed of water, glycoproteins and lipids, but it can be modified by both endogenous and exogenous factors, such as the fish developmental stage, stress and infection [13,14]. In addition, mucus is more than a simple barrier; it performs in fish a large number of functions that are crucial for ionic and osmotic regulation, reproduction, excretion, nutrition, disease resistance, communication or locomotion [6,15].

Fish skin mucus has the capacity to tolerate colonization by diverse microbial commensals [16] and, at the same time, to fight pathogen colonizers [17–19]. The adherence of bacteria to mucosal surfaces is particularly important in subsequent infection, and this union depends on the mucus status as well as the surrounding environment [15]. A variety of mucus structural molecules, such as lectins or actin, diverse enzyme activities (lysozyme, phosphatase, esterase or protease) and other stress-related proteins (heat-shock proteins, transferrin and histones) are described as innate defence mechanisms against pathogens in skin mucus [10,12,20,21,22,23,24]. Some mucus structural components, such as keratins and its enzyme-digested forms, are also related to defence mechanisms through their production of antimicrobial peptides (AMPs) [23–25]. The antimicrobial role of skin mucus appears to result from its biochemical and mechanical properties together with its renewal rate. The information available is restricted to a few fish species [22,26] and it has been demonstrated that antimicrobial activity depends not only on fish species but also on pathogen species (in some cases, even on pathogen strain).

Proteases or proteinases are enzymes that perform a large number of functions in nature: removing signalling peptides from proteins in the cell secretory pathway; removing propeptides from enzymes, hormones and receptors that are synthesized as precursors; releasing individual proteins and peptides from polyproteins; releasing bioactive peptides from protein precursors; releasing (“shedding”) proteins from the cell surface; switching off the signals that peptides and proteins initiate by degrading either themselves or the proteins they bind to; destroying potentially lethal or toxic proteins from parasites and pathogens; and releasing antigenic peptides from parasites and pathogens, among others (reviewed in Ref. [27] [28]. used zymography to report different protease activities in rainbow trout, Coho salmon and Atlantic salmon, thereby evidencing different protease families in the mucus layers. Meanwhile [29], showed selective responses of different proteases produced by infection in the skin mucus of Atlantic salmon. Esterase activity has been studied to a limited degree and is described as a defence against pathogens [22,29,30]. Recently, esterase has also been studied due to its detoxifying capacity in mucus [31,32] as assumed for other fish tissues [33,34].

The cross-linked response between fish status and welfare relies on physiological status and immune defence preparation before stressors appear. In this study, we perform broad screening of non-specific innate mucus components on three of the most important marine species in Mediterranean aquaculture: gilthead sea bream (*Sparus aurata*), European sea bass (*Dicentrarchus labrax*) and meagre (*Argyrosomus regius*). Mucus zymography was performed to evaluate the presence of different protease activities, and four different carboxylesterase (CE) activities were determined for the first time in fish skin mucus with the aim of evaluating the main esterase activities. Moreover, mucus antioxidant capacity was also measured, using the novel methodology of FRAP analysis in marine skin mucus, as a key mechanism in dealing with a state of oxidative stress that could be pernicious to the underlying live epidermal cells. Finally, we proposed as a novel approach to assess antimicrobial activities via the study of complete bacterial growth curves, when faced with *Escherichia coli*, as a non-pathogenic species for fish, and both *Pseudomonas anguilliseptica* and *Vibrio anguillarum*, as two of the main pathogens in Mediterranean aquaculture, via the study of bacterial growth curves. All the data we obtain, using a non-invasive way, could help provide a better understand of the non-specific immune defence of skin mucus and could be useful in aquaculture.

2. Material and methods

2.1. Animal conditions

Juvenile gilthead sea bream (90.7 ± 3.6 g), European sea bass (106 ± 21 g) and meagre (105 ± 2.6 g), from local providers, were acclimated at the facilities of the University of Barcelona (UB). The fish were kept for a month in 800 L fiberglass tanks with recirculating systems, and fed a standard commercial feed for each species twice a day. Rearing systems controlled solid and biological filters, while water temperature, salinity, and oxygen concentration were daily recorded (20.3 °C ± 0.6 °C, 3.7% ± 0.1 and > 95% of saturation, respectively); and furthermore, nitrite, nitrate and ammonia concentrations were periodically analysed and maintained throughout trial. All animal handling was conducted following the norms established by the Council of the European Union (86/609/EU), the Spanish government and the Catalan regional authorities; and the procedures were approved by the Ethics and Animal Care Committee of the University of Barcelona (permit no. DAAM 9383).

Skin mucus samples were collected non-invasively, following the method of [8]. Briefly, 30 fish per species 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; the skin mucus was carefully pushed and collected in a sterile tube (2 mL). The mucus volume collected per fish ranged 300 μL for sea bream and 200 μL for sea bass and meagre. Before mucus processing, the scales collected in the mucus samples were individually removed. Mechanical homogenization was performed cold using a sterile Teflon stirrer to desegregate the mucus mesh before centrifugation at 14,000 g at 4 °C for 15min. The resultant mucus supernatants were collected, avoiding the surface lipid layer. A total of 5 fish mucus samples were pooled into aliquots and stored at −80 °C for further analysis.

2.2. Protein determination

The protein concentration of homogenized mucus was determined using the Bradford assay [35] with bovine serum albumin (BSA; Sigma) as the standard. The OD was determined at λ = 596 nm with a microplate reader (Infinity Pro200 spectrophotometer, Tecan, Spain). Protein values were expressed as mg of protein · mL^{-1} of skin mucus.

2.3. Carboxylesterase (CE) activities

Multiple substrates were used for enzymatic determination of CE activities due to the occurrence of multiple isozymes that generally coexist in a single tissue homogenate and which display different substrate preference and sensitivity to potential inhibitors [36]. The activity of CEs by p-nitrophenyl acetate (pNPA), p-nitrophenyl butyrate (pNPB) and 1-naphthyl acetate (1-NA) substrates were measured and adapted for mucus samples from the method reviewed by Ref. [37]. Briefly, the hydrolysis rates of pNPA-CE, pNPB-CE and 1-NA were determined by a continuous spectrophotometric enzyme assay, performed in 50 mM phosphate buffer (pH = 7.4) containing the substrate (1 mM, final concentration) and 25 μL of sample. The formation of 4-nitrophenolate (for pNPA-CE and pNPB-CE) was monitored at 405 nm for 5 min at 25 °C and the formation of 1-naphthol (for 1-NA) was monitored at 235 nm for 5 min at 25 °C (following [34] in a microplate reader (Infinity Pro200 spectrophotometer, Tecan, Spain). Additionally, esterase activity for p-nitrophenyl myristate (pNPM-CE) substrate was determined according to the method of [29] with some modifications. Equal volumes of skin mucus and 0.4 mM pNPM-CE substrate were placed in 100 mM ammonium bicarbonate buffer containing 0.5% Triton X-100 (pH 7.8, 30 °C). The formation of p-nitrophenol was monitored at 405 nm for 15 min at 25 °C in the microplate reader. The initial rate of the reaction was used to calculate the activity.

2.4. Total protease activity

Total alkaline protease activity (TPA) was spectrophotometrically measured in the homogenates following [38]. Thus, the samples first reacted in 50 mM Tris-HCl pH 9.0 buffer containing 1% casein as proteolytic substrate. After 30 min, the reaction was stopped by adding trichloroacetic acid (TCA, 12%). The samples were then maintained for 1 h at 4 °C and centrifuged (7500 g, 5 min, 4 °C). Supernatant absorbance was measured at 280 nm. Each sample was analysed in triplicate and individual blanks were established by adding TCA solution. Bovine trypsin was used as a standard. Enzyme activity was recorded as IU · mg^{-1} of protein.

2.5. Zymography

Individual alkaline protease activities were also studied by zymograms according to the method previously established in skin mucus [24] from modifications of the methods of [39,40] for fish mucosa. To evaluate specific proteases activities the following inhibitors and concentrations were used: tosyl-L-lysyl-chloromethane hydrochloride (TLCK, 10 mM in HCl, 1 mM) and soybean trypsin inhibitor type II-S (SBTI, 250 μM) to inhibit trypsin-like activities; and tosyl phenylalanyl chloromethyl ketone (TPCK, 10 mM in methanol), *N*-benzyloxycarbonyl-L-phenylalanylchloromethyl ketone (ZPCK, 10 mM in dioxane) and phenylmethane sulfonyl fluoride (PMSF, 100 mM in isopropanol) for chymotrypsin-like activities. The selected concentrations were assayed following [40,41].

Mucus samples were mixed 4:1 with H_2O_{MQ} as a control or the corresponding inhibitor for 45 min at room temperature. Then, the control or inhibited samples were mixed 3:2 with the loading buffer (2.5 ml stacking gel buffer; SDS 10%; 2 ml glycerol; 2 mg bromophenol blue). Two mucus extracts, from the pool of 5–7 mucus samples depending on the fish species, were used to obtain both mucus caseinolytic bands and inhibitions. 30 μg of protein was loaded onto 12% polyacrylamide gel in duplicate. Electrophoresis was performed at a constant current of 15 mA per gel for 90 min (Bio Rad Mini PROTEAN Tetra Cell, 4 °C). Protease-active fractions were visualized using the method described by García-Carreño et al. (1993) where the gels were incubated at 4 °C under agitation in Tris-HCl 50 mM pH 8.2 solution containing 2% casein. After 30 min, the temperature was raised to room temperature for 90 min with shaking. The gels were washed and stained in a methanol:acetic:water solution (40:10:40) with 0.1% of Coomassie Brilliant Blue R-250 (Bio-Rad). Destaining was carried out using the same solution without colorant until the desired visualization of the digested bands was achieved. Pure trypsin was used as a positive control. To determine the molecular weight (MW) of the protease fractions, a commercial weight marker was used (RPN 800E, GE Healthcare). The gels were further scanned in an ImageScanner III (Epson J181A) and caseinolytic bands were identified.

2.6. Ferric reducing antioxidant power (FRAP)

Ferric antioxidant status is a measure of antioxidant power; it gauges 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). Following the manufacturer's instructions for plasma determinations but with slight modifications, 20 μL of mucus extract or standard solution (from 0 to 1000 $\mu M\,\mu L^{-1}$ of $FeCl_2$) was mixed with 75 μL of FRAP colour solution in triplicate and incubated for 30 min at room temperature. The OD was determined at $\lambda = 560$ nm with a microplate reader (Infinity Pro200 spectrophotometer, Tecan, Spain). Antioxidant values were expressed as nmol FRAP · mg^{-1} of protein.

2.7. Antibacterial activity

A non-pathogenic bacteria for fish, *E. coli* (DSMZ number: 423) from the German Collection of Microorganisms and Cell Cultures (Leibniz Institute DSMZ, Germany) and two pathogenic bacteria, *V. anguillarum* (CECT number: 522T) and *P. anguilliseptica* (CECT number: 899T) from the Spanish Type Culture Collection (CECT, University of Valencia, Valencia, Spain) were used in the antibacterial assay. All bacterial strains were grown from 1 mL of a stock culture. *E. coli* was cultured for 24 h at 37 °C in trypticasein soy broth (TSB, Laboratorios Conda, Spain), and the two pathogenic bacteria were cultured for 24 h at 30 °C in marine broth (MB-2216, Becton and Dickinson, USA).

Exponentially growing bacteria were centrifuged and the pellet resuspended in sterile PBS was diluted in new growth medium and adjusted to 10^6 colony forming units (CFU) · mL^{-1}. Skin mucus antibacterial activity was measured by absorbance in flat-bottomed 96-well plates. Aliquots of 100 μL of the previously cultured bacteria plus 100 μL of the medium were incubated in parallel with aliquots of 100 μL of the previously cultured bacteria plus aliquots of 100 μL of skin mucus, to study the antibacterial activity, for 24 h. The absorbance of the bacteria was measured at 400 nm every 30 min for 24 h at 25 °C in flat-bottomed 96-well plates. Triplicates of 100 μL of each fish mucus plus 100 μL of medium were used as control values and subtracted from the bacteria–mucus aliquot values.

3. Results

3.1. Enzyme activities and antioxidant capacity

Enzymatic TPA and the activities of different CEs in skin mucus are showed in Fig. 1. TPA was species-specific, with the lowest value for sea bream (1.4 ± 0.2 IU per mg of protein), followed by sea bass (5.5 ± 1.2) and the highest for meagre (19.2 ± 0.4). Four CE activities were tested using the substrates pNPM-CE, pNPA-CE, pNPB-CE, and 1-NA. Both pNPA-CE and pNPB-CE showed higher activity in mucus than pNPM-CE (around one order of magnitude) irrespective of fish species. As for TPA, meagre mucus showed the highest activities for the CEs studied and sea bream mucus the lowest. 1-NA activity was not detected in skin mucus and data are not shown. Mucus antioxidant capacity was also measured as FRAP, with the lowest activity being in meagre mucus: half that of sea bream and sea bass.

3.2. Zymography of protease activities

To characterize the alkaline protease activity pattern of skin mucus, zymographic analysis was performed using casein digestion activity. One-dimensional electrophoresis was run on 12% polyacrylamide gels and protease-active fractions were visualized after incubation with 2% casein as the substrate (caseinolytic activity) for sea bream (Fig. 2A), sea bass (Fig. 2B) and meagre (Fig. 2C). The resulting zymograms revealed the presence of different digested bands with caseinolytic activity for each species. The sea bream mucus zymograms showed three

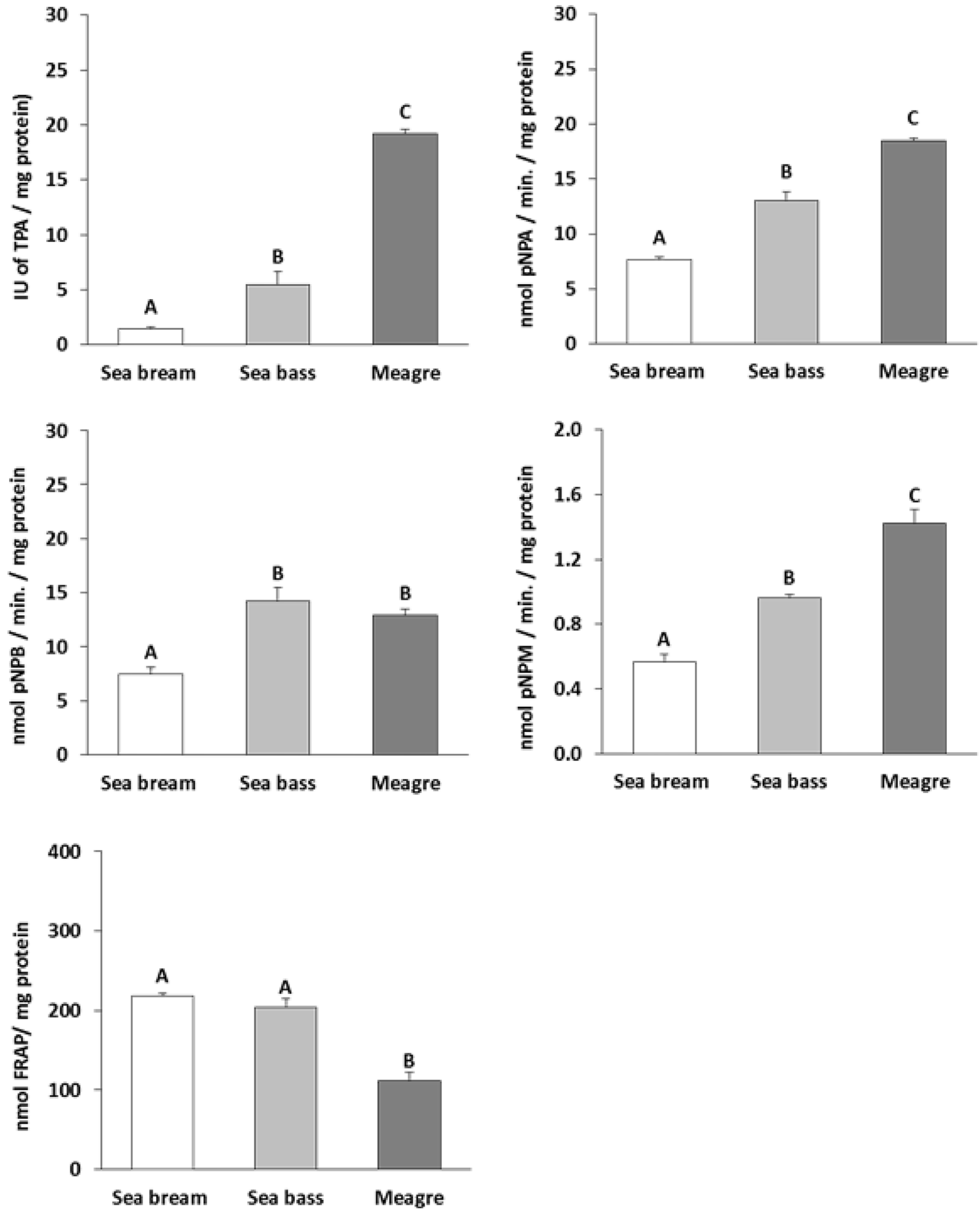

Fig. 1. Maximum enzyme activities of total protease (TPA), carboxylesterase activities (pNPA-CE, pNPB-CE and pNPM-CE) and antioxidant power of skin mucus. Values are mean ± SEM (n = 6) and different letters indicate significant groups ($p < 0.05$) from one-way ANOVA.

clear digested bands: at 12–15 kDa (low-MW band or L-band) matching the location of trypsin MW from the positive control; at 75–80 kDa (intermediate-MW band or I-band); and at 180–200 kDa (high-MW band or H-band). Moreover, other areas in the zymograms, not forming a clear band, could putatively represent caseinolytic activity (indicated by discontinuous arrows, Fig. 2A). In the zymogram of sea bass mucus, digested bands were located at the same MWs as for sea bream; however, an extra L-band appeared above the trypsin MW and an extra I-band at 100 kDa. With regard to the zymogram of meagre mucus, no H-band was evidenced under the current experimental conditions, whereas the I-band matched the sea bream and sea bass I-band, with an extra I-band in this case below 75 kDa. In contrast, an evident digested area was revealed at the trypsin MW location, extending from 15 kDa to 17 kDa. It is important to note that the same amount of protein (30 μg of protein mucus extract) was loaded for the three species and the casein incubation time was also the same (2 h), indicating that the greater area digested corresponds to higher trypsin-like activity, matching the results obtained for higher TPA in meagre mucus when measured enzymatically.

To confirm the nature of the activity of these proteolytic bands, several inhibitors of the different protease activities were assayed in respective zymograms (Fig. 3). Table 1 summarizes the inhibitory effect of each inhibitor tested (+ or - for the dose of inhibitor proposed) on each skin mucus. PMSF, TPCK and ZPCK completely halted the digestion at the H-band location in the sea bream and sea bass zymograms, demonstrating the chymotrypsin-like activity of the H-band. Likewise, TLCK and SBTI decreased digestion in the L-band in sea bream and sea bass; but the dose proposed did not affected the L-band zone of the meagre mucus zymogram. Both TLCK and SBTI inhibit trypsin-like activities and the perfect match with the trypsin-MW positive control demonstrated the trypsin activity of the L-band. With regard to I-band inhibition, the activity was not affected by any inhibitor and should be

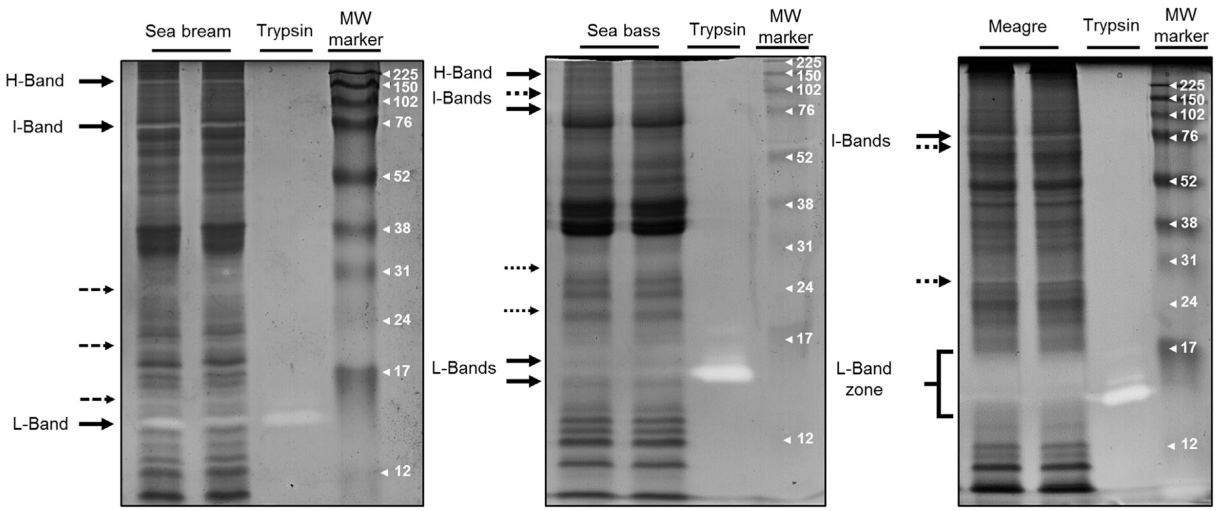

Fig. 2. Zymograms of skin mucus protease activities of gilthead sea bream (A), sea bass (B) and meagre (C). Gel zymography: electrophoresis was performed on polyacrylamide (12% acrylamide) gels using trypsin (10 ng) as a positive control (details in M&M). To determine the molecular weight of the protease fractions, a commercial weight marker was used (MW-marker lane).

considered as non-trypsin and non-chymotrypsin-like activity.

3.3. Skin mucus antibacterial activity

A non-pathogenic (*E. coli)* and two pathogenic (*V. anguillarum* and *P. anguilliseptica)* bacteria for marine species were used to evaluate the antibacterial effect of the skin mucus on bacterial growth. Fig. 4 shows the growth curves, derived from the appropriate medium for each bacterium, each 30 min for 24 h. The effects of the mucus on the bacterial growth was measured turbimetrically (OD at 400 nm) comparing "free growth" (without mucus) and "in-mucus growth". Putative antibacterial potentials were analysed via lag-period growth, log-period growth and stationary-period growth for each bacterium studied and statistical differences supplied as Supplementary Table 1. Sea bream mucus did not affect *E. coli* growth during the 24 h period. In contrast, sea bass mucus exerted a antibacterial effect at the end of log growth reducing the bacterial growth rate (significantly from 20 h onwards). Meagre skin mucus delayed and reduced total *E. coli* growth, showing both biocide and biostatic activity against this *E. coli* strain, significantly from the start of log growth and being maximum at 18–24 h period (data in Supplementary Table 1). With regard to *V. anguillarum* growth, sea bream mucus delayed log growth significantly with the maximum inhibition from 14 h to 18 h period but, at 18–19 h of co-culture, a log phase of bacterial growth re-started, resulting sea bream mucus without inhibitory effect at the end of the 24 h period. Meagre mucus exhibited a similar pattern against *V. anguillarum* as sea bream did; whereas sea bass mucus did not generally affect the log phase but the stationary phase significantly (from 22 h onwards), as it did against *E. coli*. The growth of *P. anguilliseptica* seemed to better approach linear growth without clear lag, log or stationary phases of growth (under the conditions reported in M&M). However, it was the most sensitive bacteria to co-culture with fish mucus. Sea bream and meagre mucus

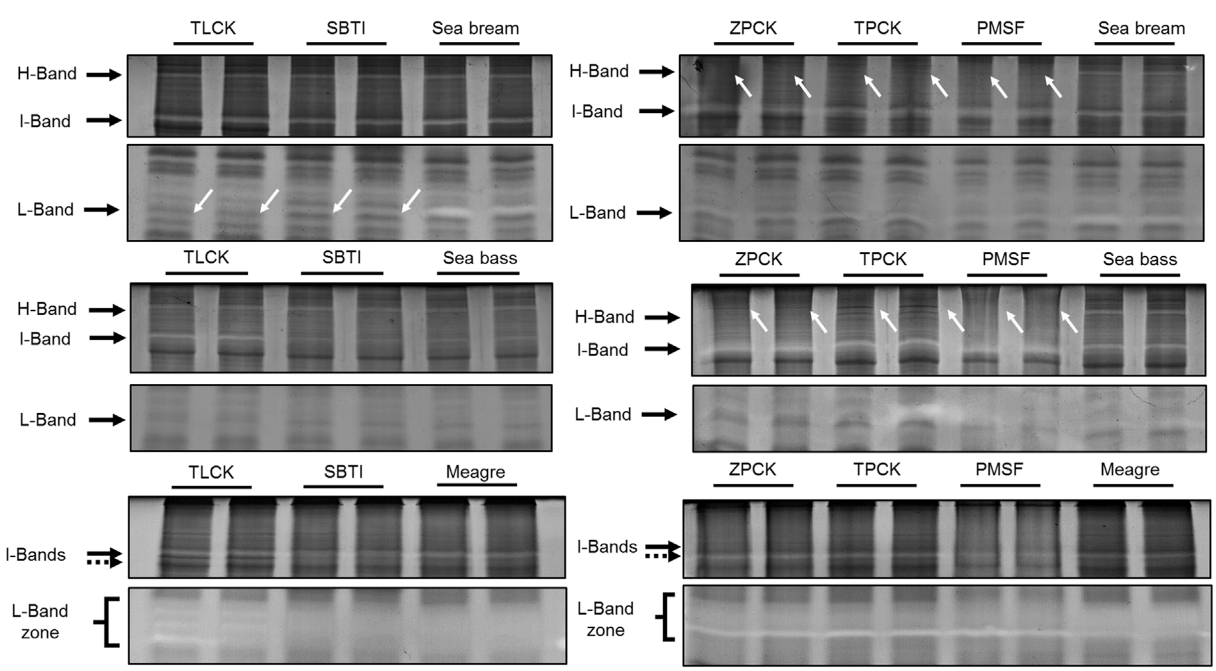

Fig. 3. Inhibitory study of caseinolytic activity in mucus zymograms. Details of the inhibitors used are in M&M. Arrows indicate evident inhibitory effects with respect to control bands for each fish species.

Table 1
Protease inhibitors related to protease specific activity.

		Trypsin		Chymotrypsin		
		SBTI	TLCK	PMSF	TPCK	ZPCK
Sea bream	H-Band	-	-	+	+	+
	I-Band	-	-	-	-	-
	L-Band	+	+	-	-	-
Sea bass	H-Band	-	-	+	+	+
	I-Band	-	-	-	-	-
	L-Band	+	+	-	-	-
Meagre	I-Band	-	-	-	-	-
	L-Band	-	-	-	-	-

Symbols represents inhibition (+) or no inhibition (−). **SBTI**: Soybean Tripsin Inhibitor; **TLCK**: Tosyl-L-lysyl-Chloromethane hydrochloride; **PMSF**: Phenylmethane Sulfonyl Fluoride; **TPCK**: Tasyl Phenylalanyl Chloromethyl Ketona; **ZPCK**: *N*-Benzyloxycarbonyl-L-Phenylalanylchloromethyl Ketona.

exhibited strong biocide activity, significantly from 5 h to 24 h of co-culture; in contrast the effects of sea bass mucus against *P. anguilliseptica* were lower in magnitude than sea bream or meagre.

4. Discussion

While traditional diagnoses of the physiological and health status in fish are provided by haematological or pathological analysis, new tools are emerging due to the necessity to produce no further stressors, mainly in aquaculture systems. This is the case of skin mucus: an interesting non-invasive tool the study of which has interested many researchers over the last decade, due to its defensive characteristic and plasticity against different physiological conditions and challenges [7–11,21,23,24]. In the present work, we selected three species cultured in southern Europe to study different non-specific innate defence parameters with diagnosis potential in mucus, such as: protease and esterase activities, antioxidant power and global antibacterial activity. Two of the species, sea bream and sea bass, lead Mediterranean fish production, to extent that their capture after reproduction has been controlled since the early 1980s [1]. Meanwhile, meagre is a good example of a new species introduced into aquaculture by fish farmers in order to obtain the same results as its predecessors. This species is less well known by the consumer and breeding it to consolidate current initial production poses new challenges.

4.1. Antioxidant power of mucus

An easy method to analyses the antioxidant capacity of biological samples is the FRAP assay [42], which has been used in fish plasma and tissues [43,44], and recently in skin mucus from trout [11]. For the marine species studied, FRAP was detected in unstressed animals, with meagre levels being half those in sea bass and sea bream. In trout skin mucus [11], proposed that there are normal oscillations in antioxidant levels around the basal level which maintain redox homeostasis in skin cells. In fact, those authors reported that in response to hypoxia the mucus antioxidant levels doubled with respect to basal levels. Those first data in trout and in the current marine species show that antioxidant levels in mucus could be a good indicator of fish status, as proposed for antioxidant levels in tissues [43,44]. However, mucus analysis is non-invasive. Although few data exist on this mucus parameter, mucus antioxidant levels are good candidates for measuring antioxidant activity as an immune defence in aquaculture conditions. Thereby, as another possibility to measure antioxidant activity, recent studies in skin mucus proposed reactive oxygen species in response to stress conditions (Mercado et al., 2018) together with other immune parameters and antioxidant enzymes as mucus oxidative markers in response to dietary supplementation with probiotics [45]. The present study demonstrates the importance of better understanding whether these three species could respond to oxidative challenges differently, as the FRAP levels suggested, via further studies.

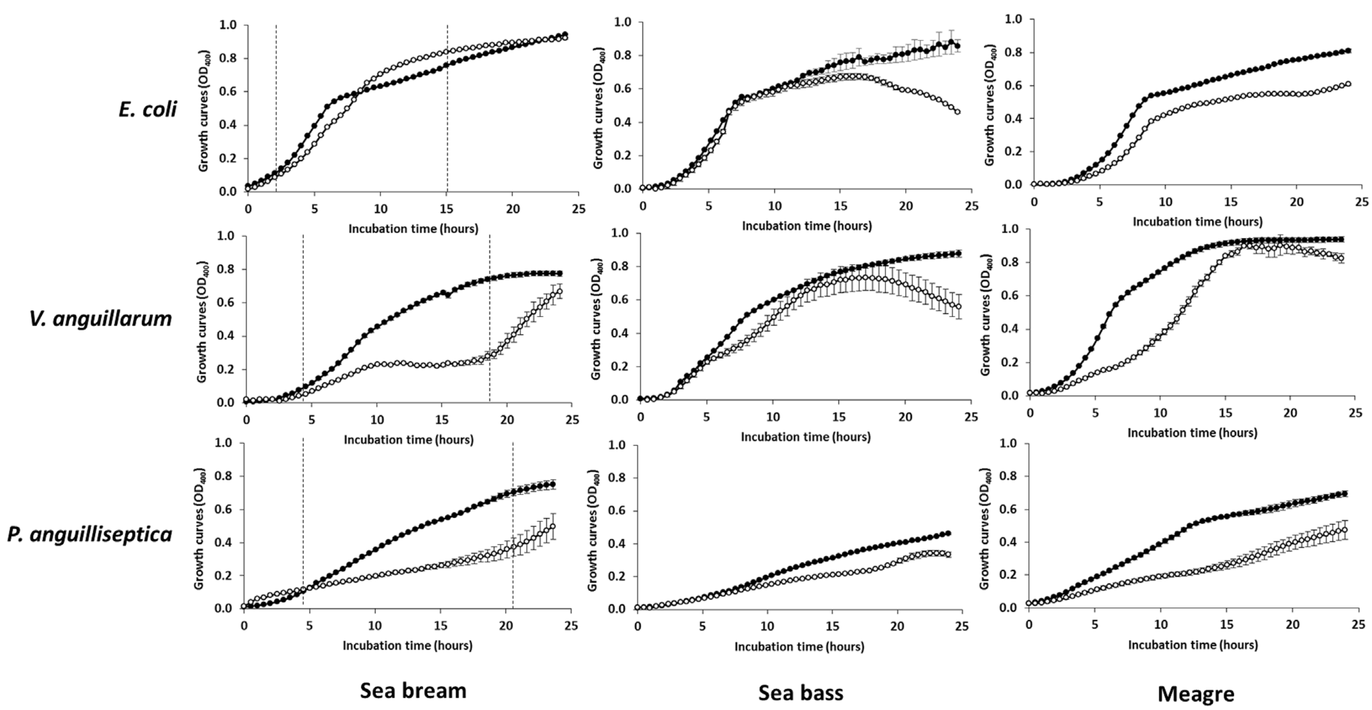

Fig. 4. Antibacterial activity study of skin mucus. Data correspond to the mean ± SEM of triplicate free growth curves (with no mucus present, black circles) and of "in-mucus" growth (with equal volumes of mucus, white circles). Mucus samples came from 3 pools of 6 individual fish. Vertical dotted lines serve to orientate the lag-, log- and stationary-growth phases for each bacteria. Details on statistical differences between bacterial growth without or with mucus presence of each fish species are provided in the Supplementary Table 1.

4.2. Defensive enzymatic activities of esterases and proteases

Mucus is the result of several enzymatic activities with a variety of functions that are not well known or defined. It has been proposed that esterases are: regulators of nerve impulse transmission; marker enzymes for glial or supportive cells, or other non-neuronal elements; and detoxifiers of many xenobiotics and endogenous compounds (revised in Ref. [34]. In fish mucus, they have mostly been studied in terms of their defensive activity against pathogens [22,29,30]. Within the esterase family, CEs are a heterogeneous group of isozymes that catalyse the hydrolysis of a wide range of esters, amides and thioesters. To the best of our knowledge, few studies have referred to the activity as CEs in fish skin mucus [31,32] and most studies only attribute esterase activity to the pNPM-CE substrate [30,46,47]; [21,22,48,49]. Our results show for the first time in skin mucus that mucus esterase activity of the three species studied has a higher specificity for the short-fatty-acid-chain conjugates pNPA-CE and butyrate (pNPB-CE) than for longer ones such as myristate (pNPM-CE). In fact, 20-fold less activity is detected for the myristate conjugate, indicating that pNPA-CE and pNPB-CE could be more appropriate substrates for the study of CEs in fish skin mucus. In spite of the same pattern being observed through substrates studied, basal levels of mucus CEs differed between species: sea bream mucus showed less CE activity for all three substrates than sea bass, in contrast to the observation by Ref. [22] using only myristate as a conjugate. In contrast, meagre mucus showed the greatest CE activity if we consider the sum of all of them. The relevance of the different CE activities in skin mucus should be further studied to better understand the role of each activity as providing non-specific immune power for each species according to, for instance, their elapsed time from domestication (culture) or their exposition to pathogens. Moreover, due to the role of CEs in detoxification processes [34], this role of CE activities should also be considered in skin mucus.

The release of proteases into skin mucus is classically described as acting directly on a pathogen or preventing pathogen invasion indirectly by modifying mucus consistency and thereby increasing mucus sloughing and the removal of pathogens from the body surface [50]. In addition, they also activate and enhance the production of various immunological components such as complement control proteins, immunoglobulins or antimicrobial peptides [6]. The analysis of total protease activity (TPA) confirmed the species dependence of that activity in fish skin mucus, as previously suggested [22]. Our results for meagre mucus showed 10-fold higher TPA than for sea bream mucus and 5-fold higher than for sea bass mucus [22]. attributed high protease and antiprotease activities to species, such as shi drum or dentex, which are more prone to suffer diseases produced by parasites than by bacteria, according to previous literature [51,52]. No similar reports existed on meagre susceptibility to bacteria or parasites with respect sea bass and sea bream. The three studied species are considered within the marine temperate species, although the recent phylogenetic classification of bony fishes from Betancur et al. (2013) separated Spariformes order, which included Sparidae family (gilthead sea bream), from the broad order of Perciformes, which included Serranidae family (European sea bass) and Scianidae family (meagre). To attribute different levels of both protease and esterase activities to phylogeny proximity seems no consistent with the "new tree of live of fishes" proposed in Betancur et al. (2013). Lower levels of these non-specific innate defences in meagre could be related to the shorter domestication time and the gradual loss of that potential in the skin mucus of sea bass and sea bream. A recent review [53] analysing the effects on fish welfare during domestication process proposed that a controversy exists between the selected production-related traits such as growth, and the negative effects on welfare-related traits, such as response to stress or infections [54]. In that line [55], reported in digestive mucosa of Eurasian perch that the domestication process results in a down-regulation of genes related to proteolysis pathways. Further studies are necessary to better know the effects of domestication process on the non-specific immune defences in fish, in general, and in skin mucus in particular.

To deepen the study of protease activity in fish skin mucus, for the first time we performed comparisons of mucus zymograms, by caseinolytic activity, for these three species that are of such importance for aquaculture, under the same protocol conditions. This zymographic evaluation showed several conserved bands as recently reported in sea bream [24] and also observed here in sea bass and meagre zymograms. A lower-MW caseinolytic band, L-band (around 15 kDa) was present for the three species, resulting in a considerable "digested area" for meagre after 2 h of casein incubation, coinciding with its higher TPA. That L-band perfectly matched the trypsin positive control MW, and it was inhibited by the specific trypsin-like activity inhibitors TLCK and SBTI in the sea bream and sea bass zymograms. The lack of inhibition observed in the meagre zymograms could be attributed to the higher caseinolytic activity and to a low inhibitor concentration in the performed protocol. A little literature exists that reports the presence of low-molecular-weight serine protease activity in fish mucus zymograms in rainbow trout [56], Atlantic salmon [29,57] and olive flounder [46], usually in response to infection challenges from Gram-positive bacteria. In agreement with those findings, our current results with the meagre mucus zymogram would indicate a greater defensive potential of this species than for sea bream and sea bass. In contrast, the seabream and sea bass zymograms presented high-MW caseinolytic bands, H-bands, at 180–200 kDa, in contrast to meagre which does not present this caseinlotyic activity. The study with PMSF, TPCK and ZPCK inhibitors, indicated chymotrypsin-like activity. Similar H-bands are also found in other marine species such as coho salmon and Atlantic salmon [28], olive flounder [46] and also in freshwater species [31] which are also increasingly exuded under infection challenges. As there was no trypsin- and chymotrypsin-like activity, the intermediate-MW caseinolytic band, the I-band at 75–80 kDa, matched in the three species. That I-band coincided with reported activity of metalloproteases in the skin mucus of trout, and salmon species [31,58]. In sea bream, a previous study demonstrated that under chronic low temperatures the caseinolytic activity of the specific I-band increased 5-fold [24]. Although further studies will be necessary in fish and in higher vertebrates, metalloprotease production has been associated with response to injury, disease or inflammation [59], activating various immune factors such as cytokines, chemokines, receptors [60] and other protease-like cathepsines, and antimicrobial peptides [61,62].

4.3. Antibacterial activity

The antimicrobial activity of skin mucus is one of the major interests in studies of mucus properties and responses that address infections, environmental challenges or nutritional improvement, such as those concerned with supplementation with pre- and pro-biotics. It is well known that the elimination of skin mucus and the consequent challenge increases fish mortality [63] or increases susceptibility to bacterial infection [64], thus evidencing the crucial role of the skin mucus in preventing fish infections. To better know the antibacterial power of the skin mucus of these three important productive species, we selected two of the most important fish pathogen main bacteria that greatly affect fish culture in farms: *P. anguilliseptica* and *V. anguillarum* (reviewed in Ref. [65]; together with a theoretical non-pathogen: *E. coli* strain for fish. We proposed a study of the putative effect of the skin on the dynamics of bacterial growth under their lag-growth, log-growth and stationary-growth phases, to elucidate antibacterial mucus activity. As the adherence of bacteria to mucosal surfaces and the subsequent infection depend on mucus [15], we recently performed a turnover study on mucus production and exudation [66] where we demonstrated that mucus is continuously produced and exuded, and mucus renewal could be a rapid process. Taking into consideration those recent insights, bacteria adhesion and growth is a constant battle between mucus properties and renewal, on the one hand, and bacteria growth capacity for colonization under mucus conditions, on the other. Thus, the different growth curves observed between pathogens and fish species responded to the different properties. If the focus of antibacterial activity was in the final 24 h final, sea bream and meagre would show a lower capacity to inhibit *V. anguillarum* growth than sea bass. However, during the log-growth phase, both sea bream and meagre greatly diminished bacteria performance, showing an evident bacteriostatic capacity, which is more in keeping with the considerable lytic activity reported in previous studies [21]. In the same way, the antibacterial study of skin mucus against *P. anguilliseptica*, subdivided in the different growth phases, highlighted both the bactericidal (lower growth) and bacteriostatic (delayed growth) roles of fish skin mucus. Evaluation of the direct lytic activity against pathogens is the most practical finding awaited by farmers [21]. The present study emphasises the importance of better understanding the time course of bacterial performance in the mucus environment, also considering the daily renewal capacity of the skin mucus components [66].

5. Conclusions

In conclusion, here we describe and compare different protease activities and specific CE activities, as well as antioxidant power for the first time in fish skin mucus. These are some of the main non-specific immune defences in skin mucus for three of the most important Mediterranean aquaculture fish species. All our results reveal that meagre shows higher levels of these non-specific defences than sea bass and sea bream. If the culture environment tends to produce poor physiological conditions for fish and increased susceptibility to infections [67], it could be hypothesized that meagre, as a more recently domesticated species in aquaculture, have conserved greater protection via esterase and protease trypsin-like activities in epidermal mucus. In contrast, "old cultured" species could present lower levels of these non-specific defences. Novelty, the antibacterial activity of fish skin mucus is shown to perform in a time-dynamics manner between bacterial growth and mucus properties, which could be conditioned by mucus renewal capacity of each species, which is still known. These non-invasive parameters will also be also very useful to study fish welfare, to understand better how fish respond to nutritional enhancements such as supplementation with pre- or pro-biotics, and even to study the effects of domestication process, scarcely known in fish, and the possible loss of non-specific immune defences in skin mucus.

Acknowledgments

This research was funded via grants from the Spanish Government (MICIN, project AGL2015-70637-R; and MAPAMA– FEMP funds, project DIETAplus).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.04.008.

References

[1] FAO, The State of World Fisheries and Aquaculture 2018 - Meeting the Sustainable Development Goals. Rome, (2018).
[2] C. Sauvage, N. Derôme, E. Normandeau, J. St-Cyr, C. Audet, L. Bernatchez, Fast transcriptional responses to domestication in the brook charr *Salvelinus fontinalis*, Genetics 185 (1) (2010) 105–112 https://doi.org/10.1534/genetics.110.115071.
[3] C.B. Schreck, L. Tort, A.P. Farrell, C.J. Brauner, Biology of Stress in Fish, ElSevier, 2016.
[4] T.C. Hrubec, J.L. Cardinale, S.A. Smith, Hematology and plasma chemistry reference intervals for cultured Tilapia (*Oreochromis* hybrid), Vet. Clin. Pathol. 29 (1) (2000) 7–12 https://doi.org/10.1111/j.1939-165X.2000.tb00389.x.
[5] M. Tavares-Dias, F.R. Moraes, Haematological and biochemical reference intervals for farmed channel catfish, J. Fish Biol. 71 (2) (2007) 383–388 https://doi.org/10.1111/j.1095-8649.2007.01494.x.
[6] M.A. Esteban, An overview of the immunological defenses in fish skin, ISRN Immunol. 2012 (October) (2012) 1–29 https://doi.org/10.5402/2012/853470.
[7] D.R. Ekman, D.M. Skelton, J.M. Davis, D.L. Villeneuve, J.E. Cavallin, A. Schroeder, K.M. Jensen, G.T. Ankley, T.W. Collette, Metabolite profiling of fish skin mucus: a novel approach for minimally-invasive environmental exposure monitoring and surveillance, Environ. Sci. Technol. 49 (5) (2015) 3091–3100 https://doi.org/10.1021/es505054f.
[8] L. Fernández-Alacid, I. Sanahuja, B. Ordóñez-Grande, S. Sánchez-Nuño, G. Viscor, E. Gisbert, M. Herrera, A. Ibarz, Skin mucus metabolites in response to physiological challenges: a valuable non-invasive method to study teleost marine species, Sci. Total Environ. 644C (2018) 1323–1335.
[9] L. Fernández-Alacid, I. Sanahuja, B. Ordóñez-Grande, S. Sánchez-Nuño, M. Herrera, A. Ibarz, Skin mucus metabolites and cortisol in meagre fed acute stress-attenuating diets: correlations between plasma and mucus, Aquaculture 499 (2019) 185–194 https://doi.org/10.1016/J.AQUACULTURE.2018.09.039.
[10] F.A. Guardiola, A. Cuesta, M.A. Esteban, Using skin mucus to evaluate stress in gilthead seabream (*sparus aurata* L.), Fish Shellfish Immunol. 59 (2016) 323–330 https://doi.org/10.1016/j.fsi.2016.11.005.
[11] E. De Mercado, A.M. Larrán, J. Pinedo, C. Tomás-Almenar, T.P. Hurst, Skin mucous: a new approach to assess stress in *rainbow trout*, Aquaculture 484 (February 2017) (2018) 90–97 https://doi.org/10.1016/j.aquaculture.2017.10.031.
[12] K.L. Shephard, Functions for Fish Mucus. Reviews in Fish Biology and Fisheries, Kluwer Academic Publishers, 1994, https://doi.org/10.1007/BF00042888.
[13] N. Blackstock, A.D. Pickering, Changes in the concentration and histochemistry of epidermal mucous cells during the alevin and fry stages of the Brown trout *Salmo trutta*, J. Zool. 197 (4) (1982) 463–471 https://doi.org/10.1111/jzo.1982.197.4.463.
[14] G. Zaccone, P. Lo Cascio, S. Fasulo, A. Licata, The effect of an anionic detergent on complex carbohydrates and enzyme activities in the epidermis of the catfish *heteropneustes fossilis* (bloch), Histochem. J. 17 (4) (1985) 453–466 https://doi.org/10.1007/BF01003205.
[15] S. Benhamed, F.A. Guardiola, M. Mars, M.A. Esteban, Pathogen bacteria adhesion to skin mucus of fishes, Vet. Microbiol. 171 (1–2) (2014) 1–12 https://doi.org/10.1016/j.vetmic.2014.03.008.
[16] D. Gómez, J.O. Sunyer, I. Salinas, The mucosal immune system of fish: the evolution of tolerating commensals while fighting pathogens, Fish Shellfish Immunol. 35 (6) (2013) 1729–1739 https://doi.org/10.1016/j.fsi.2013.09.032.
[17] R.H. Easy, N.W. Ross, Changes in atlantic Salmon (*Salmo salar*) epidermal mucus protein composition profiles following infection with sea lice (*Lepeophtheirus Salmonis*), Comp. Biochem. Physiol. Genom. Proteonom. 4 (3) (2009) 159–167 https://doi.org/10.1016/j.cbd.2009.02.001.
[18] C. Hellio, A.M. Pons, C. Beaupoil, N. Bourgougnon, Y. Le Gal, Antibacterial, antifungal and cytotoxic activities of extracts from fish epidermis and epidermal mucus, Int. J. Antimicrob. Agents 20 (3) (2002) 214–219 https://doi.org/10.1016/S0924-8579(02)00172-3.
[19] L. Tort, J.C. Balasch, S. Mackenzie, Fish Immune System. A Crossroads between Innate and Adaptive Responses vol 22, (2003) http://www.inmunologia.org/Upload/Articles/6/0/602.pdf.
[20] J. Pérez-Sánchez, I. Estensoro, M.J. Redondo, J.A. Calduch-Giner, S. Kaushik, A. Sitjà-Bobadilla, Mucins as diagnostic and prognostic biomarkers in a fish-parasite model: transcriptional and functional analysis. Edited by jean-luc desseyn, PLoS One 8 (6) (2013) e65457https://doi.org/10.1371/journal.pone.0065457.
[21] F.A. Guardiola, A. Cuesta, M. Arizcun, J. Meseguer, M.A. Esteban, Comparative skin mucus and serum humoral defence mechanisms in the teleost gilthead seabream (*sparus aurata*), Fish Shellfish Immunol. 36 (2) (2014) 545–551 https://doi.org/10.1016/j.fsi.2014.01.001.
[22] F.A. Guardiola, A. Cuesta, E. Abellán, J. Meseguer, M.A. Esteban, Comparative analysis of the humoral immunity of skin mucus from several marine teleost fish, Fish Shellfish Immunol. 40 (1) (2014) 24–31 https://doi.org/10.1016/j.fsi.2014.06.018.
[23] I. Sanahuja, A. Ibarz, Skin mucus proteome of gilthead sea bream: a non-invasive method to screen for welfare indicators, Fish Shellfish Immunol. 46 (2) (2015) 426–435 https://doi.org/10.1016/j.fsi.2015.05.056.
[24] I. Sanahuja, L. Fernández-Alacid, S. Sánchez-Nuño, B. Ordóñez-Grande, A. Ibarz, Chronic cold stress alters the skin mucus interactome in a temperate fish model, Front. Physiol. 9 (2019), https://doi.org/10.3389/fphys.2018.01916.
[25] J. Pérez-Sánchez, G. Terova, P. Simó-Mirabet, S. Rimoldi, O. Folkedal, J.A. Calduch-Giner, R.E. Olsen, A. Sitjà-Bobadilla, Skin mucus of gilthead sea bream (*sparus aurata* L.). Protein mapping and regulation in chronically stressed fish, Front. Physiol. 8 (2017) 34 https://doi.org/10.3389/fphys.2017.00034.
[26] S. Subramanian, N.W. Ross, S.L. MacKinnon, Comparison of antimicrobial activity in the epidermal mucus extracts of fish, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 150 (1) (2008) 85–92 https://doi.org/10.1016/J.CBPB.2008.01.011.
[27] K. Brix, W. Stöcker, Proteases: Structure and Function vol 9783709108, Springer Vienna, 2013, https://doi.org/10.1007/978-3-7091-0885-7.
[28] M.D. Fast, D.E. Sims, J.F. Burka, A. Mustafa, N.W. Ross, Skin morphology and humoral non-specific defence parameters of mucus and plasma in rainbow trout, coho and atlantic salmon, Comp. Biochem. Physiol. Mol. Integr. Physiol. 132 (3) (2002) 645–657 https://doi.org/10.1016/S1095-6433(02)00109-5.
[29] N.W. Ross, K.J. Firth, A. Wang, J.F. Burka, S.C. Johnson, 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. Org. 41 (1) (2000) 43–51 https://doi.org/10.3354/dao041043.
[30] K.J. Palaksha, G.W. Shin, Y.R. Kim, T.S. Jung, Evaluation of non-specific immune components from the skin mucus of olive flounder (*Paralichthys olivaceus*), Fish Shellfish Immunol. 24 (4) (2008) 479–488 https://doi.org/10.1016/j.fsi.2008.01.005.
[31] A.K. Nigam, U. Kumari, S. Mittal, A.K. Mittal, Comparative analysis of innate

immune parameters of the skin mucous secretions from certain freshwater teleosts, inhabiting different ecological niches, Fish Physiol. Biochem. 38 (5) (2012) 1245–1256 https://doi.org/10.1007/s10695-012-9613-5.
[32] A.K. Nigam, U. Kumari, S. Mittal, A.K. Mittal, Characterization of carboxylesterase in skin mucus of *cirrhinus mrigala* and its assessment as biomarker of organanophosphate exposure, Fish Physiol. Biochem. 40 (3) (2014) 635–644 https://doi.org/10.1007/s10695-013-9872-9.
[33] E. Küster, R. Altenburger, Comparison of cholin- and carboxylesterase enzyme inhibition and visible effects in the zebra fish embryo bioassay under short-term paraoxon-methyl exposure, Biomarkers 11 (4) (2006) 341–354 https://doi.org/10.1080/13547500600742136.
[34] M. Solé, L.M.G. de la Parra, S. Alejandre-Grimaldo, F. Sardá, Esterase activities and lipid peroxidation levels in offshore commercial species of the NW mediterranean sea, Mar. Pollut. Bull. 52 (12) (2006) 1708–1716 https://doi.org/10.1016/J.MARPOLBUL.2006.07.015.
[35] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1–2) (1976) 248–254 https://doi.org/10.1016/0003-2697(76)90527-3.
[36] C.E. Wheelock, B.M. Phillips, B.S. Anderson, J.L. Miller, M.J. Miller, B.D. Hammock, Applications of carboxylesterase activity in environmental monitoring and toxicity identification evaluations (TIEs), Reviews of Environmental Contamination and Toxicology, vol 195, 2008, pp. 117–178 https://doi.org/10.1007/978-0-387-77030-7_5.
[37] M. Solé, G. Rivera-Ingraham, R. Freitas, The use of carboxylesterases as biomarkers of pesticide exposure in bivalves: a methodological approach, Comp. Biochem. Physiol. C Toxicol. Pharmacol. 212 (October) (2018) 18–24 https://doi.org/10.1016/j.cbpc.2018.06.002.
[38] F.J. Moyano, M. Díaz, F.J. Alarcón, M.C. Sarasquete, Characterization of digestive enzyme activity during larval development of gilthead seabream (*sparus aurata*), Fish Physiol. Biochem. 15 (2) (1996) 121–130 https://doi.org/10.1007/BF01875591.
[39] E. Santigosa, J. Sánchez, F. Médale, S. Kaushik, J. Pérez-Sánchez, M.A. Gallardo, Modifications of digestive enzymes in trout (*Oncorhynchus mykiss*) and sea bream (*sparus aurata*) in response to dietary fish meal replacement by plant protein sources, Aquaculture 282 (1–4) (2008) 68–74 https://doi.org/10.1016/j.aquaculture.2008.06.007.
[40] I. García-Meilán, J.M. Valentín, R. Fontanillas, M.A. Gallardo, Different protein to energy ratio diets for gilthead sea bream (*sparus aurata*): effects on digestive and absorptive processes, Aquaculture 412–413 (November) (2013) 1–7 https://doi.org/10.1016/j.aquaculture.2013.06.031.
[41] F.J. Alarcón, M. Díaz, F.J. Moyano, E. Abellán, Characterization and functional properties of digestive proteases in two sparids; gilthead seabream (*sparus aurata*) and common dentex (*dentex dentex*), Fish Physiol. Biochem. 19 (3) (1998) 257–267 https://doi.org/10.1023/A:1007717708491.
[42] I.F.F. Benzie, J.J. Strain, The ferric reducing ability of plasma (FRAP) as a measure of 'antioxidant power': the FRAP assay, Anal. Biochem. 239 (1) (1996) 70–76 https://doi.org/10.1006/abio.1996.0292.
[43] E. Brucka-Jastrzebska, D. Kawczuga, A. Grzelak, G. Bartosz, Magnesium content, total antioxidant status and lipid peroxidation in rainbow trout (*Oncorhynchus mykiss walbaum*), Magnes. Res. 22 (4) (2009) 273–279 https://doi.org/10.1684/mrh.2009.0185.
[44] M.J. Sánchez-Muros, S. Villacreces, G.M. de la Lama, C. de Haro, F. García-Barroso, Effects of chemical and handling exposure on fatty acids, oxidative stress and morphological welfare indicators in gilt-head sea bream (*sparus aurata*), Fish Physiol. Biochem. 39 (3) (2013) 581–591 https://doi.org/10.1007/s10695-012-9721-2.
[45] N. Gobi, B. Vaseeharan, R. Rekha, S. Vijayakumar, C. Faggio, Bioaccumulation, cytotoxicity and oxidative stress of the acute exposure selenium in Oreochromis mossambicus, Ecotoxicol. Environ. Saf. 162 (2018) 147–159 https://doi.org/10.1016/j.ecoenv.2018.06.070.
[46] T.S. Jung, C.S. del Castillo, K.J. Palaksha, S.D. Rishikesh, S.W. Nho, S.B. Park, H.B. Jang, I.S. Cha, H.W. Sung, J. Hikima, T. Aoki, Seasonal variation and comparative analysis of non-specific humoral immune substances in the skin mucus of olive flounder (*Paralichthys olivaceus*), Dev. Comp. Immunol. 38 (2) (2012) 295–301 https://doi.org/10.1016/j.dci.2012.06.005.
[47] K. Loganathan, A. Arulprakash, M. Prakash, P. Senthilraja, Lysozyme, protease, alkaline phosphatase and esterase activity of epidermal skin mucus of freshwater snake head fish *Channa striatus*, Int. J. Res. Pharm. Biosci. 3 (3) (2013) 17–20.
[48] F.A. Guardiola, M. Dioguardi, M.G. Parisi, M.R. Trapani, J. Meseguer, A. Cuesta, M. Cammarata, M.A. Esteban, Evaluation of waterborne exposure to heavy metals in innate immune defences present on skin mucus of gilthead seabream (*sparus aurata*), Fish Shellfish Immunol. 45 (1) (2015) 112–123 https://doi.org/10.1016/j.fsi.2015.02.010.
[49] H. Cordero, M.F. Brinchmann, A. Cuesta, M.A. Esteban, Chronic wounds alter the proteome profile in skin mucus of farmed gilthead seabream, BMC Genomics 18 (1) (2017), https://doi.org/10.1186/s12864-017-4349-3.
[50] F. Aranishi, N. Mano, H. Hirose, Fluorescence localization of epidermal cathepsins L and B in the Japanese eel, Fish Physiol. Biochem. 19 (3) (1998) 205–209 https://doi.org/10.1023/A:1007779600183.
[51] F.M. Rueda, F.J. Martínez, A review on the biology and potential aquaculture of *dentex dentex*, Rev. Fish Biol. Fish. 11 (1) (2001) 57–70 https://doi.org/10.1023/A:1014276700138.
[52] I.N. Vatsos, K.D. Thompson, A. Adams, Colonization of rainbow trout, *Oncorhynchus mykiss* (walbaum), eggs by *flavobacterium psychrophilum*, the causative agent of rainbow trout fry syndrome, J. Fish Dis. 29 (7) (2006) 441–444 https://doi.org/10.1111/j.1365-2761.2006.00735.x.
[53] J.L. Saraiva, M.F. Castanheira, P. Arechavala-López, J. Volstorf, B.H. Studer, Domestication and Welfare in Farmed Fish, IntechOpen, 2018, https://doi.org/10.5772/intechopen.77251.
[54] T. Takahara, T. Minamoto, H. Doi, T. Ito, Z. Kawabata, Differences between domesticated Eurasian and Japanese indigenous strains of the common carp (*Cyprinus carpio*) in cortisol release following acute stress, Ichthyol. Res. 61 (2) (2014) 165–168 https://doi.org/10.1007/s10228-013-0385-4.
[55] X. Chen, J. Wang, L. Qian, S. Gaughan, W. Xiang, T. Ai, Z. Fan, C. Wang, Domestication drive the changes of immune and digestive system of Eurasian perch (*Perca fluviatilis*), PLoS One 12 (3) (2017) e0172903https://doi.org/10.1371/journal.pone.0172903.
[56] K. Hjelmeland, M. Christie, J. Raa, Skin mucus protease from rainbow trout, *Salmo gairdneri* richardson, and its biological significance, J. Fish Biol. 23 (1) (1983) 13–22 https://doi.org/10.1111/j.1095-8649.1983.tb02878.x.
[57] R. Braun, J.A. Arnesen, A. Rinne, K. Hjelmeland, Immunohistological localization of trypsin in mucus-secreting cell layers of atlantic Salmon, *Salmo salar* L, J. Fish Dis. 13 (3) (1990) 233–238 https://doi.org/10.1111/j.1365-2761.1990.tb00778.x.
[58] K.J. Firth, C.J. Stewart, N.W. Ross, Characterization of proteases in the skin mucus of atlantic Salmon (*Salmo salar*) infected with the Salmon louse (*Lepeophtheirus Salmonis*) and in whole-body louse homogenate, J. Parasitol. 86 (6) (2000) 1199–1205 https://doi.org/10.1645/0022-3395(2000)086[1199:COPITS]2.0.CO;2.
[59] J.F. Woessner Jr., Matrix metalloproteinases and their inhibitors in connective tissue remodeling, FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. 5 (8) (1991) 2145–2154 http://www.ncbi.nlm.nih.gov/pubmed/1850705.
[60] L.J. McCawley, L.M. Matrisian, Matrix metalloproteinases: they're not just for matrix anymore!, Curr. Opin. Cell Biol. 13 (5) (2001), https://doi.org/10.1016/S0955-0674(00)00248-9.
[61] J.H. Cho, I.Y. Park, H.S. Kim, W.T. Lee, M.S. Kim, S.C. Kim, Cathepsin D produces antimicrobial peptide parasin I from histone H2A in the skin mucosa of fish, FASEB J.: Off. Publ. Fed. Am. Soc. Exp. Biol. 16 (3) (2002) 429–431 https://doi.org/10.1096/fj.01-0736fje.
[62] J.H. Cho, I.Y. Park, M.S. Kim, S.C. Kim, Matrix metalloproteinase 2 is involved in the regulation of the antimicrobial peptide parasin I production in catfish skin mucosa, FEBS (Fed. Eur. Biochem. Soc.) Lett. 531 (3) (2002) 459–463 https://doi.org/10.1016/S0014-5793(02)03584-6.
[63] T. Kanno, T. Nakai, K. Muroga, Mode of transmission of vibriosis among ayu *plecoglossus altivelis*, J. Aquat. Anim. Health 1 (1) (1989) 2–6 https://doi.org/10.1577/1548-8667(1989)001%3c0002:MOTOVA%3e2.3.CO;2.
[64] C. Lemaitre, N. Orange, P. Saglio, N. Saint, J. Gagnon, G. Molle, Characterization and ion channel activities of novel antibacterial proteins from the skin mucosa of carp (*Cyprinus carpio*), Eur. J. Biochem. 240 (1) (1996) 143–149 https://doi.org/10.1021/om7005276.
[65] M.L. Kent, T.T. Poppe, Infectious diseases of coldwater fish in marine and brackish water, Diseases and Disorders of Finfish in Cage Culture, 61–105, CABI, Wallingford, 2002, https://doi.org/10.1079/9780851994437.0061 , Accessed date: 10 November 2018.
[66] A. Ibarz, B. Ordóñez-Grande, I. Sanahuja, S. Sánchez-Nuño, J. Fernández-Borràs, J. Blasco, L. Fernández-Alacid, Using Stable Isotopes Analysis to study skin mucus exudation and renewal in fish, J. Exp. Biol. (2 April 2019), https://doi.org/10.1242/jeb.195925 jeb.195925.
[67] E. Mehana, A. Rahmani, S. Aly, Immunostimulants and fish culture: an overview, Annu. Res. Res. Biol. 5 (6) (2015) 477–489 https://doi.org/10.9734/ARRB/2015/9558.